

Differential Proteomic Profiling

Towards Elucidation of TB-IRIS Pathogenesis

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Abstract

Background Up to 59% of tuberculosis (TB)/human immunodeficiency virus (HIV) co-treated patients develop paradoxical TB-associated immune reconstitution inflammatory syndrome (TB-IRIS) after addition of antiretroviral (ARV) therapy to anti-tuberculous therapy (ATT). The course can be prolonged and the average mortality rate is 2% (75% for TB-IRIS involving the central nervous system (CNS)). Immune elements – including neutrophils - involved in the anti-*Mycobacterium tuberculosis* (Mtb) response are implicated in pathogenesis, which remains incompletely understood. Diagnosis is one of exclusion, no reliable laboratory markers exist, corticosteroid-mediated prophylaxis and therapy are only partially effective, and no treatment targets tissue damage. Disentangling cause and effect in complex disorders such as TB-IRIS requires techniques capable of interrogating complex biological systems. Neutrophils are the major circulating leukocyte population, the earliest innate system responders, and exhibit various unusual immunometabolic functional specialisations. Proteins represent the most functionally-proximal and commonly pharmacologically-targeted cellular biomolecules. Label-free high-performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) is well-suited to differentially profiling the ex vivo neutrophil proteome in an unbiased manner, in order to investigate TB-IRIS predisposition and pathogenesis.

Methods Applying first principles to existing human literature, the most parsimonious holistic hypothetical model regarding paradoxical TB-IRIS predisposition and pathogenesis was inferred. A clinical cohort of control (CTRL) and matched TB-IRIS case (IRIS) study participants was assembled. Demographic, clinical, and biochemical characteristics were analysed for statistically-significant differences and to identify potential risk/protective factors (relative risk (RR) with 95% confidence interval (CI)). A small group (n = 9) of TB-HIV- healthy volunteers (HVs) was also assembled. Phlebotomy occurred at two time-points: just prior to ARV initiation (week 0) and at the typical time of IRIS manifestation (week 2). Neutrophils were isolated and lysed, proteins underwent on-filter protein trypsinisation, peptide salts and detergents were removed, and neutrophil-optimised HPLC-MS/MS was conducted. Spectra were submitted to MaxQuant for parent protein identification and quantitation. Comparisons of (a) CTRL0 and IRIS0 to HV (and resultant differences) identified class-differential impacts of partial ATT-treated co-infection (IRIS predisposition) and of (b) CTRL2 to CTRL0 and IRIS2 to IRIS0 (and resultant differences) identified class-differential impacts of ARV therapy (IRIS pathogenesis). Class-discriminating proteomic differences were visualised using principal components analysis (PCA), protein differential expression analysis was performed (including for detectable/undetectable and significantly differentially-expressed (SDE) proteins), and results informed differential functional profiling via gene ontology over-representation analysis (GO-ORA) and pathway activation state prediction. To address shortcomings of current knowledgebases and automated tools, a novel deep manual analysis approach focused on key inference-friendly proteins, convergent findings, and neutrophil-specific functional modules. Integrated findings extended the literature-derived TB-IRIS model, generating testable novel hypotheses, one of which was partially validated using live-cell fluorescence microscopy. Proteomic data were additionally analysed to detect Mtb proteins, preliminarily analyse variable post-translational modifications (PTMs) of interest, and identify candidate prognostic and diagnostic biomarkers. Finally, mechanistic hypotheses facilitated identification of novel potential prophylactic and therapeutic targets.

Results (1) Literature suggests advanced TB/HIV-coinfection (including a higher Mtb/antigen load) as the major TB-IRIS risk factor. Attendant significant immunometabolic state perturbations include myeloid overactivation, metabolic stress (possibly including adaptogen depletion), a lack of regulatory receptors, impaired pro-inflammatory signal transduction, and impaired antigen clearance. These likely predispose to lytic cell death - including release of host- and pathogen-derived inflammatory/cytotoxic molecules and proteolytic enzymes - and less restrained/more abnormal inflammation as well as tissue damage, on restoration of HIV-suppressed inflammatory signalling pathways by ARV therapy. **(2)** Regarding the clinical cohort, a sample size of > 42 participants per characteristic-matched comparison class provides > 95% power to detect a two-fold change with 99% confidence. Cases exhibited known TB-IRIS risk factors, and largely expected white cell count (WCC), body mass index (BMI), and C-reactive protein (CRP) level changes in response to ARV therapy (e.g. WCC increase and BMI decrease). None of the few participants on alternate (efavirenz (EFV)- or tenofovir (TDF)-lacking) regimens developed TB-IRIS. Pre-ARV prednisone (or incidental antihistamine/anti-fungal) use was associated with a non-significantly decreased TB-IRIS risk. Interestingly, smoking is associated with a significant decrease in TB-IRIS risk by 60%. **(3)** Regarding sample processing and analysis, the average

sample collection to neutrophil isolation interval was within recommended limits. Isolation yield exceeded 15×10^6 and 25×10^6 per sample in the CTRL and IRIS groups, respectively. Isolated neutrophil purity exceeded 80% in both groups; the few low-purity samples were excluded from subsequent proteomic analysis. Lysates from 5×10^6 neutrophils routinely yielded over 100µg total protein, tryptic digestion was efficient (on average < 96% missed cleavages), and equivalent peptide injection volumes yielded comparable total ion chromatogram (TIC) profiles and intensities. An average 23% spectral identification rate resulted in a total of 2532 protein group identifications, the deepest neutrophil proteome coverage achieved to date without pre-fractionation, representing ~12% of human protein coding genes, and ~25% of the detectable human proteome. Samples were analysed in two (randomised) batches, producing independent datasets A (N = 37) and B (N = 74). Within-dataset technical replicates exhibit excellent agreement in protein identities and quantities; between-dataset protein identities and functional inferences also exhibit excellent agreement. We identify a number of proteins apparently not known to be expressed by human neutrophils, as well as one predicted human protein never before observed empirically. Overall, parent pathways of level-altered proteins suggest perturbation of nine major neutrophil function modules at both time-points: (a) signal transduction, (b) pattern recognition receptor (PRR) and cytokine signalling, (c) the eicosanoid cascade, (d) neutrophil antimicrobial functions, (e) carbon-energy metabolism, (f) protein homeostasis, (g) integrated nitrogen-sulfur-B-vitamin and redox/xenobiotic/glyoxal metabolism, (h) gene expression, and (i) cytoskeletal dynamics. **(4)** Regarding impacts of partially ATT-treated co-infection (week 0), neutrophil proteomic profiles successfully distinguish between HV and IRIS0 or CTRL0. Many differences from HV are shared between IRIS0 and CTRL0 (i.e. driven by partially ATT-treated co-infection), but some are class-unique (i.e. driven by factors predisposing to or protecting from TB-IRIS). Findings are supported by a head-to-head comparison of the CTRL0 and IRIS0 proteomes, including changes suggesting: more prevalent type I IFN, TGFβ, and Th2-type cytokine signalling; poorer capacity for restraint of alternate complement activation; mitochondrial and oxidative stress (including proneness to necrosis); impaired function (e.g. microbicidal, TLR/IL-1R-MyD88-NFκB signalling, and caspase 1-mediated IL-1β and IL-18 maturation) of activated neutrophils; and enhanced lipid and upstream (but inhibited downstream) isoprenoid synthesis (including decreased steroidogenesis). Candidate biomarkers distinguish CTRL- and IRIS-class partially ATT-treated neutrophils from HV neutrophils and from each other. **(5)** Regarding impacts of ARV therapy (week 2), both IRIS and CTRL neutrophil proteomes exhibit significant changes in response to ARV therapy. Many changes are shared between IRIS and CTRL (i.e. driven by ARV therapy and declining viral load (VL)), but some are class-unique (i.e. driven by factors preventing/contributing to TB-IRIS pathogenesis). Findings are supported by a head-to-head comparison of the CTRL2 and IRIS2 proteomes, including changes suggesting: a slower decline in type I IFN signalling; increased inflammatory cytokine (e.g. IL-6, TNFα, and IFNγ) signalling and protease (e.g. MMP-8) activity; decreased sensitivity to immunoregulatory glucocorticoids and vitamin A; and increased mitochondrial, endoplasmic reticulum (ER), and oxidative stress. Candidate biomarkers distinguish CTRL- and IRIS-group ARV-exposed neutrophils from baseline and from each other. **(6)** Live-cell fluorescence microscopy of HV neutrophils suggests that *in vivo*-equivalent levels of EFV rapidly alter mitochondrial, lysosomal, and aggresomal architecture in a manner consistent with organelle and protein folding stress, and suggesting cell death commitment. **(7)** Integrated neutrophil immunometabolic changes suggested by proteomic findings support and extend the biologically compelling literature-derived model. Model highlights include more advanced baseline TB/HIV (including higher type I IFN, TGFβ, and possibly Th2-type cytokine levels), with consequent impaired myeloid-mediated Mtb antigen clearance and depletion of cellular adaptogens. The resultant abnormal immunometabolic state produces myeloid cells less able to counteract metabolic stress and primed for less-restrained inflammation. Introduction of mitotoxic ARV drugs and rapid lifting of HIV-mediated immune embargoes escalates myeloid metabolic (including oxidative) stress and overactivation (including via NLRC4, CASP4/5, TLR/IL-1R-MyD88-NFκB, and MAPK-AP1 signalling), producing - instead of Mtb clearance - inflammatory cell death with release of immune-activating and tissue-damaging host- and Mtb-derived molecules. Reactivation of Mtb lymphocyte memory responses likely only produces clinically-apparent inflammation (TB-IRIS) when multiple simultaneous but incompatible immune programmes (e.g. overzealous myeloid activity, Th1, Th2, Th17, and Treg) coexist. Based on this model, existing compounds with the potential for rational, safe, effective TB-IRIS prophylaxis/therapy are identified (e.g. glutathione, vitamins B-complex and A/D/E, rapamycin, and metformin) which may assist in restoring system homeostasis.

Conclusions An improved understanding of TB-IRIS immunopathogenesis – especially of the most proximal triggering molecules and their target cell and receptor types – will facilitate more rational and

effective risk stratification, prophylactic, prognostic, diagnostic, and targeted (e.g. immunometabolic modulatory) therapeutic strategies. This includes candidate biomarker and putative treatment target identification, and repurposing of existing safe pharmacological agents. Appropriate clinical cohorts were assembled and suitable systems biology research and statistical/computational techniques were applied. Proteomic findings were integrated with existing literature to extend a biologically compelling, comprehensive, and parsimonious model in order to generate testable hypotheses regarding TB-IRIS predisposition and pathogenesis. One hypothesis (contribution of ARV drugs to myeloid metabolic stress) was partially orthogonally validated. Candidate risk-stratification and diagnostic biomarkers were identified, as were strategies and compounds with the potential for safe and effective TB-IRIS prophylaxis/therapy. Research techniques are available to validate all major hypotheses, assess biomarker/biomarker panel performance in a larger independent cohort, and assess strategy/compound performance in preventing/treating TB-IRIS both pre-clinically and (should any show promise) via clinical trials. To the best of our knowledge, this is the first study to: (a) identify a potential link between smoking and protection against TB-IRIS, (b) deeply interrogate neutrophil metabolic function, including regarding how this relates to TB-IRIS predisposition and pathogenesis; and (c) investigate the impact of EFV on neutrophils.

Chapter 1

Literature Review: Paradoxical TB-IRIS in Context

*"Medical research in the 20th century mostly takes place in the lab.
[During] the Renaissance ... researchers went first ... to the library"*

- Peter Lewis Allen

Abstract

Background Up to 59% of TB/HIV-coinfected and co-treated patients develop paradoxical TB-IRIS after addition of ARV therapy to ATT. Morbidity decreases quality of life, the course can be prolonged (up to months), and the average mortality rate is 2% (increasing to 75% for TB-IRIS involving the central nervous system). Immune elements involved in the anti-Mtb response are implicated, but pathogenesis is incompletely understood. For example, the most proximal triggers and resultant immunopathogenic cascades remain unknown. Diagnosis is one of exclusion (often complicating confident diagnosis in resource-limited settings), no reliable laboratory markers exist, prevention/treatment (corticosteroids) is only partially effective, not specifically licensed for this indication, and may target downstream effectors of clinical manifestation rather than upstream mediators of pathogenesis, and no treatment currently targets tissue damage. **Methods** We review the most relevant paradoxical TB-IRIS literature (epidemiological, clinical, and biochemical) to construct more holistic hypotheses regarding TB-IRIS pathogenesis. Dynamics of epidemiological, clinical, cellular, and biochemical findings are summarised and evaluated in context of what is known to occur during TB/HIV-coinfection and ATT as well as ARV therapy, and inferences are made to construct a current and comprehensive pathogenic model. **Results & Discussion** More advanced TB/HIV-coinfection (including a higher Mtb/antigen load) is likely the major risk factor for TB-IRIS, suggesting significant immunometabolic state perturbations, some of which are reflected in the baseline literature. We infer that aberrant myeloid activity (including metabolic stress, impaired PRR signalling, and impaired Mtb/antigen clearance), representing a state predisposed to more unrestrained inflammation on restoration of pathways embargoed by one or both co-infecting pathogens. After ARV therapy initiation, this abnormal state also appears to result in simultaneous induction of multiple incompatible adaptive immune programmes (including failure of normal regulatory mechanisms), likely due to a combination of decreasing viral load and rapidly-increasing availability of both host- and Mtb-derived inflammatory molecules, mimicking new infection. We hypothesise that ARV therapy – initiated on a background of metabolic abnormality (e.g. possible glutathione depletion) – results in co-ordinated inflammatory death of within-tissue myeloid cells, with consequent liberation of host- and pathogen-derived inflammatory molecules, as well as proteolytic enzymes. Together, enzymes, TNF α , complement, and cytotoxic leukocytes and their mediators are likely responsible for tissue damage. **Conclusions** An improved understanding of TB-IRIS immunopathogenesis – especially of the most proximal triggering molecules and their target cell types and receptors – will facilitate more rational and effective risk stratification, prophylactic, prognostic, diagnostic, and targeted (e.g. immunomodulatory) therapeutic strategies. This includes candidate biomarker and putative treatment target identification, and repurposing of existing safe pharmacological agents. Given the significant implication of myeloid cells (especially neutrophils) in TB-IRIS predisposition and early pathogenesis, we investigate how ARV therapy differentially impacts IRIS developer versus non-developer direct ex vivo neutrophil proteomes, both to investigate the above hypotheses, and to generate testable novel hypotheses regarding TB-IRIS pathogenesis.

1.1. Paradoxical TB-IRIS: Definition

Paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) is a clinical syndrome arising during tuberculosis (TB)/human immunodeficiency virus (HIV) co-treatment. It manifests as worsening TB with inflammatory signs and symptoms (incidentally including biochemical and histologic features of both) despite improvement on anti-tubercular therapy (ATT) prior to antiretroviral (ARV) therapy initiation and no histologic evidence of productive infection.

1.2. TB/HIV-Coinfection and Co-treatment: Epidemiology

Of a global population of approximately 7.6 billion, over 10 million (0.001%) have active TB [1] (with ~10 million new infections in 2018 [2], down from 12 million in 2012 [3]), and over 36 million (0.005%) are HIV-infected [4] (with 2.1 million new infections in 2015 [5]). Furthermore - albeit based on tuberculin skin-test (TST) positivity – an extrapolated one third of the population is latently infected with *Mycobacterium tuberculosis* (Mtb), representing a massive reservoir of this pathogen [6].

After vascular disease, infectious disease is the second leading cause of death worldwide. Despite effective public health interventions, TB and HIV remain leading causes of morbidity and mortality. In fact, they are the top two leading infectious causes of death, respectively accounting for an annual 1.4 million [2] and ~1 million [7] deaths annually.

South Africa (SA) exhibits the highest TB incidence and HIV prevalence. The most recent reliable source for TB/HIV-coinfection prevalence [8] estimates that 41% of HIV-positive individuals also have TB. Of an estimated 36.7 million individuals living with HIV in 2015, 25% were estimated to have latent TB, and up to 1.17 million new incident TB cases occurred among individuals living with HIV in 2015 [9]. The region with the highest HIV prevalence among incident TB cases is SA (fig. 1), with Sub-Saharan Africa accounting for approximately 80% of HIV-associated TB [10].

Estimated HIV prevalence in new and relapse TB cases, 2018

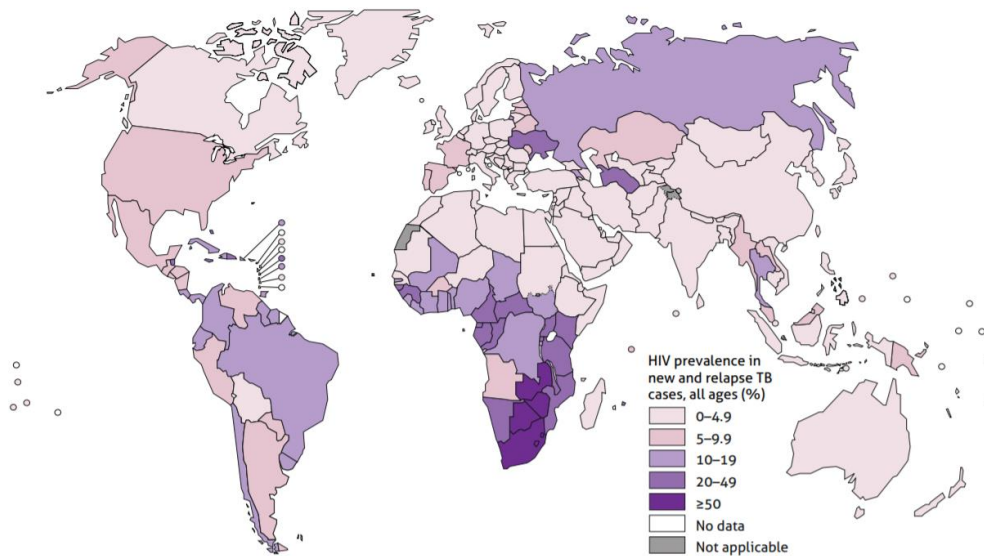


Figure 1 Estimated 2018 HIV prevalence among incident TB cases [2]

Host immune responses to bacteria (TB) versus to viruses (HIV) differ significantly in quality, including certain synergies and incompatibilities. Pathogen-host interactions during co-infection thus result in a relatively abnormal immune state. Each pathogen drives progression of the other, ultimately worsening host outcomes. For example, HIV (in part via decreasing CD4 count and disrupting granuloma architecture) increases Mtb incidence, re-activates latent Mtb, and increases severity of existing TB. It is the leading risk factor for developing active TB. “The risk of developing TB is estimated to be ... 16-37 times greater in people living with HIV” [1]: the lifetime risk of developing active TB in a latently-infected HIV-negative individual is ~10%, while the risk in HIV-positive individuals is ~10% per year [11]. Indeed, TB is the primary cause of mortality in individuals living with HIV, responsible for 25-33% of HIV-associated deaths [12] [13]. Similarly, Mtb infection (partly via leukocyte - and thus HIV replication - activation, e.g. via NFκB and/or AP-1 [11]) drives progression of HIV, including to acquired immune deficiency syndrome (AIDS). Clinically, too, co-infection complicates every aspect from presentation through treatment [14].

The SA first-line ATT regimen consists of a two-month intensive phase of combined rifampicin (rif), isoniazid (INH), pyrazinamide (PZN), and ethambutol (EMB) aimed at sputum smear conversion (to minimise transmission), followed by a four-month continuation phase of only rif and INH (aimed at sterilisation [15], though efficacy is controversial). Successful ATT reduces the Mtb load, in the process making available a wider variety of Mtb-derived molecules (eventually cleared from the system, although at differing rates between patients) [16] [17]. Six possible classes of antiretrovirals exist for HIV treatment: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, CCR5 co-receptor antagonists, and integrase inhibitors [18]. The SA first-line regimen (for non-pregnant, non-breastfeeding patients) during the study period comprised two NRTIs (tenofovir: TDF, emtricitabine: FTC) and an NNRT (efavirenz: EFV) [19]. This remains the current regimen of choice for TB-coinfected patients. Successful ARV therapy significantly suppresses viral load (VL) and reduces all-cause mortality in HIV-positive individuals [10] [18]. However, although ARV therapy significantly reduces the risk of TB (unsurprisingly by ~70-90% [20]), TB/HIV-cotreatment is complicated by various factors [10]: protracted course, poor tolerance due to increased rates of adverse drug reactions (cumulative toxicity), pharmacological considerations regarding drug-drug interactions, lower plasma drug concentrations, the potential for development of drug resistance due to inadequate adherence (and even despite adherence), and paradoxical TB-IRIS [21] [10] [14].

Previously, the diagnosis of TB during HIV infection was an indication to initiate ARV therapy [22]; but since 2016 SA public sector guidelines have made ARV therapy accessible to all individuals living with HIV: it is now preferred that ARV therapy be initiated within one week of diagnosis and counselling [23].

In co-infected patients, ATT is initiated first. To minimise the risk of shared drug toxicity and IRIS without increasing the risk of progression to AIDS or mortality, SA evidence-based guidelines [23] suggest that ARV initiation timing is best determined by CD4 count: if it is ≤ 50 cells/ μL , ARV should be started as soon as possible (after two weeks of tolerated and effective ATT), whereas if it is > 50 cells/ μL , ARV may be delayed (until ATT intensive phase completion, unless other serious HIV-related conditions are present). However, should TB meningitis (TBM) occur, ARV must be initiated four to eight weeks after TBM diagnosis [23]. A review of eight relevant randomised, controlled clinical trials (RCT) [24] evaluating the impact of early, late, or deferred ARV therapy initiation (1-4 or 8-12 weeks post-ATT initiation, versus post-ATT completion) on all-cause mortality and TB-IRIS confirmed only the early-start recommendation for those with low CD4 counts. Specifically, early ARV therapy initiation decreases mortality in those with a CD4 count < 50 cells/ μL (albeit correlated with a two-fold increase in TB-IRIS frequency). Early ARV therapy (~2 weeks post-ATT initiation) in these patients is thus associated with a significant increase in TB-IRIS risk [24].

Indeed, TB-IRIS is regularly diagnosed in regions where co-infection is common [25]. Given the high prevalence and incidence of TB and HIV in SA, increased access to screening and treatment for both (mandatory screening of all TB cases for HIV and vice versa) - especially in more developed provinces such as the Western Cape – and early initiation of ARV therapy in co-infected individuals with a low CD4 count (i.e. already at high risk for TB-IRIS), the absolute TB-IRIS incidence will continue to increase (at least transiently, until overall TB and HIV prevalence decreases), reflecting similar situations in other urban centres of Sub-Saharan Africa [26].

1.3. Evolving IRIS Terminology Has Hindered Study Comparability & Integration

Unexpected exacerbation of inflammation in response to therapy has been reported since at least the 1970s [27] [28] [29]. With reference specifically to TB, exacerbation of lymphadenopathy and intracranial lesions during ATT [27] [28], and – during HIV coinfection - ARV therapy-induced restoration of TST responses (with a tendency towards pyrexia in those with the most pronounced responses) [30] have been reported. The latter situation, deterioration in mycobacterial-coinfected ARV-naïve patients following ARV therapy initiation, was originally termed immune restoration syndrome (IRS), and became very common in TB/HIV-coinfected patients (incidence 29-36%) in the highly-active antiretroviral therapy (HAART) era [31], prior to optimising the interval between ATT and ARV therapy initiation.

Most broadly, the phenomenon (exacerbation of any infection in various therapeutic settings) was termed immune reconstitution disease (IRD). Under this umbrella, specifically in the context of any HIV-associated opportunistic infection (OI) or immune disorder (e.g. autoimmunity/neoplasia), the term immune reconstitution inflammatory syndrome (IRIS) describes clinical deterioration after ARV therapy initiation [32] (with mycobacterial OIs comprising a large proportion of these: 40% of cases described up to 2002; this may be due to persistence of mycobacteria and capsule products in host tissues for weeks even after initiating therapy [33]). Within OI-IRIS, two distinct clinical IRIS entities exist: unmasking (undiagnosed sub-clinical OI suddenly manifests) and paradoxical (sudden exacerbation of an OI previously responding to therapy).

Currently, however, the term IRIS is applied most broadly: to exacerbation of any infection or immune disorder arising in various different settings of recovery from relative immunosuppression [34]. Such settings include but are not limited to: correction of pathogen-induced immunosuppression by antimicrobial therapy, haematopoietic stem cell/bone marrow transplant in SCID mice [35], solid organ transplant [34], withdrawal of immunosuppressive medication (e.g. TNF blockade, $\alpha 4$ integrin blockade, corticosteroids, etc.) [36], in women during the post-partum period, and in rapid recovery from neutropaenia [37]. Although IRIS is thus described in HIV-uninfected individuals experiencing recovery from other immunosuppressed states, it is most well-studied in HIV-infected individuals after initiation of ARV therapy [38].

TB is the OI most frequently associated with OI-IRIS [38] (approximately 15.7% of cases are due to Mtb co-infection [25], varying by geographic location). This may reflect the continued persistence of mycobacteria and their products within host tissues for weeks after initiation of ATT [39]. Given the SA public health context (Section 2.2), this thesis focuses specifically on paradoxical exacerbation of partially-treated TB during ARV therapy: paradoxical TB-associated IRIS (TB-IRIS). The high frequency of

TB-IRIS relative to other forms of OI-IRIS likely simply reflects the fact that TB is the most prevalent OI among people living with HIV ("and the most common presenting illness in newly-diagnosed HIV patients") [9], combined with increasing access to ARV therapy in regions where TB/HIV-coinfection is a frequent occurrence [38].

Typically conceptualised as resulting from immune alterations, each OI-IRIS is believed to have a distinct molecular and cellular etiology. In the absence of conclusive proof, this hypothesis is based on observations that different leukocytes and soluble immune mediators are associated with each OI-IRIS. However, it is unlikely to be a coincidence that a similar clinical syndrome arises in association with so many different OIs, and interesting similarities exist (e.g. some pathogens – such as *Cryptococcus neoformans* and *Mtb* – can alter immunity in very similar ways, both able to induce Th2 and nTreg, and the time-frames for cryptococcal IRIS and TB-IRIS onset post anti-fungal therapy/ATT are similar [37], although the former occurs slightly later). Furthermore, given that immune responses are tailored to inciting pathogen class (e.g. neutrophilic-dominant responses to extracellular fungi, cytotoxic-dominant responses to intracellular viruses, etc.), observed immunological differences – such as CD8⁺ T-cell-dominated infiltrates in viral OI-IRIS versus Th1-type CD4⁺ T-cell-dominated granulomatous responses in fungal and mycobacterial OI-IRIS [25] – are unsurprising and do not preclude a common underlying mechanistic trigger (even if incorporating different molecules) for the OI-IRIS phenomenon. Finally, differential cytokine responses likely represent distal consequences rather than proximal triggers of IRIS, although a subset of these are also likely responsible for clinical IRIS manifestations (although this is not yet proven [40] [41] [42]). It is my contention that predisposing factors and the most proximal inciting molecular factor are likely common (or at least may be conceptualised as translatable) among different forms of paradoxical OI-IRIS. Although distinct OI-IRISes should be studied in isolation to provide the cleanest results, literature discussing these various forms may provide additional clues to TB-IRIS pathogenesis.

1.4. TB-IRIS Epidemiology, Presentation, Diagnosis, and Treatment

The incidence of TB-IRIS varies widely, from 0.6% [43] to 59% [44], with differential risk perhaps due to socioeconomic [25], geographic/ethnic, clinical, and study design contexts [38]. For example, known risk factors commonly differ between clinical studies, and pickup sensitivity may vary between centers.

When it does arise, TB-IRIS usually occurs *early*, on average 14 days after ARV therapy initiation [45] [46] [47] [48] (Appendix A). Studies reporting a shorter median time to IRIS onset (11 days to within 2 months; fig. 2, blue markers) represent populations originating from SA [49] [50] [51], Uganda [47] [48], Thailand [52] [53] (retrospective) [46], USA [54] [55] (retrospective), India [56] [57] (retrospective) [58] (with the exception of Kumarasamy et al. 2004, a retrospective study), and the UK [59] (retrospective). However, a subset of patients experience delayed (late-onset) TB-IRIS, presenting 98d after ARV therapy initiation [60]. Studies reporting a much longer median time to IRIS onset (fig. 2, red markers) represent populations originating from Brazil [61] (retrospective) [62] (the latter featuring a 6mo follow-up period), Serbia [63] (retrospective), Ethiopia [43] (retrospective), and Australia [64] (retrospective). However, rather than geographically-determined factors (e.g. patient genotype, *Mtb* lineage, HIV-1 subtype, national ARV regimen, vitamin D status, etc.), longer intervals likely reflect either the longer 'follow-up' period of four of the latter studies (or reporting of the mean instead of the median interval in two of these), although it is interesting that intervals are similar for both Brazilian studies (only one of which is retrospective). Intervals are not reported in an additional, smaller, Brazilian study [65]. Four additional small TB-IRIS case-reports also report longer ARV-IRIS intervals: 60d and 15d [66], 70d [67], 30d [68], and 60d and 35d [69]. The two most recent 12-week prospective studies (representing populations derived from SA, and from which samples for this study derive) report short median times from ARV therapy initiation to TB-IRIS onset of six and eight to ten days [44] [70]. The most recent retrospective study from India reports only that of 12 affected patients, 66.7% developed TB-IRIS within three months of ARV therapy initiation, while the remaining 33.3% developed TB-IRIS after this three-month period [71].



Figure 2 Time to IRIS onset by geographic location. Blue markers indicate shorter intervals. Red markers indicate longer intervals.

Clinical presentations of TB-IRIS are diverse, potentially including constitutional symptoms (e.g. fever, weight-loss, night sweats), inflammatory lymphadenopathy, and worsening of organ-specific (e.g. respiratory) TB symptoms previously responding to ATT (including incidental worsening pulmonary radiographic features) [12]. Fever $\pm$ rigors (shivering) and lymphadenopathy are the most common manifestations [58] [72], but symptoms vary in severity, and - like TB itself - can affect almost any organ system (including meningitis/space-occupying lesions, chest pain/cough, abdominal pain and diarrhea, hepatosplenomegaly, epididymo-orchitis, etc.) [14]. Thus, the more frequent TB-IRIS symptoms typically localise to original sites of TB presentation (e.g. lymph nodes, pulmonary), but systemic inflammatory features are common, and multi-system involvement (including previously apparently- unaffected anatomical sites) is not infrequent [73] [38].

TB-IRIS is a diagnosis of exclusion requiring experience and skill, as presentation is non-specific and no specific and reliable laboratory markers exist [74]. Differentials include: non-compliance or treatment failure (including drug-resistant Mtb), drug toxicity/adverse drug reaction, or new infection/neoplasm/autoimmune reaction [21] [75]. As such, introduction of the INSHI consensus case definition [21] (Chapter 2, Appendix C) has helped standardise both diagnosis and research. Exclusion of radiological and laboratory parameters from this definition (without compromising case pickup) also makes it appropriate for use in low-resource settings.

TB-IRIS increases morbidity, decreasing the ability to engage in various domains of functioning and thus also quality of life (QoL) [52] [76], and increases the burden on healthcare services [49]. The course can be prolonged (> 90 d in 40% of patients), with lymph node involvement during TB-IRIS independently increasing the risk of prolongation [77]. One study reports a median time to resolution of 2.53 months [59], although the interval can be much shorter or longer. The hospitalisation rate is 25%, with 17% requiring procedures (e.g. surgical) [38]. Although relatively uncommon, life threatening presentations of TB-IRIS include acute respiratory distress syndrome and acute renal failure. The most severe TB-IRIS sub-types are respiratory and neurological. Central nervous system (CNS) TB-IRIS occurs in 13% of cases [78]. The relatively low CNS TB-IRIS rate may reflect underlying rates of CNS TB, and/or the relatively antigen-/immune-privileged nature of the CNS. General TB-IRIS/IRD-attributable mortality rate is on average 2-3.2% [25] [38] [74] (corresponding to an upper-bound estimate of approximately 128 000 of all patients currently co-infected with HIV and TB), although CNS TB-IRIS mortality rate is 13-75 % (with a long-term disability rate of 25%) [79]. Overall, morbidity and mortality are amplified in resource-poor settings where diagnostic and management options are more limited [76]. Furthermore, a prospective SA study observed that ATT resistance rates are higher (relative to background MDR prevalence) among patients presenting with suspected TB-IRIS [45].

Across IRIS settings, attempted immunomodulatory management strategies have included corticosteroids, NSAIDs (e.g. aspirin, meloxicam), anti-TNF monoclonal immunoglobulins and other intravenous immunoglobulins, leukotriene inhibitor montelukast, statins (to discourage Th1/Th17-type inflammation), vasoactive pentoxifylline, CCR5 receptor antagonist maraviroc, hydroxychloroquine, and even thalidomide [80] [14]. However, efficacy of many of these remains to be formally assessed,

although the CADARIS trial proved that maraviroc does not prevent IRIS, nor impact its timing/severity [81], likely because other chemokine receptors (e.g. CCR2/3) sensitive to overlapping stimuli compensate for loss of CCR5 function.

Regarding TB-IRIS, specifically, delaying ARV therapy initiation can decrease TB-IRIS risk, but – as discussed above – the latter must be weighed against the risk of increased HIV-related all-cause mortality. A 2013 prospective study [82] noted that baseline steroids nonsignificantly decrease TB-IRIS risk. A 2018 SA RCT [70] determined that co-initiation of prednisone (40mg/day for two weeks days, then 20mg/day for two weeks) with ARV therapy decreases the risk of TB-IRIS by 30%, without increasing the incidence of severe infections or adverse events. Management of existing TB-IRIS depends on presentation and severity, but constitutes continuation of ATT to decrease the antigenic load, use of anti-inflammatory agents, and, ideally, continuation of ARV therapy [76]. Interruption of ARV therapy is only infrequently considered, in potentially-fatal situations not responding to other treatment options (e.g. life-threatening CNS TB-IRIS) [38]. A 2010 SA RCT [83] found that prednisone (1.5mg/kg for two weeks, then 0.75mg/kg for two weeks) reduces morbidity and shortens the course of confirmed paradoxical TB-IRIS, while also decreasing hospitalisation rate [80]. However, steroid administration can alter histopathological features of other emerging disorders (e.g. lymphoma), complicating diagnosis [84]. Furthermore, until specific TB-IRIS differential diagnoses (e.g. new infection) are ruled out, corticosteroids must be dispensed cautiously [45].

The anti-inflammatory effect of NSAIDs may be of benefit in TB-IRIS: they “may be as effective as corticosteroids for treatment of non-life-threatening TB-IRIS” [83] [85]. Although an SA trial (NCT02060006) evaluating the efficacy of meloxicam for TB-IRIS treatment was planned for 2014 – with a projected 2015 completion date – no status update has been posted, nor have findings been published. It has also been suggested that the MMP inhibitor doxycycline may be able to prevent IRIS or associated tissue damage [44] [74].

Generally speaking, corticosteroids are broadly and potently anti-inflammatory (e.g. favouring tolerogenic dendritic cell (DC) and regulatory T-cell (T_{reg}), but opposing inflammatory T-cell, activity [86]). They are thus commonly employed in inflammatory conditions (e.g. infection, autoimmunity), including respiratory disorders “such as asthma, bronchiolitis, cystic fibrosis and TB” (“significantly reduc[ing] mortality in all forms of TB” [87]. However, corticosteroids are less effective in certain inflammatory conditions (e.g. severe neutrophilic asthma, asthmatic smokers, and exacerbations of chronic obstructive pulmonary disease) [88]. The function of the glucocorticoid receptor (GR) is decreased in the presence of “pro-inflammatory cytokines, growth factors, viruses, double-stranded RNA, bacterial products, and oxidative stress”, with PI3K and MAPK pathways (the latter regulated by DUSP1, which contributes to corticosteroid-mediated “repression of inflammatory gene expression”) implicated in corticosteroid resistance [88].

Mechanisms of corticosteroids' beneficial effects (e.g. improved chest radiography) during TB-IRIS are incompletely understood, but apparently – instead of inhibiting the Mtb-specific T-cell response – they rapidly and significantly decrease levels of soluble PRR/cytokines/chemokines elevated during TB-IRIS (CRP, IL-6, IL-10, IL12p40, TNF α , IFN γ , and IFN γ -inducible IP-10), and thus presumably the effector activities of these molecules; the two cytokines most consistently elevated during TB-IRIS are IL-6 and TNF α [83] [89] [40] [85] [87]. Although the maximal benefit of prednisone occurs within the initial two to four weeks of treatment, some individuals require ongoing treatment: once steroids are withdrawn, a subset of patients (~20%) experience symptom relapse [90]. Thus, premature steroid withdrawal may result in symptom recrudescence [58] [72], indicating that steroids may not necessarily resolve the underlying mechanism (proximal cause) of TB-IRIS, despite masking symptoms (the ability of steroids to help prevent TB-IRIS doesn't necessarily suggest otherwise, since they may not suppress proximal cause, but only clinical manifestation-inducing effectors). This effect may be dependent on when during the TB-IRIS pathogenic cascade steroids are introduced. Moreover, as indicated above, steroids fail to prevent TB-IRIS in 70% of cases.

Endogenous corticosteroids exhibit three major anti-inflammatory mechanisms: glucocorticoid-responsive element (GRE)-mediated impact on gene expression (delayed), transcription factor (e.g. NF κ B) interaction-mediated impact on gene expression (delayed), and alteration of signal transduction (rapid) [87]. Once corticosteroids bind the GR, it is liberated from Hsp90 and translocates to the nucleus to recruit transcriptional coactivators/repressors to GRE, inducing “transcription of anti-inflammatory genes such as annexin-1, IL-10, and I κ B α (inhibitor of NF κ B)” and repressing “transcription of genes

associated with hypothalamic pituitary-adrenal (HPA) axis function and inflammation" [87]. At lower steroid concentrations, the corticosteroid-GR complex - in co-operation with coactivators such as CREB-binding protein - directly antagonises immunomodulatory transcription factors (e.g. NFκB, AP-1), opposing transcription of "cytokines, chemokines, adhesion molecules, and [production of] eicosanoids" [87] [88]. Via GR, corticosteroids have various effects on leukocytes: suppressing DC maturation/reprogramming DC toward a tolerogenic phenotype inducing CD4⁺ and CD8⁺ T_{reg}; reprogramming monocytes/macrophages toward a pro-phagocytic, pro-efferocytic, anti-inflammatory phenotype (via inhibiting caspase function), and - via induction of IL-10-producing T_{reg} - favouring Th2- over Th1-type immunity [91] [37] [87]. Finally, corticosteroids passively diffuse into the cell or are taken up by receptor-mediated endocytosis for intracellular ligation of G protein-coupled receptors (GPCR), inhibiting (for example): pancreatic insulin release (at higher doses and longer exposures) [92], endothelial NO production, and adipocyte histone deacetylase complex assembly [87]. Corticosteroids thus have multiple general effects on soluble immune mediators (Table 1), although these effects are moderated by disease context and route of administration.

Table 1 General Effects of Corticosteroids on Selected Soluble Mediators

Suppressed	Enhanced	References
IL-1, -3, -5, -6, -8, -12, -16, -18	IL-1Ra, IL-4*, -13*	[93] [88] [94]
IFNγ, TNFα	IL-10 (indirect), TGFβ*	[93] [95] [88] [94]
GM-CSF, MCP, MIG, MIP, eotaxin, IP-10	CCL20 (neutrophil-recruiting)	[93] [96] [97]
cPLA ₂ , inflammatory eicosanoids 15-PGDH	ANXA1 (PLA ₂ inhibitor), pro-resolving lipid mediators, ALOX5, LXA4 receptor (ALX)	[93] [98]

*Studies report opposing findings. Corticosteroids also enhance expression of the β-adrenergic receptor, while inhibiting expression of adhesion molecules (E-selectin, ICAM, and VCAM) [93].

Thus, corticosteroids are not only anti-inflammatory, but also pro-resolving. They "spare, and even augment, innate immune responses" [88]. In addition, their effects are partly dependent on contextual factors (e.g. dose, receptor expression level). While clinical doses of corticosteroids have immunosuppressive and anti-inflammatory effects [86], "low, physiological-range levels enhance macrophage phagocytic and efferocytotic capability" [91], thereby enhancing clearance of apoptotic cells and pathogen-derived molecules. Further, corticosteroids are directly pro-apoptotic for mature DC, macrophages, NK cells, T-cells, and B-cells, but have an anti-apoptotic effect on neutrophils [99]. Finally, corticosteroids also interact with ATT, for example decreasing INH concentration, its own more rapid metabolism by Rif-induced CYP450s (e.g. CYP3A4, which decreases corticosteroid concentrations by ~50%), and favouring increased pleural but not serum PZN concentration ("in the absence of Rif") [87].

Given morbidity, difficulty in diagnosis, complicated management, a potentially protracted course, recrudescence in a subset of steroid-managed patients, and mortality, there is a need to elucidate immunopathogenic molecular mechanisms underlying TB-IRIS at the molecular and cellular levels [39] [73] [38] [100]. This will contribute to identifying putative risk stratification and prophylactic strategies, specific and reliable biomarkers (for risk stratification, diagnosis, prognosis, and treatment monitoring), and more specific and effective therapeutic strategies (e.g. identifying novel treatment targets or repurposing existing compounds/biologics for earlier interruption of the pathogenic cascade, perhaps including taking a more host-directed immunomodulatory approach).

1.5. Current Understanding of TB-IRIS Pathogenesis

Because TB-IRIS occurs in response to ARV initiation, it is conceptualised as dysregulated immune recovery causing an overzealous inflammatory response to residual Mtb antigens. At least for ARV-induced IRD, it was originally believed that the unmasking form was due to viable pathogen, while the paradoxical form was due to residual antigens [25].

However, despite two decades of original research (including case studies, over 40 clinical studies, at least 50 ex vivo immune studies) and a number of excellent reviews (including three systematic reviews and meta-analyses (SRMAs)), the underlying immunopathogenic mechanisms of paradoxical TB-IRIS remain incompletely understood. This is partly attributable to poor consistency between studies (e.g. whether all IRD or only unmasking/paradoxical TB-IRIS subsets are reported, and different case

definitions, ARV regimens, follow-up interval, clinical/biochemical/immunological parameters reported, and statistics analyses). Remaining questions include the reason underlying differential susceptibility to paradoxical TB-IRIS, the most proximal post-ARV immunological inciting factor(s), ensuing pathogenic biological pathway cascades resulting in clinical manifestation, and which biological phenomena underlie which clinical manifestations.

To examine whether new mechanistic hypotheses can be inferred from first principles, as well as to inform interpretation of current study findings (demographic, clinical, and biochemical/immunological), we first perform a fresh and detailed review of known TB-IRIS-relevant clinical and immune parameters. Regarding the latter, we focus heavily on *in vivo* rather than *ex vivo* ($\pm$ restimulation) findings, which are more difficult to interpret, as well as preferring convergent findings from multiple studies (rather than isolated findings from single studies).

1.5.1. Integrating TB-IRIS Epidemiological and Clinical Clues

The first prospective cohort study was conducted in the late 1990s [54]. Although many early clinical studies focused on US and European populations, studies are now more common in TB-endemic areas, including Africa; the first African cohort study became available in 2007 [101]. Many international case reports and > 50 clinical cohort studies of varying quality report inconsistent findings. For consensus, we turn to the single systematic review [39] and three SRMAs published to date [73] [25].

Lawn et al. (2005) reviewed 166 published cases of mycobacterial-associated IRD [39], detecting latent data patterns and drawing insightful and clear conclusions. The first SRMA, by Leone et al. (2010), collated data from 51 clinical studies (including individual case studies and case-series) of ARV- and TB-associated IRIS, but did not adhere to Cochrane recommendations, and employed relatively rudimentary statistical analyses [73]. The second SRMA, by Muller et al. (2010), collated data from 54 clinical studies (longitudinal cohort studies/abstracts/letters to the editor featuring at least 10 adult patients) of IRD [25]. Although 16 of these studies focused on paradoxical TB-IRIS, it has been suggested that the authors may have misinterpreted the findings of some studies, and that not differentiating between IRIS sub-types may have skewed statistical results (e.g. producing extreme outliers) [102]. These SRMAs were complicated by inconsistent target study populations (e.g. differential timing of ATT and ARV therapy relative to each other, including some participants initiating ARV prior to ATT) and methodologies (reporting and analysing distinct aspects), and did not adequately distinguish between IRIS sub-types.

The third SRMA, by Namale et al. (2015), is the only one to date focusing specifically on TB-IRIS [38], and is well designed and of a high quality. The authors collated data from 40 clinical cohort studies (the maximal number of identifiable studies that met inclusion criteria, over the entire history of TB-IRIS publications, including both prospective and retrospective studies featuring at least five adult patients); they also include RCTs (SAPiT, TIME, CAMELIA, and STRIDE) investigating optimal timing of ARV therapy in relation to ATT, but which report on TB-IRIS as an endpoint. A fifth included RCT (CARINEMO) investigated the comparative safety and efficacy during rifampicin-based ATT of EFV- versus NVP-based ARV regimens. All three SRMAs were complicated by differential case definitions used in target studies, and even Namale et al. (2015) specify that "included studies often did not report sufficient detail regarding selection and reporting of outcomes to enable meaningful assessment of relevant biases" (often necessitating descriptive rather than quantitative comparisons). Nonetheless, to date these reviews provide the most reliable estimates of TB-IRIS epidemiological/clinical characteristics (Table 2) and risk factors (Table 3).

Table 2 TB-IRIS Consensus Epidemiological and Clinical Characteristics

	Lawn 2005	Leone 2010	Muller 2010	Namale 2015	Proposed Consensus	Mechanistic Inference(s)
study focus	mycobacterial-associated IRD	TB-associated IRIS	ARV-induced OI-associated IRD	paradoxical TB-IRIS		
incidence (%)	NR	11 – 45	<10 - >50 TB-IRD: 15.7	4 – 54 (pooled: 18% [100])	<10 - >50 [87]	wide range perhaps due to differential host, pathogen, or therapeutic factors (e.g.

			(highly variable)			host/pathogen genotype, S.E.S., lifestyle, timing of infections relative to each other, differential risk factors, therapeutic regimen and timing, and pickup sensitivity)
ARV-IRIS interval (weeks)	median 4 (majority within 3mos)	average 4 (some delayed)	NR	within 4	within 4 (average ~2)	despite a relatively slow onset, this (and lymphocyte expansion within SLO to produce lymphadenopathy and hepatosplenomegaly) is consistent with the Mtb recall response, potentially modulated by depletion of the Mtb-specific TCR repertoire and/or ATT-induced availability of <i>different</i> Mtb antigens
commonest manifestations	Mtb-associated IRD: fever lymphadenopathy respiratory	fever lymphadenopathy respiratory	NR	lymphadenopathy respiratory	fever lymphadenopathy respiratory	fever: hypothalamic action of endogenous pyrogens (IL-1, PGE2, TNFa, MIP-1), suggesting innate leukocyte activation (including inflammasome activity) lymphadenopathy: lymphocyte proliferation stimulated by antigens passing through/depositing or infection; hepatosplenomegaly in certain patients: may indicate known deposition of circulating free/immune-complexed Mtb antigens in these organs during attempted clearance respiratory: airway inflammation; radiography and histology suggests this involves: leukocyte infiltration, matrix destruction, and fibrosis symptoms preferentially affecting previously TB-affected organs suggests a response to residual Mtb antigens (e.g. tissue-sequestered, including within granulomas)
management	corticosteroids	NSAIDs corticosteroids surgery	NR	corticosteroids NSAIDs	NA	corticosteroids likely alleviate symptoms, via effects on inflammatory and metabolic effectors; TB-IRIS-prone patients may exhibit pre-existing relative steroid-resistance; symptom return on early withdrawal suggests amelioration of distal processes rather than proximal triggering mechanisms (or perhaps even new steroid-withdrawal IRIS)
duration (days)	?	NR	NR	19 – 87	19 – 87 (up to 2.53mos) [59]	acute inflammation aims to clear inciting stimulus/resultant debris (minutes to days); prolongation indicates persistence of inciting stimulus/antigens, or abnormal transition to chronicity (e.g. failure of resolving mechanisms, resulting in attempts at resolution/healing against a background of ongoing inflammation)

Namale et al. [2015] observes that 62% of reviewed studies report an incidence between 11 and 30%, but experienced local clinicians may agree on an estimated incidence of 30-50% in Western Cape study populations. Cevaai et al. (2019) report a pooled incidence of 18%, but specifies that this may be higher in high-risk cohorts [100]. IRIS is also associated with non-clinical observations: intrathoracic/abdominal lymphadenopathy, worsening pulmonary infiltrates on chest radiography [73] and “unexpectedly florid cell-mediated immune responses” on histologic analysis of tissue samples [39]. ? unclear comparability. NR not reported.

In an independent review of the literature, we find incidence ranging from 0.6-59% (average $29 \pm 16\%$ across prospective and $20 \pm 13\%$ across retrospective studies), with a median ARV to TB-IRIS interval of approximately 14 days across prospective studies and 22 days across retrospective studies (Appendix A). We also find general agreement that the commonest TB-IRIS presentations include constitutional symptoms (most often fever) and lymphadenopathy, with or without worsening respiratory symptoms

(and radiographic worsening, although this is non-specific for TB-IRIS) (Appendix A). An additional biochemical characteristic of TB-IRIS manifestation is elevated CRP (also non-specific for TB-IRIS) [103] [104] [4] [105], including in a study incorporating unmasking TB-IRIS [106].

The most consistent clinical characteristics of TB-IRIS reported by available SRMAs and confirmed by an independent literature review therefore appear to be: heterogeneous incidence across study populations, ARV-TB-IRIS interval of approximately 14 days, presentation including fever and lymphadenopathy, and likely elevated CRP. It is notable that lymphadenopathy may be cervical/axillary or intrathoracic/-abdominal (and thus not necessarily clinically-apparent even when present), suggesting the possibility that all TB-IRIS actually does exhibit lymphadenopathy. Additionally, similar changes in chest radiography and laboratory investigations occur in both mild and severe TB-IRIS, and occasionally there is radiographic worsening in the absence of overt symptoms [72]. Although TB-IRIS is defined as a clinical syndrome, and only clinically-apparent TB-IRIS impacts morbidity and mortality, clinically-unapparent molecular and cellular changes underlying TB-IRIS may occur in many more TB/HIV-coinfected patients initiating ARV therapy (only manifesting clinically when sufficiently pronounced).

Table 3 TB-IRIS Clinical and Biochemical Risk Factors

	Lawn 2005	Leone 2010	Muller 2010	Namale 2015	Likely Risk Factor?	Hypothesised Reason
baseline extrapulmonary TB (EPTB) or disseminated TB	?	NR	NR	uni- and multi-variate positive association with incidence	Y	more advanced TB (likely due to more advanced HIV); higher bacillary burden (antigen load); since Mtb preferentially disseminates during primary infection rather than reactivation, this may also suggest HIV infection preceding Mtb infection
median baseline CD4 count (cells/μL)	typically <50	55	< 50 (exponential increase in incidence as CD4 count declines)	< 50 (uni- and multi-variate negative association with incidence, incl. RCTs)	Y (CD4 < 50)	more advanced HIV: more pronounced/permanent alteration in immune state (e.g. more pronounced type I IFN response, decreased TCR repertoire diversity, abnormal Th polarisation, depletion of leukocytes required to prevent/resolve inflammation, etc.)/ poorer Mtb control higher antigen load
median baseline VL (copies/ml)	?	5.6log10	NR	higher VL increases risk	Y (higher VL)	
median ATT-ARV interval (days)	?	87 (*no significant negative linear association with incidence)	NR	shorter (uni- and multivariate negative association with incidence, incl. RCTs)	Y (shorter interval)	insufficient time to clear higher Mtb antigen load and/or recover immune homeostasis after ATT-induced lifting of Mtb-mediated immune modulation
CD4 response to ARVs	preceding immunological recovery	*no significant positive linear association with incidence	NR	more pronounced recovery (uni- and multivariate positive association with incidence, incl. RCT)	Y (more pronounced recovery)	rather than only 'recovery', likely also represents new inflammation and activated (preferential CD4 ⁺ rather than CD8 ⁺) T-cell mobilisation (effect rather than cause)
VL response to ARVs	preceding virological suppression	*no significant negative linear association with incidence	NR	associated with earlier and more pronounced virological suppression	Y (more pronounced suppression)	more rapid lifting of HIV-mediated immunosuppression and antiviral (e.g. type I IFN) immune responses (supported by CD4:CD8 ratio increase)

*Inappropriate statistical analyses employed by this study [73] may account for these outcomes. In addition, conclusions are drawn in the absence of aggregate statistics regarding EPTB, though other studies identify EPTB as an independent risk factor for IRIS (e.g. via multivariate analysis [107]). Additional evidence of IRIS-preceding immune "restoration" includes more pronounced restoration of cutaneous delayed-type hypersensitivity (TST responses) and increased *in vitro* T-cell proliferation (*vide infra*, next section), both in response to mycobacterial antigens (PPD, MAC) [30] [54] [108]. Even at three months post-IRIS, elevated TST responses persist [109]. Note that a minority of studies find no association between ATT-ARV interval, CD4 recovery, and VL suppression and risk of TB-IRIS [110] [38]. Although three RCTs (SAPIT, CAMELIA, and STRIDE) demonstrate that lower baseline CD4 and higher VL are independent risk factors, some of these encompassed randomisation of participants with significantly lower CD4 counts to the shorter-interval arm (i.e. a significant interaction existed between interval and CD4 count) [38]. However, SAPIT, at least, demonstrates that a shorter ATT-ARV interval also produces more severe IRIS of longer duration.

The most comprehensive review to date (employing formal statistical analysis of aggregated data) [38] concludes that the main TB-IRIS risk factors are indeed baseline EPTB/disseminated TB, lower baseline CD4 count, higher baseline VL, and shorter ATT-ARV therapy initiation interval. Our independent literature review (Appendix A) finds that the most frequently-reported risk factors are more pronounced

virological/immunological response to ARV therapy (in 86% of seven prospective and 80% of five retrospective studies reporting this parameter), baseline EPTB/disseminated TB (in 67% of six prospective and 50% of six retrospective studies reporting this parameter), shorter ATT-ARV interval (in 43% of seven prospective and 60% of five retrospective studies reporting this parameter), and lower baseline CD4 count (in 20% of ten prospective and 43% of seven retrospective studies reporting this parameter). Some evidence also exists for higher baseline VL (in 25% of eight prospective but 0% of four retrospective studies reporting this parameter). Four studies (one of which includes unmasking and paradoxical TB-IRIS cases) report low baseline haemoglobin (Hb) as a risk factor (Appendix A). Four studies report high baseline CRP (Appendix A). Three studies report baseline urine LAM positivity as a risk factor [10] [47] [44]. Four studies report low baseline BMI as a risk factor, but another reports increased baseline BMI as a risk factor (Appendix A). Two studies report an association with ethnicity: black [111] and non-Hispanic [112]. Additional potential risk factors reported by single studies: gender (female [113] and male [114], however) and elevated baseline heart rate and respiratory rate (Appendix X). Findings regarding age are also in opposition, with one study suggesting age > 35 is a risk factor [115], but another study suggesting younger age as a risk factor.

Again, baseline LAM positivity argues in favour of a higher Mtb antigen load (as well as possibly slower antigen clearance rates in TB-IRIS-prone individuals, and – in combination with MMP findings (*vide infra*) – perhaps matrix turnover-mediated liberation of previously-inaccessible Mtb antigens). Indeed, single studies have suggested that smear-positive TB [113] and higher sputum culture grade [72] are risk factors for TB-IRIS. Low baseline BMI, too, suggests more advanced TB/HIV-coinfection (and thus more pronounced immunological alteration and higher Mtb antigen load), and anemia likely represents anemia of chronic disorders due to iron sequestration (again arguing in favour of longer-duration/more advanced TB/HIV-coinfection). Splenic activation by (especially chronic) granulomatous inflammation can also result in hypersplenism, which may contribute to such anemia [116], particularly in IRIS patients. Both iron sequestration and hypersplenic anemia result in higher iron loads within leukocytes (monocytes, macrophages, and neutrophils) or the reticuloendothelial system, which impacts their function.

One study suggested boosted protease inhibitor (PI) use is associated with TB-IRIS risk [107] (perhaps because PIs enhance macrophage IL-6 and TNF α production [117]). However, despite this suggestion of PI involvement, a multi-centre retrospective study of three types of OI-IRIS (including TB-IRIS) reports that different ARV regimens do not alter risk [114], and one study reports that NVP-containing regimens do not increase risk [118]. There are also suggestions of a more pronounced response to ATT prior to ARV initiation [119], which – similarly to many of the other risk factors – likely indicates more advanced baseline TB, a higher Mtb antigen load, and rapid lifting of Mtb-induced immunosuppression (i.e. alteration of the immunological state). Although speculative, it is also not currently possible to exclude the possibility that such patients achieve higher circulating or intracellular concentrations of ATT or ARV drugs (this has not been formally assessed). A single study [72] reports a non-significant trend towards higher pre-IRIS aspartate aminotransferase (AST).

The most consistent TB-IRIS risk factors thus appear to be EPTB/disseminated TB, low CD4 count (<50 cells/mm³), higher VL, shorter ATT-ARV therapy initiation interval, more pronounced immunological/virological response to ARV therapy, low Hb, urine LAM positivity, and possibly low BMI. With the exception of urine LAM positivity and low BMI, this is generally in agreement with previous clinical reviews of TB-IRIS [107] [120] [121] [80] [122] [14] [74], as well as with Namale et al. (2015)'s excellent systematic review and meta-analysis. All suggest that more advanced TB/HIV-coinfection and higher antigen loads are what predispose only certain patients to TB-IRIS (discussed below).

1.5.1.1. Higher Baseline Mtb Antigen Load & Possibly Delayed Clearance

Multiple epidemiological, clinical, and biochemical/immunological features of TB-IRIS thus suggest more advanced (and reciprocally co-exacerbating) HIV and TB disease at baseline, resulting in a higher Mtb antigen load. Indeed, as early as 2008, Manabe et al.'s rabbit model first suggested IRIS dependence on antigen load at the time of immune reconstitution [123]. Based on disseminated TB, lower CD4 count, and shorter ATT-ARV interval being TB-IRIS risk factors, Namale et al. (2015) also conclude that a higher Mtb "antigen load ... appears to be an important driver of ... inflammation".

The most abundant Mtb cell wall component is the glycolipid lipoarabinomannan (LAM) [124] [125], and as an Mtb antigen is likely a proxy for general Mtb bacillus/antigen load. It is shed not only from infected alveolar macrophages and metabolically active mycobacteria, but also from mycobacterial fragments [125]. It is immunosuppressive, favouring Mtb survival (including via scavenging reactive oxygen species (ROS), inhibiting PKC, modulating macrophage cytokine production, inhibiting transcription of IFN γ -induced genes, and inhibiting T-cell proliferation), but simultaneously immunogenic, eliciting antibodies (some of which may be protective) during natural Mtb infection [124]. Regarding LAM dynamics in mice, intravenously-administered LAM deposits mainly in splenic marginal zone (but also hepatic) macrophages, although prior administration of anti-LAM IgM leads to rapid circulating LAM clearance via mainly hepatic (but also splenic) deposition [124]. Ultimately, LAM is cleared from circulation via both hepatobiliary and renal routes [124] [125].

Urine LAM positivity is associated with sputum smear and culture positivity, HIV infection and low CD4 count (even though the latter two themselves correlate *inversely* with sputum smear positivity), and mycobacteriuria (but not Mtb strain, proteinuria, or treatment outcome) [126]. As mentioned above, a single study [113] identifies baseline smear-positivity as a TB-IRIS risk factor, perhaps more reflective of a higher Mtb antigen load than of HIV-mediated immune suppression (though the two are related): innate immune-mediated liquefaction, cavitation, and tissue erosion are required for Mtb airway access [127], and smear-positivity is less (although miliary TB is more) common during advanced HIV.

Generally speaking, degree of urine LAM positivity remains unchanged during the initial two weeks of ATT, but then levels steadily decline (at least over a 24-week study period) [126]. In a more recent study monitoring urine LAM throughout ATT (and in which degree of LAM positivity - from low to high - was graded from 1 to 5), "the percentage of LAM-positive participants decreased significantly from baseline (32%) to two months (16%) and ... 6-months (10%) [128]. Multivariate analyses demonstrate that LAM positivity and grade preferentially decreased among sputum culture-positive patients but did not change among sputum culture-negative patients [128], suggesting that HIV infection or non-pulmonary TB are associated with *prolonged/delayed antigen clearance*. Patients with LAM grade remaining at ≥ 2 after two months' ATT exhibited "a significantly greater risk of mortality" (5.6-fold higher) [128]. As mentioned, there is a tendency towards urine LAM positivity in those going on to develop TB-IRIS: in an early study, all TB/HIV-coinfected patients ($n = 5$) who went on to develop TB-IRD exhibited baseline urine LAM positivity [129], pre-ARV therapy urinary LAM presence is associated with TB-IRIS risk (sensitivity 80.8%; specificity only 52.4%) [47], and anti-LAM IgG levels are higher during ARV therapy in TB-IRD developers [130].

Urine LAM positivity at the time of IRIS is also associated with higher circulating MMP levels [44], which may suggest increased liberation of previously-inaccessible Mtb antigens from the extracellular matrix or from granulomas. On initiation of ATT, patients with a higher initial Mtb burden will likely require more extended periods to achieve antigen clearance. Furthermore, significant inter-individual differences in post-ATT initiation Mtb antigen clearance rates are known to exist, even in HIV-negative patients (fig. 3). Such differences may contribute towards differential susceptibility to TB-IRIS.

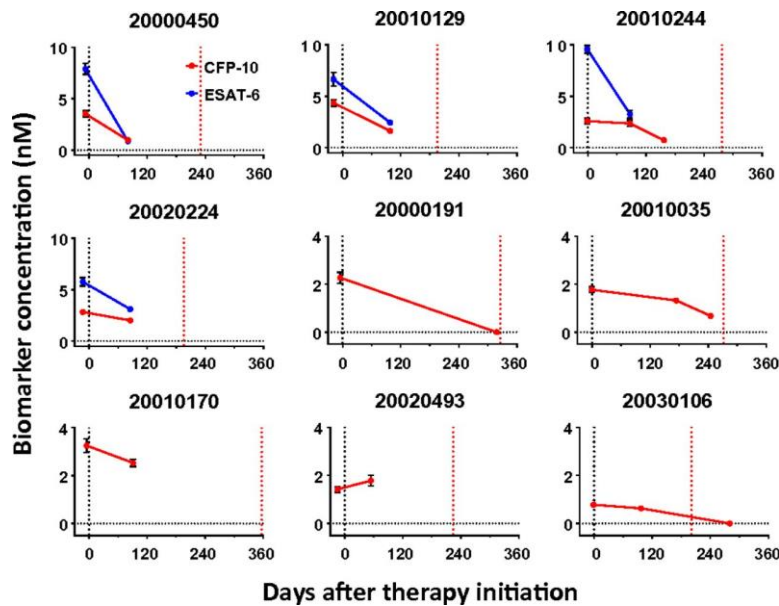


Figure 3 Differential Inter-Individual Circulating Mtb Antigen (CFP10, ESAT-6) Clearance Rates During ATT [131]

1.5.1.2. Not Just Antigens: Hypothetical Immunological Consequences of Treating More Advanced Disease

However, a higher Mtb antigen load may not be the only factor predisposing to TB-IRIS. Initiation of a primary inflammatory response requires a certain minimum set of stimuli, including but not limited to *productive infection and/or host tissue injury*, i.e. a *rapid increase* in detectable and sufficiently-immunogenic pathogen-derived molecules featuring pathogen-associated molecular patterns (PAMPs) and antigens, usually accompanied by host cell stress and host-derived molecules - featuring homeostasis-associated and danger-associated molecular patterns (DAMPs) - and alarmins. Immunogenicity of these molecules may or may not require complexing with specific host factors, and (at least for antigens) requires the presence of cognate lymphocyte specificities (i.e. not depleted during HIV infection). Barriers to initiation of secondary response are much lower, however.

As mentioned, those who go on to develop TB-IRIS exhibit various features suggestive of more advanced TB/HIV-coinfection at baseline. Apart from leading to higher overall Mtb antigen loads within previous sites of disease (e.g. granuloma-resident leukocytes or cores), another consequence is more profound and/or irreversible immune dysregulation. This includes certain common immune embargoes independently instituted by both pathogens, and immature granulomas which are less prone to liquefaction/cavitation [132] [44] (i.e. which sequester rather than releasing antigens). For example, *HIV inhibits TLR/IL1R-MyD88-IRAK4 signalling and (downstream of TLR-4 activation) ERK1/2 phosphorylation and signalling, impairing production of cytokines and chemokines such as TNF α and MCP-1* [133] [134] [135]. Therefore, more advanced disease states present greater potential for lifting of HIV- and Mtb-imposed embargoes (and therefore significant immunological change) in response to pharmacological therapy. Such responses could also conceivably be due to achievement of higher circulating/intracellular ATT and ARV drug - or drug metabolite - concentrations in IRIS-prone patients.

In response to ATT, patients with more (relative to less) advanced TB actually experience an opposite initial response in levels of some cytokines, for example an increase - instead of decrease - in TNF α , IFN γ , and IL-5 [136]. On subsequent initiation of ARV, dual lifting of shared immune embargoes may include a decrease in HIV-stimulated early autophagy and type I IFN production, both of which typically oppose inflammasome activation and IL-1 β -mediated inflammation (while autophagy - specifically mitophagy - also increases cellular robustness to mitochondrial stress-induced cell death). It is plausible that ARV drugs (known to be mitotoxic) - at the site of granulomatous disease - may induce or exacerbate myeloid cell stress (e.g. mitochondrial stress, as indicated by elevated circulating baseline mtDNA levels in patients who go on to develop TB-IRIS [137]) and possibly co-ordinated myeloid cell death (perhaps including granuloma liquefaction and cavitation), with release of Mtb antigens as well as host tissue

DAMPs (e.g. HMGB1, mtDNA) and alarmin cytokines, thereby *mimicking* the soluble milieu of a productive, damaging infection.

Apart from mitophagy-mediated opposition of mitochondrial stress, another factor depleted by more advanced disease that may predispose cells to ARV-induced cell death is glutathione. Among other crucial functions, glutathione plays an important role in protecting cells from oxidative stress, but becomes depleted during states of high energy demand associated with cachexia-like metabolism (high rates of glycolysis despite decreased oxidative phosphorylation capacity), e.g. inflammation, HIV/AIDS, sepsis, over-training, and starvation. Indeed, during both HIV and *Mtb* infection, elevated circulating TNF α and ROS increase antioxidant requirements [138] [139], depleting macrophage, NK cell, and T-cell intracellular glutathione levels [138] [140] [139] [141]. In an attempt to regulate inflammation, T_{reg} also inhibit DC glutathione synthesis to induce a redox environment unfavourable for Th proliferation (furthermore, while effector T-cells direct cysteine towards glutathione and protein synthesis, T_{reg} consume cysteine for conversion to sulfate) [142]. PBMC from TB patients display significantly lower glutathione levels [143]. Thus, more advanced co-infection likely results in severe glutathione depletion. NK cell glutathione levels have a role in controlling intracellular *Mtb* growth in various cell types; for example, NK cell glutathione depletion disrupts NK-DC cross-talk, contributing to increased *Mtb* multiplication within monocytes [144]. Lack of glutathione also makes mitochondria significantly more vulnerable to mtDNA oxidative damage and thus mitochondrial and cellular dysfunction during inflammation/metabolic dysfunction-induced redox stress [145]. Thus, intracellular glutathione status significantly contributes to determining the mode of cell death (e.g. apoptosis versus necrosis). In other contexts, oral N-acetylcysteine (NAC) supplementation provides cysteine to help boost intracellular glutathione regeneration [144] (enhancing bacteriostasis, and – *ex vivo* – lowering IL-1, IL-6, TNF α , and IL-10 levels [143] [144]). Other compounds which can boost glutathione levels include L-cysteine, active vitamin D (ARV therapy is known to decreased vitamin D levels [146]), and SAME.

Thus, we hypothesise that advanced baseline disease may represent an immunometabolic state specifically primed for TB-IRIS in response to ARV-induced immune/virological responses or host cell stress, and a shorter ATT-ARV interval may also provide insufficient time (apart from clearing *Mtb* antigens) to recover immunometabolic homeostasis prior to another significant perturbation. It is possible that while both advanced HIV and TB are active, the immunometabolic effects of HIV predominate, and that when ARV therapy inhibits viral replication the immunometabolic effects of TB become predominant.

1.6. Integrating TB-IRIS Cellular and Biochemical Clues

1.6.1. Predisposition: Baseline Immunological State Prior to TB-IRIS (relative to non-IRIS developers)

1.6.1.1. Baseline Innate Myeloid Cell Activity

Elevated circulating **neutrophil** and **CD14⁺CD16⁻ (classical) monocyte** counts likely indicate the chronic inflammation of more advanced coinfection (perhaps with skewing towards myeloid immaturity/MDSC, with impaired functionality, including IL-8R expression and chemotaxis, antigen clearance, and survival [147] [148]). Myeloid-derived **MPO** levels are also **increased** [105]. Neutrophilia is known to delay *Mtb* clearance, being independently associated with pulmonary *Mtb* burden [149].

These elevated counts occur despite **lower** levels of **all three myeloid colony-stimulating factors (IL-3, G-CSF, GM-CSF)** examined [150] [118], suggesting that other factors must mobilise myeloid cells from the marrow. Such factors may include marrow endocannabinoids, ROS/hypoxia-mediated activation of HIF-1 α , and sphingosine-1-phosphate - S-1-P, an endoplasmic reticulum (ER) lysophospholipid - none yet investigated in the context of TB-IRIS. While **G-CSF** is the major elicitor of granulopoiesis and rapid neutrophil mobilisation from the marrow, various other chemotactic factors (e.g. LTB₄, C5 α , IL-8, KC, MIP-2, etc.) can rapidly induce mobilisation and neutrophilia [151]. Indeed, **C5** production and circulating **IL-8** are **elevated** [152] [153]. Unusually, circulating levels of the major classical monocyte mobiliser, **MCP-1** are, however, **decreased** across three independent cohorts [154]; monocytes can also be mobilised during inflammation by MCP-3 and during hyperlipidemia (which also induces expansion of neutrophils and classical monocytes) by CXCL1 (GRO α) [155], neither yet investigated in this context. HIV- and ARV-induced insulin resistance and dyslipidemias – e.g. hypertriglyceridemia and decreased HDL levels – are common [156], but have not been investigated in context of TB-IRIS.

However, these effects are most strongly associated with protease inhibitors, and tend to occur later post-ARV initiation than does TB-IRIS. Circulating levels of monopoiesis-eliciting M-CSF have also not been investigated in this context (but IL-3, G-CSF, and GM-CSF all contribute to both granulopoiesis and monopoiesis) [157].

At this time, **classical monocytes** (and **mDC**) may exhibit **higher TLR-2** expression (perhaps due to coinfection-mediated inhibition of TLR signalling), though findings are mixed [158]. Enhanced TLR-2 expression should sensitise cells to TLR-2 ligands, resulting in a lower threshold of activation and/or increased production of inflammatory cytokines (e.g. TNF α) in response to TLR-2 ligands (unless the reason for increased expression is decreased efficacy of TLR signalling). Inflammatory stimuli (including TLR ligands and inflammatory cytokines), too, are known to enhance expression of various PRR, including TLR-2 (and its LPS co-receptor CD14), TLR-4 (and its LPS co-receptor Ly96), CD163 (the phagocyte Hb scavenger receptor, which can also act as a bacterial PRR), and TREM-1 (whose ligation enhances TLR signalling), including ADAM-mediated shedding of sCD14, sCD163, and sTREM-1.

Baseline circulating **sCD163** is **elevated** (**sCD14** findings are **mixed**, but **often elevated**) [4] [104] [119] [105], again suggesting myeloid cell activation and more advanced co-infection (during which elevated sCD14 and sCD163 levels are known to be associated with all-cause mortality). Regarding CD163, ligation by host-derived HMGB1 or complexed Hb-haptoglobin would elicit cytoprotective IL-10 and HMOX-1 (catabolises heme to biliverdin), while ligation by bacteria would elicit inflammatory cytokines.

Equivalent LPS as well as **endoCAb (anti-LPS antibody)** levels [150] suggests non-contribution of microbial translocation to innate leukocyte activation. However, free levels of the positive acute-phase reactant **LBP** are **depressed** [150], suggesting increased complexing with a cognate molecule. Although only Gram-negative bacteria (and not Mtb) produce LPS, mycobacterial LAM physicochemically resembles LPS, including binding to LBP and LPS-recognising PRR. Recall that a higher baseline frequency of urine LAM positivity indeed suggests higher baseline LAM loads.

A pro-inflammatory **intermediate monocyte subset (CD14⁺CD16⁺)**, i.e. expressing low-affinity activating Fc γ RIIIa) exhibits **increased transcription** of inflammatory genes, including those encoding **PRR** and **complement**, and those representing **PPAR signalling**, **LXR/RXR activation**, and **IL-6 and IL-10 signalling** [159] [160] (though circulating levels of **IL-6** are **mixed**, those of **IL-10** are indeed **higher**). **Increased** expression of **C1q** (A, B, and C) and **C5** (though the latter trend is non-significant) by these monocytes suggests increased activation of C1 by antigen-antibody/pentraxin (e.g. CRP, PTX3) complexes (classical pathway), and indeed inhibition of this process by **C1-INH** is **down-regulated** in these monocytes [159] [160]. Both HIV and Mtb stimulate classical and lectin pathways of complement activation, with the C3 receptor apparently accounting for ~80% of Mtb phagocytosis by monocytes/macrophages. However, CR-mediated uptake induces a less robust anti-mycobacterial response (including Th1 induction) than does uptake via Fc γ R, and can even bypass anti-mycobacterial responses altogether.

Activation of myeloid cells by other means (e.g. NLRP3 inflammasome activation) also induces production of C1q, however. Assembly of the NLRP3 inflammasome activates CASP-1 to cleave IL-1 β and IL-18 to their mature forms. Indeed, at baseline, a **higher percentage of circulating monocytes expresses CASP-1** [137], and levels of **IL-1 β** and **IL-18** are **elevated** [152] [161] (this could also be the result of dying cells releasing their immature forms, which can be activated by other myeloid proteases extracellularly). This may reflect various possible NLRP3-activating signals, including cytoplasmic nucleic acids/LPS/PDG/cathepsin, extracellular ATP/K⁺ flux through P2X7, or (IFN-induced) AIM2 sensing of cytoplasmic dsDNA. Indeed, HMGB1 (via the AGEs receptor) induces lysosomal cathepsin B (CTSB) leakage, facilitating NLRP3 inflammasome assembly. Activated CASP-1 also cleaves gasdermin D, and both contribute to pyroptosis with release of DAMPs (e.g. HMGB1). The other IL-1 family cytokine (apart from IL-1 and IL-18) is IL-33 (constitutively produced in endothelial nuclei, already in its mature form [162] [163]), but this has not been investigated in context of TB-IRIS. This cytokine is released by stressed and necrotic cells, likely acting as an alarmin which can enhance both Th1- and Th2-type immunity, depending on the prevailing cytokine milieu [162] [163] [164] [165]. While elastase and cathepsin G from activated neutrophils processes mature IL-33 to forms with ~10-fold higher activity (including in a murine model of acute lung injury associated with neutrophil infiltration), CASP-1 actually inactivates it [163].

Apart from inflammasome-matured IL-1 β and IL-18, myeloid phagocytes activated by intracellular-type pathogens (via surface and intracellular PRR) typically also secrete pro-inflammatory IL-6, TNF α , IL-8, IL-12, IL-15, IL-23, IL-27, MCP-1, MIP-1 α and β , and type I IFN. Although largely *antiviral*, type I IFN are also produced during intracellular *bacterial* infection, induced by intracellular TLR and cGAS ligation. Genetic **polymorphisms involving IL-18, IL-6, TNF α , and IL-12** are known to **modulate TB-IRIS risk** [166] [167] [168], but this may be indirect (i.e. via their effects on HIV and TB progression rates). In this context, both the presence of intracellular pathogens and observed inflammasome activation suggest myeloid cell activation, but at baseline many of the above cytokines and chemokines – expected to be up-regulated during myeloid activation – actually exhibit apparently *lower* levels (though findings are mixed), apart from **IL-8** and **IL-27**, whose levels are **elevated** (Table 4). The former cytokine recruits and activates neutrophils. The latter cytokine is produced mainly by activated myeloid phagocytes/APC [169] (e.g. neutrophils, monocytes/macrophages, and DC), for example in response to type I IFN and/or IFN γ $\pm$ TLR ligation (and/or S-1-P imbalance) [170] [171] [172]. Unsurprisingly, IL-27 is elevated during Mtb infection, including in granulomas and pleural fluid [172] (IL-27p28 – whose production is dependent on MyD88, and partly on NF κ B [170] – is significantly elevated during sepsis [169]). It enhances neutrophil IL-1 β production, and favours Th1 polarisation and (innate and adaptive T-cell-mediated) cytotoxicity, including IFN γ production by Th1 and Tc1, but also inhibits IL-2, IL-6, possibly TNF α , IL-12, COX-mediated prostaglandin production, and autophagy (via mTOR activation), opposes Th2, Th9, and Th17 responses, and induces Tr1 (which produce IL-10) in an effort to regulate immune responses (including IFN γ production), though IL-10 also interferes with antigen clearance by inhibiting phagosome maturation [172]. Although baseline levels of type I IFN have not been investigated specifically in the context of TB-IRIS, higher circulating IFN α 2 is “associated with a greater risk of subsequent IRIS” in the setting of multiple other HIV co-infections [173]. Furthermore, type I IFN and IL-27 (both antiviral, e.g. IL-27 suppresses monocyte SPTBN1 expression and thus HIV entry, and inhibits within-monocyte and –T-cell HIV replication) reciprocally induce each other’s expression during HIV infection [174] [175].

The abovedescribed generally low production of pro-inflammatory myeloid-derived cytokines (except IL-8) at baseline in those who go on to develop TB-IRIS (Table 4) may be attributable to: activation (e.g. of inflammasomes) by metabolic stress (HAMPs) or host-derived alarmins/DAMPs *rather than* pathogen-derived TLR ligands, TLR signalling inhibition by HIV and Mtb even in the presence of pathogen products (**likely control-equivalent** baseline **TNF α** responses despite elevated myeloid TLR-2 expression supports both of these hypotheses), or inhibitory effects of elevated IL-10. Although elevated IFN α 2A would decrease e.g. monocyte pro-inflammatory cytokine production, this is specific to IL-1 β , IL-6, and TNF α [176]. Inflammatory situations in which both IL-1 β and IL-8 (but not necessarily IL-6 or TNF α) are elevated are unusual, since myeloid cell activation via PRR typically results in production of *all* of these pro-inflammatory cytokines. However, certain contexts *can* give rise to isolated IL-8 expression.

For example, during ischemic optic neuropathy, circulating IL-8 (but neither IL-6 nor TNF α) become elevated [177], suggesting that isolated IL-8 elevation may indeed be more characteristic of tissue damage-mediated *sterile* (rather than infectious) inflammation. Additionally, at least in retinal pigment epithelia, exogenous S-1-P (which extracellularly modulates cytokine secretion) induces IL-8 (but not IL-6 or MCP-1) secretion [178]. Indeed, in mice S-1-P also suppresses myeloid IL-12p70 production [179], and *in vitro* S-1-P diminishes DC IL-12 and IL-23 production, while elevating IL-27 secretion [180].

Membrane sphingolipids are major contributors to lung epithelial mucous barrier function, and the balance of their consecutive metabolites ceramide and S-1-P (produced by stress-activated sphingosine kinase-1) form one of many cellular rheostats determining cell fate (e.g. in the context of infection and immunometabolism) [181]. For example, ER stress increases production of pro-apoptotic/-senescence ceramide and anti-apoptotic/pro-proliferative S-1-P, both of which favour autophagy [182] [183]. Mtb, HIV, and ARV therapy all modulate sphingolipid metabolism [184], altering the balance between sphingomyelin (the major membrane sphingolipid [185]) breakdown product ceramide and its metabolite S-1-P [186]. Mtb “exploits sphingolipid metabolism” for its survival: TB patients exhibit decreased lung and circulating S-1-P levels (which may inhibit macrophage-mediated Mtb control and opposition of inflammatory lung damage) [181]. Finally, ligation of a *single* TLR induces IL-1 β , followed by IL-1Ra (setting up a balanced ratio required for regulated inflammation), but *simultaneous* ligation of TLR-2, TLR-4, and TLR-7/8 produces oxidative stress in excess of antioxidant system capacity, causing ATP externalisation, inflammasome activation, higher IL-1 β and TNF α production, but also inhibition of protein synthesis (and thus inhibition of IL-1Ra and IL-6 production),

leading to inflammatory imbalance [187]. Apparently neither EGFR nor ROS are involved in the S-1-P-induced IL-8 production pathway [188]. While ATT significantly lowers IL-1Ra in TB/HIV-coinfected patients, baseline **IL-1Ra** level is comparable between TB-IRIS developers and non-developers [119].

Observations in context of the above knowledge support the idea that isolated elevation of inflammasome-matured cytokines and IL-8 more likely represent inflammation triggered by host cell metabolic/oxidative stress or damage rather than by productive infection. Indeed, intra- or extracellularly generated ROS trigger signal transduction cascades which activate IL-8 and ICAM-1 (CD54), favouring leukocyte extravasation [189]: "oxidant stress is an important [inducer] of IL-8 gene expression" [190]. At least in bovine chondrocytes, IL-1 β (and, less markedly, IL-6), too, dysregulates antioxidant systems, enhancing Mn-SOD and GPX expression and activity, but simultaneously decreasing Cu/Zn-SOD, EC-SOD, and CAT expression [191]. In a rodent model, mitochondrial-protective antioxidants alleviate oxidative stress and also lower IL-6 levels [192]. Many other cytokines (e.g. IL-33, IL-21, IL-25, etc.) have not yet been investigated in the context of TB-IRIS.

Typically, myeloid phagocyte activation also results in activation of NOX2 (contributing to respiratory burst-mediated ROS and RNS production) and iNOS. Both NOX2 and iNOS convert L-arginine and O₂ into NO (a vasodilatory gaseous free radical signalling molecule produced by many cell types) and L-citrulline. Although NO-inhibiting IL-6 and TNF α levels are not clearly elevated at baseline, **NO** production is apparently **lower** [137] (perhaps due to intracellular glutathione depletion [193], or to lower NO-boosting IL-17 levels [194]). Lower NO may also compromise intracellular mycobacterial killing and antigen clearance. Interestingly, NO and inflammasomes reciprocally regulate each other [195], so lower NO levels may result from increased inflammasome activation, or may contribute to insufficient inflammasome regulation.

Table 4 Baseline Levels and Functions of Selected Cytokines Produced by Activated Myeloid Cells

Soluble Factor	Level Relative to Non-IRIS-developers	Selected Functions	Predictions	References
IL-6	mixed	with IL-1 β (higher) and TNF α (equivalent): elicits hepatic APR incl. $\uparrow$ CRP, LBP, haptoglobin, C3, Hsp, etc. $\downarrow$ CYP450, RBP, CBG, lactoferrin, transferrin with TGF β (not investigated) elicits Th17 with IL-12 (lower) elicits Th1 helps differentiate B-cells to IgA/G ⁺ plasmablasts	if lower: lower positive APR levels (though CRP is higher [50]) higher negative APR levels	[196] [105]
TNFα	mixed	with IL-1 β (higher) and IL-6 (lower): initiates HPA axis (stress response), fever, anorexia, malaise enhanced phagocyte phagocytosis microbicidal (e.g. cathelicidin, Fc γ RI, and iNOS expression, CYP27B1-mediated vitamin D activation, NOX2 recruitment)	if equivalent: equivalent symptoms, cortisol/adrenaline levels, and phagocyte microbicidal	[197] [198] [105]
IL-8	higher	neutrophil recruitment and priming can recruit basophils and T-cells	increased activated neutrophil infiltration	[199]
IL-12	lower	with IL-18 (higher), IL-15 (lower), and type I IFN (unknown, likely higher): favours IFN γ production by and cytotoxicity of innate and adaptive T-cells (IFN γ enhances phagocyte TNF α production) with IL-18 (higher) and IL-15 (lower): helps activate T _{mem} APC maturation and T-cell recruitment for synapsing	decreased stimulus for APC-mediated T-cell activation, Th1-type response, and T _{mem} activation (though IL-27 sensitises T-cells to lower IL-12 levels)	[200]
IL-15	lower	as for IL-12 neutrophil and innate T-cell maintenance and cytotoxicity (incl. neutrophil phagocytosis) NK cell proliferation NK cell and $\gamma\delta$ T-cell IL-22 production elicits GM-CSF, IL-1, IL-6, IL-8, TNF α , IFN γ , MIP (thus favours neutrophil and monocyte recruitment) with IL-7 (trends higher): memory generation (incl. KLRG1 expression) and maintenance with IL-12 (lower), IL-15 (lower): NK cell production of HIV-restricting factors with IL-21 (unknown): B-cell differentiation into follicular plasmablasts with IL-3 (lower): promotes mast cell and basophil IL-4 and IL-13 production	as for IL-12 decreased neutrophil and monocyte recruitment decreased neutrophil and innate T-cell maintenance and cytotoxicity lower GM-CSF, IL-1, IL-6, IL-8, TNF α , IFN γ , MIP, and IL-22 elicitation ?decreased memory/KLRG1 induction and maintenance ?decreased antigen clearance (as for lower IL-15 levels during cryptococcal IRIS)	[201]
IL-23	unknown	induces Th17 responses	unknown	[202]
IL-27 (mostly from neutrophils and mono/mac)	higher	decreases neutrophil oxidative burst, but enhances neutrophil IL-1 β and (persistently) TNF α production sensitises T-cells to lower IL-12 levels, supporting Th1 induction (including cytotoxicity) opposes T-cell IL-2 production inhibits Th2 and Th17 activity strongly induces IL-10, opposing antigen processing supports Tr1 (CD4 ⁺ FoxP3 ⁺ IL-10 ⁺ T _{reg}) activity	increased neutrophil inflammatory activity (IL-1 β , TNF α production), but decreased microbicidal increased IL-10, decreased antigen presentation favouring of Th1 and T _{reg} responses opposition of Th2 and Th17 responses	[169] [203] [170] [204] [105]
MCP-1 (also induced by IL-4 and IL-6)	lower	major mono/mac-recruitment and activation chemokine also enhances neutrophil recruitment and LTB4/cytokine/chemokine production	lower levels associated with accumulation of apoptotic neutrophils and damaged cells, delayed antigen clearance, and	[205]

13)		helps maintain T-cell survival	exacerbated inflammation/I-R-induced tissue injury	
MIP-1a and β	trend lower	with RANTES (unknown): recruits neutrophils, monocytes, NK cells, and αβT-cells as part of Th1-type immunity (IFNγ boosts MIP-1a receptor expression by neutrophils) with TNFα (equivalent): MIP-1a enhances neutrophil LTB4 production (thus enhancing MIP-1β production)	decreased recruitment of neutrophils, monocytes, NK cells, αβT-cells (though higher levels of MIP-1a receptor due to IFNγ) decreased enhancement of neutrophil LTB4 production and myeloid MIP-1β production	[206]
type I IFN	unknown (higher baseline VL and active TB both suggest higher levels)	reciprocally antagonise IL-1, inhibiting NLRP1/3 inflammasome activation and increasing IL-1Ra expression support Th1 response (incl. IFNγ production, but esp. cytotoxicity) enhance production of MIP-1a and β and RANTES induce antiviral type I ISG induce IL-10 production alter response to TLR ligands enhance T-cell maintenance oppose macrophage production of Th2-recruiting chemokines, inhibit T _{reg}	may support Th1-type response and cytotoxicity should enhance MIP-1a and β production, but these trend lower may oppose inflammasomes/IL-1 may induce ISG (incl. type II IFN-induced) may induce IL-10 may oppose Th2 and T _{reg} responses	[207]

Furthermore, lone IL-1β (without IL-12) would favour Th17 induction, whereas lone IL-18 (without IL-12, IL-15) would promote Th2 induction. Cytokines still present (albeit at lower levels) may still exert effects, depending on the cytokine and on target cell sensitivity. Neutrophilia may argue against a Th2-type response. During both HIV infection and TB levels of IL-12p70 and IL-15 are depressed (including during co-infection), suggesting their lower levels observed here are due to more advanced co-infection.

Even though only IL-1β (but neither IL-6 nor TNFα) of the systemically-acting cytokines are elevated, **CRP** (PTX1) is **elevated** at baseline [50] [105]. Since both DAMPs and IL-1β rapidly induce neutrophil, macrophage, and DC expression of PTX3 [208] [209] [210] [211], this pentraxin is likely also expressed at baseline, although it has not been investigated in this context. Pentraxins act as soluble PRR, enhancing opsonic phagocytosis (e.g. via high-affinity FcγRI and low-affinity FcγRII for CRP, and low-affinity FcγRIII for PTX3) [212] [213] [214] [215] [216] [211], among other immunomodulatory functions. At least on **monocytes** (CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺, and CD14⁺CD16⁺⁺ subsets), **FcγRI expression is higher** at baseline [137].

Various circulating MMPs are also elevated at baseline, either due to secretion by activated viable myeloid cells in response to more advanced TB, or release from lysed/necrotic myeloid cells. Specifically, levels of **MMP-3** and **MMP-7** (likely from monocytes/macrophages [217]) are **higher** in TB-IRIS patients also exhibiting a positive urine LAM assay [44]. Indeed, levels of both **MMP-8** (likely from neutrophils [217] and which degrades type I-III collagens) and **PIIINP** (the collagen breakdown product procollagen III N-terminal peptide) are generally **higher** at baseline (MMP-8 especially so when urinary LAM is present) [44]. MMPs facilitate extravasation and interstitial migration, and have additional complex pro- and anti-inflammatory roles, including in tissue remodelling or fibrosis during healing processes [217]. Another ECM component involved in inflammatory responses is hyaluronic acid, whose deposition and accumulation increases in injured/inflamed organs (including the lungs), and which can create a leukocyte-recruiting and -activating scaffold [218]. Concomitantly, circulating levels increase during inflammation [219]. At baseline in TB-IRIS patients, circulation **hyaluronic acid** levels are **elevated** [105].

Activated cells (e.g. endothelia, myeloid) also produce tissue factor (TF: the major initiator of coagulation), with > 90% typically attributable to activated monocytes [220]. At baseline, **circulating TF is elevated** [104], likely favouring a hypercoagulable state (indeed, baseline levels of the fibrinogen breakdown product, **D-dimer**, are also **elevated** [4] [105], suggesting increased thrombosis and fibrinolysis), which can also favour inflammation (e.g. intra-thrombus monocytes express much higher levels of IL-8 and MCP-1 [221]). Thrombosis is initiated by the intrinsic pathway (factor XII activation by a negative surface e.g. activated platelet polyphosphates) or the extrinsic pathway (factor VII activation by TF on injured vessel walls, activated endothelia, monocytes, and microparticles – both of which activate factor X to convert prothrombin into thrombin, which cleaves fibrinogen into fibrin [222]). Additionally, hypoxia due to stagnant blood-flow (e.g. venous stasis, due to inactivity) can result in increased ROS production, mast cell and complement activation, endothelial and leukocyte (including neutrophil) activation (including directly by ROS and by activated platelet products), and leukocyte activity (e.g. inflammasome activation and NETosis) which contributes to initiation of thrombosis [222]. Inflammation itself (e.g. resulting from HIV and Mtb infection) typically also enhances coagulability via various pathways, constituting Virchow's triad. For example, complement activation by von Willebrand's factor derivatives; conversely, both C3 and C5a are associated with increased thrombotic risk.

Regarding lipid mediators of inflammation, **LTA4H mutants** (CT/TT) **exhibiting increased activity** are associated with **more severe TB-IRIS** manifestations, but (although corticosteroids inhibit ALOX5) not with altered responses to corticosteroid treatment [14]. Eicosanoids such as leukotrienes are implicated in TB pathogenesis, and an LTA4H SNP is apparently associated with the TBM response to dexamethasone, suggesting that the polymorphism may enhance binding of dexamethasone to this gene's GRE to suppress LTA4H expression [80]. At baseline, activation of the eicosanoid cascade LOX arm is characterised by **elevated HETE** [223] (perhaps rather than LTB₄) production, possibly due to HIV-induced altered lipid metabolism, and/or lower MCP-1 levels. HETEs, via TRPV1 and PPAR (respectively) sensitise nociceptors and modulate LXR activity (and therefore cholesterol homeostasis) as well as increasing expression of the fatty acid translocase, CD36 [224]. This translocase is implicated in type II pneumocyte surfactant production and Mtb-permissive foamy macrophage development [225] [226]. It binds oxLDL and β -amyloid peptides, inducing assembly of TLR-4/6, priming the NLRP3 inflammasome for activation, and favouring sterile inflammation [227].

1.6.1.2. Baseline Innate Lymphocyte Activity

Two innate lymphocyte subsets - $\gamma\delta$ T-cells and iNKT cells - respond to CD1-presented lipid antigens (e.g. isoprenoids and glycolipids, respectively), while the third - NK cells - responds to missing self (although all can also be activated by DAMPs). High levels of the host-derived isoprenoid, isopentenyl pyrophosphate (IPP, an intermediate in the cholesterol biosynthetic pathway), can activate $\delta 2$. During TB/HIV-coinfection, preferential $\delta 2$ depletion causes inversion of the $\delta 2/\delta 1$ ratio, and their functions (including IFN γ production) become impaired. Although baseline circulating proportions of **$\gamma\delta$ T-cells** are **comparable** between TB-IRIS developers and non-developers, baseline **elevation of HLA-DR expression** on these cells (including the $\delta 2$ subset) and of the **$\delta 2$ proportion lacking inhibitory receptors** [228] [229] suggests increased (capacity for) activation, including cytokine production and cytotoxicity (although the level of **inhibitory receptor expression on $\gamma\delta$ T-cells overall is higher** [229]). At baseline, TB-IRIS developers display **NK cell frequencies** (and **NK receptor – activating NKG2C, activating NKp80, and inhibitory CD158a - expression** levels) more similar to healthy controls (i.e. **elevated**) than to non-developers [230]. Baseline **elevation of the NK proportion expressing IL-18R** [137] (i.e. sensitised to IL-18, already present at elevated levels) and **elevated degranulation markers** [231] also suggest activation. Baseline circulating proportions/frequencies of **iNKT cells** are **comparable/increased** between TB-IRIS developers and non-developers [229] [232], but baseline iNKT activity has not been studied in this context. High levels of IL-18 in the presence of IL-12 sensitisation (by elevated IL-27) will favour IFN γ production and cytotoxicity mediated by $\gamma\delta$ T-cells and NK cells (IL-18 also promotes NK cell expansion, amplifies NK cell function, and facilitates NK cell mTORC1 activation). High levels of isolated IL-1 β and IL-23 (not investigated, but possibly elevated in this context) would favour Th17-type responses (e.g. production of IL-17 and IL-22 to boost mucosal immunity), but given available information (i.e. IL-1 β in the presence of – albeit low-level – IL-12, and unknown IL-23 levels) a Th1-type response seems more likely. Type II IFN induce expression of a large set of IFN-stimulated genes (ISGs), such as IP-10 (which recruits activated macrophages, DC, NK cells, and T- and B-lymphocytes) [233] and MIG. Indeed, baseline levels of **IFN γ** (but not TNF α) may be **elevated** [153] [161] [234] [105], inducing **elevated** levels of **IP-10** [161] [173] [235] [105] (MIG levels remain unknown but no differential ex vivo production occurs in response to PPD/RD1 antigens [235]). Baseline levels of **IL-17** are **depressed** [118] (therefore IL-17-mediated enhancement of neutrophilic inflammation will be less pronounced), possibly due to more advanced HIV infection. Baseline levels of cytotoxic mediators (perforin, granzymes, granulysin, etc.) remain uninvestigated in this context.

Essential cytokines for lymphocyte development and homeostasis include IL-2, IL-7, and IL-15 (with both IL-2 and IL-15 favouring Th1-type responses, including cytotoxicity). Mycobacterial infection (e.g. of macrophages) usually elicits both IL-7 and IL-15 secretion [236], and HIV-induced lymphopenia results in elevated IL-7 which can support T-cell proliferation [237], maintenance, and full lymphocyte activation via T-B interactions. Both cytokines are crucial for the maintenance of $\gamma\delta$ - and $\alpha\beta$ T-cell responses [236]. Although IL-7 is insufficient to drive T-cell expansion on its own, it can do so in the presence of antigenic stimulation. At baseline, levels of NK and NKT cell-activatory **IL-2** levels are **low**, as are levels of NK- and NKT-maintaining **IL-15**, [118] the latter also suggesting a lower stimulus for IL-22 production and KLRG1 expression/memory induction. Constitutive production of IL-7 by non-leukocyte stromal cells during conditions of T-cell depletion (i.e. decreased IL-7 consumption by its receptor) likely lead to the **higher-**

trending levels of **IL-7** [238] (a non-redundant requirement for T-cell maintenance) observed at baseline. This cytokine also supports KLRG1 expression/memory induction, reactivation of pathogen-specific IL-2⁺IFN γ ⁺TNF⁺ T_{mem} [239], and enhances T-cell sensitivity to lower Mtb product levels [240].

1.6.1.3. Baseline Adaptive T-cell Activity

At baseline, lymphocyte counts are typically lower [44], with lower CD4/CD8 ratios [72] [241]. Indeed, **CD4 counts** are typically **lower**, although **IL-2⁺IFN γ ⁺TNF⁺ and T_{mem} subset percentages** are **equivalent** to those of controls. Findings regarding baseline CD4⁺ T-cell activation state are mixed. While baseline **CD8 counts** are technically **equivalent** to those of controls (although one study finds a non-significantly higher CD8 count at baseline [72] and another finds that CD8 count ≤ 500 cells/mm³ is a risk factor [242]), the **proportions of chronically-activated (KLRG1⁺PD-1⁺) cells** [243] and **Fas⁺ T_{mem}** are **elevated** (though these cells express lower levels of other activation markers than do those of controls, and the number of activated CD8⁺ T-cells is lower [60]). One study finds that **carriage of the HLA-B*41 allele** is a genetic risk factor for TB-IRIS [242], suggesting that preferential presentation of certain antigens may be involved in pathogenesis.

Despite lower baseline levels of IL-2 (due to decreased T-cell activation or counts), an **increased** level of **sCD25** (the soluble IL-2 receptor) is observed [238], thus perhaps deriving from activated innate rather than adaptive T-cells (e.g. NK cells). Indeed, both IL-18 and IL-12 induce T-cell CD25 expression [244], and T-cell activation induces CD25 shedding.

As mentioned above, baseline levels of Th1-type cytokines (IFN γ) are elevated, while baseline levels of Th17-type cytokines (IL-17) are depressed. Indeed, elevated IL-27 levels (as observed here) oppose T-cell IL-2 production as well as antagonising Th2 and Th17 polarisation, and may be responsible for such effects in this context. Although tissue damage-induced DAMP/alarmin release is a potent inducer of Th2 responses (e.g. in the context of parasitic infection), neither Th2-type cytokines nor Th9-type cytokines have been examined at baseline in this context (but neutrophilia and neutrophil infiltration would typically argue against Th2-type inflammation, although does not rule it out). Interestingly, in general: ATT has been shown to reverse Th2-mediated immunoregulation, causing inflammatory reactions [37], and following initiation of ARV therapy ex vivo T-cells demonstrate a switch from Th2 to Th1 type cytokine production [245].

Regulatory leukocytes (including myeloid cells and CD4⁺ or CD8⁺ T_{reg}) produce anti-inflammatory IL-10 during inflammation. Baseline **IL-10** levels are significantly **elevated** [152] [105], possibly partly due to being strongly elicited by IL-27. Although TGF β becomes elevated during both Mtb and HIV infection (during the latter derived in part from CD8⁺ T_{reg}), its baseline level has not been investigated in the context of TB-IRIS.

Baseline B-cell counts/activity have not been determined in this context, but **anti-PGL-Tb1** antibody levels are **lower**, while titres of **antibodies against** other Mtb antigens (**ManLAM**, **ESAT-6/CFP10**) are **equivalent** (relative to controls) [246]. Although (as mentioned above) monocyte Fc γ R expression is higher [137], an ex vivo proteomic study of unstimulated PBMC suggests **relative under-utilisation of Fc γ R-mediated phagocytosis** (suggesting impaired antigen clearance) [247]. PGL-Tb1 is a membrane branched phenolic glycolipid (non-protein antigen) and virulence factor overexpressed by hypervirulent Mtb. Its low level in TB-IRIS patients is independent of TB clinical presentation and very unlikely to reflect a lower Mtb load; furthermore – although such antibodies are less common during HIV co-infection [248] – apparently neither represents more advanced HIV, being independent of CD4 count. In mice and rabbits, expression of PGL-Tb1 by a clinical Mtb isolate is linked to decreased Th1 response efficiency and enhanced virulence [249]. Anti-PGL-Tb1 antibodies may actually be protective *against* TB-IRIS [246] or may indicate some difference in Mtb metabolism/host response reflective of the factors predisposing to TB-IRIS. It is similarly unlikely that equivalent levels of anti-ManLAM and –ESAT-6/CFP10 antibodies suggest no difference in Mtb antigenic load, but rather that these antibody responses are already maximal.

1.6.1.4. Baseline Immunological State: Conclusions

Despite more broadly elevated myeloid counts, activation, and PRR expression, baseline inflammation appears largely characterised by DAMP/IL-8-induced neutrophilia (arguing against a Th2-type response) with (secreted or necrotically-released) MMP-8-mediated collagen degradation, and an activated CD14⁺CD16⁺ monocyte subset undergoing relatively unopposed classical complement activation (fig. 4). Such activation likely occurs due to higher levels of Mtb antigen-antibody/pentraxin complex levels, due to a higher Mtb burden at baseline, as well as apparently poorer antigen clearance. Activated complement likely contributes to neutrophil recruitment. Higher baseline mtDNA levels (perhaps due to more advanced HIV, and possibly contributing to type I IFN responses [250] and – via induction of IL-8 – to neutrophilia [251] as well as to monocyte activation [252]) support increased host cell metabolic stress (and thus mitochondrial dysfunction) and death; observed inflammasome activation is likely mediated by host cell metabolic stress or host-derived DAMPs/alarmins. Indeed, mitochondrial dysfunction may result from increased vulnerability to mtDNA oxidation in the presence of oxidative stress/absence of glutathione, and higher levels of circulating mtDNA are known to be associated with more severe presentations and worse outcomes in many inflammatory disease settings (including being able to cause acute organ injury in healthy animals) [252] [253]. Oxidised mtDNA, especially, powerfully stimulates type I IFN production via cGAS-STING.

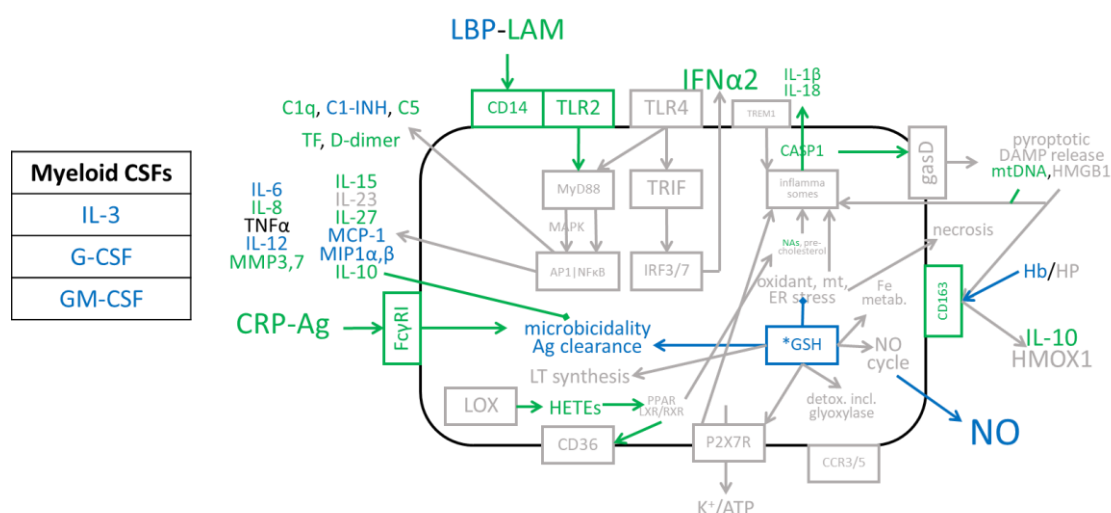


Figure 4 Baseline (Pre-ARV Therapy) Monocyte Immunometabolic Model of TB-IRIS. Host genotypes predisposing to TB-IRIS risk include SNPs in IL-18, IL-6, TNFα, and IL-12, but these effects may be indirect via impacts on TB/HIV progression. Additionally, a SNP which elevates LTA4H activity increases TB-IRIS severity. The single study examining Mtb genotype found that it had no effect on TB-IRIS risk. HIV strain has not been examined in this context. Epidemiological and clinical data suggest that more advanced HIV (incl. higher VL) and TB (incl. higher Mtb load) predispose to TB-IRIS. The diagram represents findings regarding monocyte subsets and circulating factors at baseline (i.e. prior to ARV therapy initiation). Green represents elevated/activating, blue represents depressed/inhibiting, black represents equivalent, grey represents unknown (only GSH levels are indirectly inferred from non-IRIS studies; marked with an asterisk). Classical monocytopenia and neutrophilia are baseline features, the latter likely due to elicitation/mobilisation by C5α and IL-18 (given that levels of all myeloid CSFs are lower than in controls). Higher circulating MMP-8 levels likely derive from these activated neutrophils, and in general higher activated myeloid MMP (as well as PIINP) levels suggest ECM turnover. Activated myeloid cells also shed CD163 and CD14 in their soluble forms. Higher C1q activation (unopposed by C1-INH) and C5 production suggest increased uptake via CR-mediated (i.e. less microbicidal) pathways. Although FcγRI and CRP expression levels are higher, this may just reflect a higher Mtb load (and perhaps sensitisation of hepatocytes to lower IL-6 by higher IL-1β levels [254]), since proteomic studies suggest under-utilisation of FcγR-mediated (i.e. more microbicidal) uptake pathways; this may be due to increased complement-mediated uptake and/or inhibition of phagosome maturation by IL-10. Levels of LPS and anti-LPS antibody being equivalent to controls suggest no contribution of microbial translocation to TB-IRIS, while there is a tendency towards urine LAM positivity in those going on to develop TB-IRIS (consistent with the concept of higher Mtb load/slower antigen clearance in these patients). Although TLR-2-mediated signalling should activate NFκB and thus expression of all listed cytokines (as well as complement components, TF, and iNOS), the unusual pattern of elevated cytokines suggests either the influences of IL-10 and IL-27 (the latter also favours IL-1β induction), or a more sterile inflammatory picture (due to host metabolic stress activating inflammasomes and cytoplasmic TLRs). Indeed, circulating mtDNA levels are elevated, and more advanced TB/HIV-coinfection would suggest depletion of intracellular GSH, thereby predisposing cells to oxidant, mitochondrial, and ER stress (as well as affecting a number of other cellular pathways, including Fe metabolism). Indeed, lower baseline Hb suggests sequestration of heme-derived Fe in myeloid networks, preventing its usage by pathogens but also increasing host cell oxidative stress. Apart from the possible effects of higher HETE levels on cholesterol homeostasis, type I IFN also significantly impact this cellular functional module.

While there is comparatively less TLR-mediated myeloid activation (as interpreted by lower levels of IL-6 and the Th1-inducing cytokines IL-12 and IL-15) – perhaps due to HIV/Mtb-mediated embargoes on TLR signalling, relative absence of TLR ligands, or the potential presence of HIV/Mtb-induced ER stress-elevated S-1-P levels – IL-27 does sensitise T-cells to the lower IL-12 levels. Along with elevated IL-1β, IL-18, and IL-27 levels, then, the presence of IL-12 likely favours Th1-type responses, with innate and adaptive

T-cell IFN γ production and cytotoxicity (though IL-27 somewhat suppresses activated myeloid cell functions, including antigen clearance). This agrees with the observed higher sCD25 levels and elevated proportion of CD8⁺Fas⁺ T_{mem}. Although CD4 count is lower at baseline and no differences in activation are evident (and the low IL-2 level is likely due to suppression of IL-2 production by IL-27), higher IL-7 levels may promote formation (or create ideal conditions for reactivation) of antigen-specific KLRG1⁺ T_{mem} (including IL-2⁺IFN γ ⁺TNF α ⁺ T_{mem}). Furthermore, IL-27 directly opposes Th2-, Th9-, and Th17-type responses (which may be responsible for lower IL-2- and Th17-type cytokines), and strongly induces Tr1-mediated IL-10 production (which – probably along with regulatory myeloid cell types - likely contributes to high IL-10 levels). Th17 opposition and possibly-decreased LTB4 production suggests little enhancement of neutrophilic inflammation at this stage (as well as likely decreased IL-21 and IL-22 production), while elevated HETEs likely significantly alter cholesterol and lipid metabolism, including via CD36-mediated favouring of inflammasome activation.

Finally, results suggest elevated baseline sequestration of haemoglobin (likely mediated by activated phagocytes), and hypercoagulability (which can both cause and result from inflammation). Baseline Th2/Th9/Treg-type cytokines and B-cell activities remain to be determined (fig. 5).

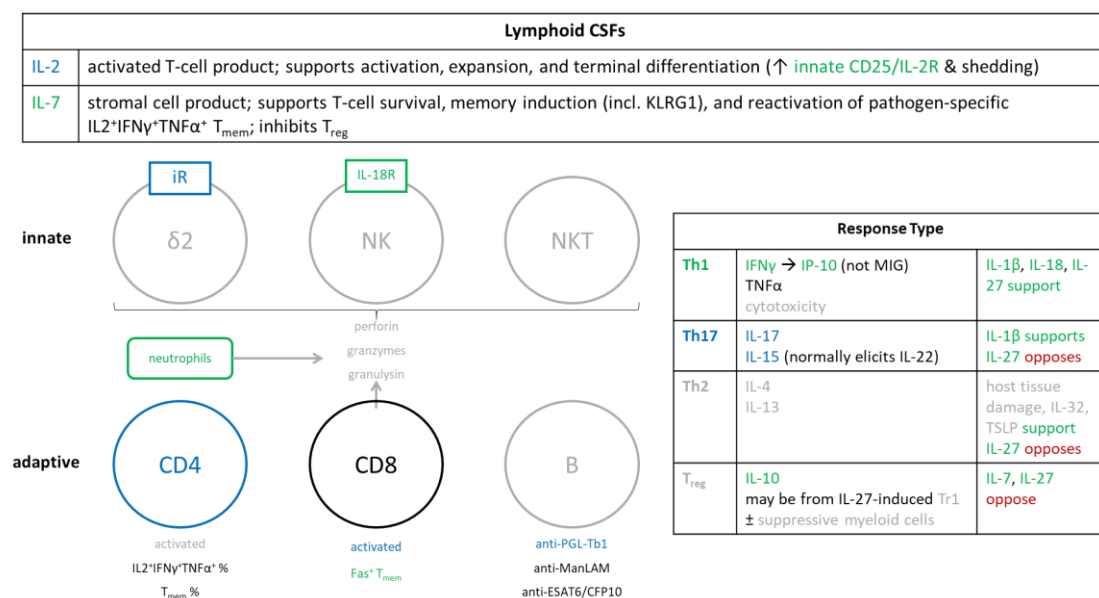


Figure 5 Baseline (Pre-ARV Therapy) Lymphocyte Immunological Model of TB-IRIS. Blue indicates decreased/inhibited, green indicates increased/enhanced, black indicates equivalent, and grey indicates unknown (iR inhibitory receptor, IL-18R IL-18 receptor).

1.6.2. Pathogenesis: Immunological State During TB-IRIS Development and Manifestation

1.6.2.1. Innate Myeloid Cell Activity During Pathogenesis

Initiation of ARV therapy triggers a **greater increase** in **G-CSF** [118], likely helping to drive **increasing neutrophil counts**, and levels of **both are higher at the time of TB-IRIS** [44] [150]. Indeed, higher G-CSF levels are associated with an increased risk of mortality in this setting [118]. Neutrophils are activated, leading to **lower** levels of cell **surface L-selectin** (due to shedding) at the time of TB-IRIS [255]. At least during TB-IRD, such neutrophils express **increased** (non-significant trend) levels of **chemokine receptors (CCR3, CCR5)** [256] covering MCP-1-4, MIP-1 α and β , β -defensins, and RANTES, suggesting active recruitment to sites of disease. Indeed, TB-IRIS patient **necrotic lymph node areas stain intensely for the neutrophil marker CD15** [255]. Those who go on to develop TB-IRIS also exhibit **a lesser ARV-induced decrease** in **GM-CSF** [118] (the trophic factor which drives myeloid mobilisation during infection). Although monocyte counts post-ARV initiation now only non-significantly trend higher in TB-IRIS patients [104], at least during TB-IRD monocyte expression of **chemokine receptors (CCR3, CCR5)** are also elevated [256]. There is an overall **decreased DC proportion** in **ex vivo PBMC at the time of TB-IRIS** [228],

which could indicate decreased production, recruitment into tissues, or 'editing' (destruction) by innate T-cells.

ARV initiation also triggers a **further increase** in **MMP-8** (likely derived from activated/necrotic neutrophils) and **MMP-9**; **MMP-8** and **PIIINP** levels are also **higher at the time of TB-IRIS** [257] [44]. **At the time of TB-IRIS, MMP-7** (likely derived from activated monocytes/macrophages) also remains **higher**, and levels are (non-significantly) **suppressed by corticosteroids** concomitantly with symptom reduction [258]. Post-ARV initiation IL-3 dynamics have not been determined.

Monocyte counts, too, are **increased at the time of IRIS** [44]. In response to ARV therapy initiation, both **sCD14** and **sCD163** (possibly from neutrophils and/or monocytes) **increase, and are higher at the time of IRIS**, indicating myeloid cell activation [104] [119]. Interestingly, initiation of ARV therapy apparently causes **monocyte TLR-2 and TLR-4** expression to **decline** (though TLR-2 expression remains elevated) [158] [243], despite the likely continued presence of inflammatory stimuli and myeloid activation, perhaps indicating lifting of HIV-induced TLR/IL1R-MyD88-IRAK4 signalling inhibition (i.e. increased signalling pathway potency, requiring fewer surface TLR to retain responsiveness to ligands), or the provision of TLR ligands (i.e. cells may then decrease TLR expression-mediated sensitivity to such ligands, which are no longer in short supply). Simultaneously, **TLR-2** expression only **on mDC increases** in response to ARV therapy initiation, though overall DC TLR-2 expression is equivalent to that of controls at the time of TB-IRIS (**post-IRIS, mDC TLR-2 expression normalises**) [158].

All TLR (as well as the IL-1 and IL-18 receptors) signal via the MyD88 adaptor protein to activate NFκB, except for TLR-3 (which signals via TRIF to activate IRF3) and, partly, TLR-4 (which also signals via MAPK to activate AP-1 and via TRIF to activate IRF3) [259] [260] [261] [262]. The **blood transcriptome at the time of TB-IRIS** indicates **over-representation** of transcribed genes belonging to **TLR/MyD88 signalling and TREM-1 activation of inflammasome pathways** [263] (though it is unknown which cell types these extracellular transcripts originate from; transcripts may also be released by lysed/necrotic myeloid cells, e.g. neutrophils). Indeed, ARV therapy initiation leads to **increased transcription of AIM2, NLRP1, NLRP3, and NLRC4** (as well as **higher levels of AIM2, NLRP1, and NLRP3 transcripts at the time of IRIS**) [137], and **increasing** circulating levels of **IL-1β and IL-18** [263] (again possibly originating from lysed/necrotic cells rather than being secreted) which **peak at the time of TB-IRIS** [31]. Indeed, **at the time of TB-IRIS**, plasma concentrations of **CASP-1 and -5** are also **elevated** [263]. Unsurprisingly, *ex vivo* **inhibition of MyD88 or group 1 caspases** markedly **decreases pro-inflammatory cytokine production (including IL-1)**, but **only in PBMC from TB-IRIS patients** [263]. TLR-4 is a key PRR involved in Mtb clearance, contributing significantly to modulating the balance between infected cell apoptosis and necrosis. Interestingly, autophagy opposes inflammasome activity, inflammation, and fibrosis. The decrease in VL induced by ARV therapy thus likely removes embargoes on TLR and inflammasome activity, and likely also results in decreasing production of type I IFN (which otherwise engages in strong reciprocal counter-regulation of IL-1 production and activity), although circulating type I IFN levels are apparently equivalent to those of controls at the time of TB-IRIS [263]. At the time of TB-IRIS, **IL-1Ra** levels are (unsurprisingly) **elevated** [119].

Similarly to baseline, **LPS and endoCAB** levels **do not differ** from those in controls during the course of TB-IRIS, though **I-FABP** exhibits a **greater decrease** [150], likely reflecting more rapid recovery of gut mucosal integrity in response to ARV therapy. **At the time of TB-IRIS**, the free **LBP** level is **elevated** [150], indicating either decreased LPS/LAM-mediated consumption of it, or increased production as part of a new acute-phase response (the latter being more likely). Although urine LAM has not been monitored during the course of ARV therapy in TB-IRIS developers, a TB-IRD study (any IRD) [130] does report that anti-LAM IgG levels are higher during ARV therapy, but equivalent (to control levels) at the time of TB-IRD. As mentioned above, prior administration of anti-LAM IgM causes rapid LAM clearance from circulation, via decreased splenic and increased hepatic deposition [124]. This suggests that higher levels of anti-LAM IgG during ARV therapy may favour deposition in hepatic rather than splenic macrophages, although the ultimate significance of this is unknown.

Although initiation of ARV therapy induces **further increased C5** expression by the intermediate monocyte subset (including a **higher level at the time of TB-IRIS**), differences from controls remain **non-significant due to high variability** [159]. Even more so than at baseline, **intermediate monocytes at the time of IRIS** exhibit **increased inflammatory gene transcription**, still including **PRR and complement (including higher C1q and lower C1-INH transcript levels)**, and reflecting **dysregulation of pathways involved in hematopoiesis (including B-cell development), primary immunodeficiency, innate-adaptive**

communication (including T-cell signalling), and tryptophan degradation (including NAD biosynthesis from tryptophan) [160]. At the time of TB-IRIS, both transcription [263] and monocyte (CD14⁺⁺CD16⁻, CD14⁺⁺CD16⁺, and CD14⁺CD16⁺⁺) expression [137] of FcγRI increases (active TB is known to enhance FcγRI expression and FcγRI signalling, while successful ATT usually decreases FcγRI expression [264] [89] [265] [266]). Although FcγRI-mediated uptake *should* result in increased phagocyte microbicidal activity and Th1 response induction (as compared to uptake via complement receptors), an ex vivo proteomic study of unstimulated PBMC [247] suggests that TB-IRIS continues to be characterised by **relative under-utilisation** of leukocyte **FcγR-mediated phagocytosis**, suggesting impaired Mtb and antigen clearance.

As mentioned, myeloid phagocytes activated by exposure to intracellular pathogens produce not only mature IL-1β and IL-18, but also IL-6, TNFα, IL-8, IL-12, IL-15, IL-23, IL-27, MCP-1, MIP-1, RANTES, and type I IFN, among other cytokines and chemokines. After initiation of ARV therapy, **greater increases in circulating IL-1β, IL-18, IL-6, TNFα, and IL-8** occur in those who go on to develop TB-IRIS, and **their levels are higher at the time of TB-IRIS** [267] [137] [161] [146] [118] [257] [268] [41] [238] [153] [118]. With the **exception of IL-8 (not investigated), these five cytokines become suppressed by corticosteroids** concomitant with symptom reduction (Table 5) [41] [269]. ARV therapy also **increases** the levels of **IL-12, possibly of IL-23, and of MCP-1** [41] [103]; dynamics of the other cytokines remain largely uninvestigated (Table 5). As corticosteroids decrease IFNγ levels, it logically follows that levels of type II ISG (e.g. IP-10 and MIG) will also decrease, and indeed **corticosteroids decrease IP-10 levels** in the context of TB-IRIS [269]. **At the time of TB-IRIS, NO** levels, surprisingly, remain **lower** [137], perhaps due to iNOS inhibition by IL-6 and TNFα or ROS, or due to intracellular glutathione depletion.

Table 5 Dynamics of Selected Activated Myeloid Cell Cytokines During the Course of TB-IRIS

Factor	Effect of ARVs	Level at Time of TB-IRIS Relative to Non-IRIS-developers	Effect of Corticosteroids	Inferences
IL-6	greater increase (higher increase associated with earlier TB-IRIS)	higher	suppression	significantly involved in clinical presentation
TNFα	greater increase	higher	suppression	increased myeloid microbicidal activity increased but undirected myeloid motility increased TNFR-mediated cell death
IL-8	greater increase	higher	ND	further increased neutrophil recruitment and priming
IL-12	increase	peaks	suppression	acting on IL-12-sensitised T-cells, this sudden increase may over-stimulate Th1 and cytotoxic activity
IL-15	ND	ND	ND	NA
IL-23	ND	higher IL-12p40 (also belongs to IL-12)	ND	synergy with IL-6 to promote a Th17 response including production of IL-17 and IL-22 (from neutrophils, γδT-cells, NKT cells, NK cells, and Th17) and production of IL-21 by Th17
IL-27p28	ND	ND	ND	NA
MCP-1	increases	peaks lower (ex vivo stimulation: higher production)	ND	increased monocyte recruitment and antigen clearance, but still poorer than in controls
IP-10	increases	higher	suppression	enhanced target leukocyte recruitment
MIG	increases	peaks	ND	enhanced target leukocyte recruitment
MIP-1	ND	ND	ND	NA
type I IFN (IFNα2)	ND (likely decreases with VL)	non-significant difference [263]	ND	decreased IL-1 counter-regulation decreased stimulus for IL-10 production decreased opposition of antigen presentation and Th1 activity

Altogether, the above findings suggest continued higher inflammasome activation at the time of TB-IRIS, perhaps due to further induction of host cell stress/death and release of alarmins/DAMPs (e.g. ATP, altered K⁺/Ca²⁺ signalling, mtROS, mtDNA, or HMGB1, among others), much more prominent TLR signalling than at baseline (perhaps in part due to a decrease in counter-regulation by Mtb- and/or HIV), and increased availability of Mtb antigens (e.g. lipomannans signalling via TLR-2/CD14) as well as Mtb or HIV nucleic acids (signalling via intracellular TLR or AIM2/NLRP3). Indeed, literature appears to support that cytoplasmic nucleic acids (perhaps including host mtDNA) contribute to inflammasome/cGAS/TLR activation, and that extracellular/endocytosed Mtb antigens (e.g. LAM) rather than LPS contribute to TLR activation of innate myeloid cells (probably enhanced by TREM-1), leading to production of ROS and RNS (but not NO), inflammatory cytokines, IL-10, MMPs, and TF. Indeed, it appears that both Mtb-derived TLR-2/CD14 agonists and TREM-1 ligands are proximal triggers of the ARV-induced inflammation underlying TB-IRIS, although additional proximal triggers (e.g. viral or

host antigens) may also exist/contribute. Ultimately, the identities of such proximal post-ARV therapy initiation inflammasome-, TLR-, and cGAS-activating signals remain to be confirmed.

Regarding lipid mediators, both the LOX and COX arms of the eicosanoid cascade are active at the time of TB-IRIS, as evidenced by **increased** levels of ALOX5-derived linoleic acid product **9-HPODE** (and its product **9-HODE**) [223] and by **increased** expression of **COX-1/PTGS1** [255]. Although the LOX arm is activated, it may preferentially metabolise linoleic acid (rather than arachidonate), and therefore perhaps continues not to favour (LTA4H-mediated) LTB4 production (despite neutrophilic inflammation). The oxidised lipid product 9-HODE is known to bind the oxidised fatty acid receptor GPR132 to activate PPAR γ (and thus modulate LXR, cholesterol homeostasis, and CD36 expression), causing secretion of PAI-I/SERPINE1, the major inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA/PLAU), both of which convert plasminogen to fibrinolytic plasmin (thereby favouring maintenance of a thrombotic state). The urokinase receptor (PLAUR) associates with CR3, and their expression increases on activated myeloid cells. During ARV therapy, circulating **TF levels elevate further**, and are **higher both at the time of TB-IRIS and after** [104] (D-dimer dynamics have not been studied in this context). Taken together, this suggests further enhancement of hypercoagulability during development and manifestation of TB-IRIS. Fever observed at the time of TB-IRIS indicates hypothalamic action of endogenous pyrogens (e.g. IL-1, PGE2, TNF α , MIP-1, etc.), further supporting innate leukocyte activation (including inflammasome activity).

1.6.2.2. Innate Lymphocyte Activity *During Pathogenesis*

During ARV therapy, there is a further **increase** in the **percentage of $\delta 2$ lacking inhibitory receptors**, and levels of these are also **higher at the time of TB-IRIS** [228] (though the ex vivo PBMC proportion of $\gamma\delta$ T-cells trends downwards) [51]. It is possible that $\delta 2$ are expanding in response to Mtb lipids, elevated host isoprenoid levels, or a combination of both (perhaps due to especially-delayed clearance of mycobacterial phosphoantigens, or to elevated host cell IPP biosynthesis). Similarly, **NK cell CD56** (lineage and **cytotoxicity marker**, lost when cells become anergic) **and IL-18R expression increases**, and **NK counts are higher at the time of TB-IRIS** (still containing an **elevated percentage of IL-18R $^{+}$** cells, although **activation marker expression is lower** and **degranulation marker** expression levels **match those of controls** [231]). Thus, the elevated baseline NK cell degranulation capacity that is a risk factor for TB-IRIS development does not persist at the time of TB-IRIS. Another study finds no difference in ex vivo PBMC NK cell proportions at the time of TB-IRIS [51], supported by a second study reporting no difference in NK cell subsets [230]. During TB-IRIS onset, iNKT cells are depleted in the CD4+CD8- subset, and exhibit signs of degranulation [232]. However, in ex vivo PBMC from patients at the time of TB-IRIS, proportions of iNKT cells are significantly elevated, but these cells are indeed a potential "source of perforin" [51]. They may also contribute to GM-CSF production in response to Mtb antigens [270] during TB-IRIS.

The activity of $\delta 2$ against intracellular pathogens (including mycobacteria) is well known, including potent antiviral functions (e.g. lysis of HIV-infected cells and production of replication-regulating cytokines) [271]. However, HIV induces significant changes in the composition and function of circulating $\gamma\delta$ T-cells, which may facilitate opportunistic infection and neoplasia [272] [273] [271] [274]. Both $\gamma\delta$ T-cells and NK cells influence DC activity, thereby modulating the $\alpha\beta$ T-cell response [271]. For example, NK cells lacking inhibitory KIR can 'edit' immature DC via an NKp30-dependent process [275]; perhaps this is also true of $\gamma\delta$ T-cells lacking inhibitory receptors. At least during advanced macaque SIV/BCG-coinfection, ARV-induced restoration of BCG phosphoantigen-induced $\gamma\delta$ T-cell expansion and function also requires expansion of competent PPD-specific IFN γ^{+} Th1 [276]. Similarly, during human HIV-monoinfection, effective ARV restores the TCR repertoire and the *in vitro* ability of $\delta 2$ to produce IFN γ in response to phosphoantigen challenge [276]. Furthermore, relevant **genetic risk factors** for TB-IRIS include: **carriage of the KIR2DS2 or KIR2DS1 + HLA-C2 pair**, and **non-carriage of the KIR2DL3 + HLA-C1/C2 or KIR2DL1 + HLA-C1/C2 pairs** [242].

As TB-IRIS develops, the cytokine milieu changes from that at baseline, thereby influencing leukocyte (including T-lymphocyte) polarisation and phenotypes: IL-1 β and IL-18 remain high (though counter-regulation of IL-1 β by type I IFN is decreased), and IL-6, TNF α , and IL-12 become elevated (in the presence of cells extra-sensitised to IL-12; IL-27 may remain high but has not been investigated in this context). Although no studies have examined the *in vivo* IL-15 response to ARV therapy, including in

context of TB-IRIS, ex vivo PBMC from ARV responders during HIV-monoinfection recover normal levels of IL-15 production [277], so elevation of IL-15 may occur. Similarly, levels of IL-27 decline during HIV infection (though during advanced chronic infection IL-27 levels are indistinguishable from HIV-negative subjects) and normalise during ARV therapy [278] [279] [280], so ARV therapy may further elevate IL-27 levels.

The likely cytokine milieu thus continues to suggest support/induction of a Th1-type response, including production of IFN γ and TNF α to enhance phagocyte microbicidal activity. Indeed, at the time of TB-IRIS both NK and Th1 IL-18 receptor expression peaks [137], and production of both IFN γ and TNF α , and the Th1-type response chemokine MCP-1, all increase (though MCP-1 still remains lower at the time of TB-IRIS) (*vide supra*, Table 5).

Simultaneously, elevated IL-1 β and IL-6 – and possibly IL-23 – may also favour concomitant induction of a Th17-type response, with production of IL-17 and IL-22 from neutrophils, $\gamma\delta$ T-cells, NKT cells, NK cells, and Th17 (including production of IL-21 by NKT and Th17). Although gut mucosal Th17_{mem} become significantly depleted during HIV infection, IL-22 supports mucosal integrity, IL-17 (as part of this) supports enhanced neutrophilic inflammation, and IL-21 further supports Th17 induction and B-cell activity, including memory induction and B_{mem} reactivation). Indeed, levels of **IL-17 do increase** after ARV initiation [118] [153] [41], and **higher circulating IL-22 levels are present at the time of TB-IRIS** [281], probably produced by NK cells (since their count is higher at the time of TB-IRIS, and since they are typically the major producers of IL-22). Too, IFN γ – elevated at the time of TB-IRIS – induces epithelial IL-22R expression. NK cells may thus also contribute significantly to IL-17 in this setting. Indeed, Xu et al. (2016) report that “NKT-like” cells (in addition to Th17) are a major IL-17 source during pulmonary TB [282]; these may in fact represent NK cells.

During ARV therapy, **IL-2 levels increase, peaking at the time of TB-IRIS** (including higher levels of production by ex vivo stimulated PBMC) [153] [267] [41]; this indicates production by activated T-cells, supporting their expansion (especially terminal differentiation rather than memory) and induction (especially with IL-15) of Th1-type cytotoxicity (though IL-15 would also favour memory generation, maintenance, and reactivation). Similarly, **IL-7 levels remain higher at the time of TB-IRIS** [150] (despite concomitant expansion of T-cells bearing IL-7-consuming IL-7R), suggesting enhanced T-cell (including memory, especially in combination with IL-15) maintenance.

Cytotoxic mediators are released from the granules of cytotoxic (innate and adaptive) lymphocytes to induce apoptosis of infected target cells. Perforin is produced by neutrophils, NK cells, and CD8⁺ T-cells and facilitates delivery of cytotoxic granule contents (e.g. granzyme B, secreted by cytotoxic lymphocytes as well as by neutrophils – *vide infra*) into target cells [51]. The serine protease granzyme B, once it has accessed the target cell's cytoplasm, initiates apoptosis [51] and pathogen clearance (e.g. via interaction with substrates such as pro-CASP-3 [283]). Innate lymphocytes (NK cells, $\gamma\delta$ T-cells, and NKT cells) can recognise non-peptide antigens and contain granule-associated perforin and granzyme B [51]. Regarding cytotoxic mediators which may be produced by such innate T-lymphocytes (or neutrophils), **granzyme B levels are elevated at the time of TB-IRIS**, and are **suppressed by corticosteroids** concomitantly with symptom reduction [51]. All that is known regarding **perforin** is that **ex vivo stimulation TB-IRIS patient PBMC results in more pronounced production** [51]. Granulysin levels have not been investigated in this context. These (as well as baseline) findings support a contribution by increased cytotoxic activity to TB-IRIS pathogenesis, though cytotoxic mediator sources remain unclear. Given the decreased proportion of $\gamma\delta$ T-cells, they may not be a major source [51] (unless their decreased proportion reflects redistribution from circulation to sites of disease). Both CD4⁺ and CD8⁺ T-cells are sources of perforin in TB-IRIS cases, including iNKT cells, whose ex vivo proportions among PBMC are elevated at the time of TB-IRIS [51]. Though ex vivo Mtb-stimulated PBMC from TB-IRIS patients (relative to non-IRIS) also produce relatively higher levels of both perforin and granzyme [51], this could perhaps derive largely from CD8⁺ T-cells rather than iNKT (given the relative frequency of iNKT versus CD8⁺ T-cells).

However, neutrophils – whose levels are significantly elevated at baseline, become further increased during ARV therapy, and are elevated at the time of TB-IRIS – also secrete both perforin and granzyme B on activation, including in response to Mtb antigens at the site of disease (e.g. pulmonary granulomas) [284] [283]. Indeed, large numbers of granzyme B-expressing neutrophils are present in granulomas of Mtb-infected macaques and humans, and contain higher numbers of granzyme B⁺ granules than do local T-cells [283]. Furthermore, elevated neutrophil (but not T-cell) granzyme B expression is associated

with a higher bacterial burden [283] (indeed, neutrophilia has been previously associated with delayed antigen clearance). Although neutrophils employ various mechanisms to kill pathogens (e.g. ROS/RNS, AMPs, NO, proteases, NETosis, etc.), they are also able to target host cells for killing, for example via ADCC [285]. FcγRIII are involved in ADCC, with FcγRII being the major ADCC-mediating receptor in neutrophils [286]. Unlike cytotoxic T-cells (which use granule-dependent killing) and macrophages (which use phagocytosis-dependent killing), the neutrophil mechanism of ADCC is a unique process called trogocytosis, in which neutrophils mechanically disrupt target cell plasma membranes through active endocytosis of cytoplasmic fragments, leading to target cell lytic (necrotic) death [286]. While it is unclear whether perforin and granzymes are involved in trogocytosis, neutrophils may nevertheless represent a major source of perforin and/or lytic granules during TB-IRIS pathogenesis

1.6.2.3. Adaptive Lymphocyte Activity During Pathogenesis

At the time of TB-IRIS manifestation, lymphocyte counts are generally lower [44]. Dynamics of specific subsets are covered below.

CD4⁺ T-cells

Initiation of successful ARV therapy lowers the VL and **increases the CD4 count**, especially so in TB-IRIS developers (who typically have lower baseline CD4 counts). Indeed, in such patients, **CD4 expansion co-occurs with conversion of T_{cm} to T_{em}** (including activated Fas⁺ cells expressing the CXCR3 receptor for IP-10 and MIG), as well as an **expansion of Mtb-specific IL-2⁺IFNγ⁺TNF⁺ T-cells (only associated with TB-IRIS if IL-6 levels rise concomitantly**, however). This suggests reactivation of Mtb-specific T_{mem} and homing to the site of disease (i.e. suggestive of TB-IRIS being the manifestation of a recall rather than a primary response). However, the Mtb-specific Th1 'explosion' and simultaneous high IFNγ levels also occur in a subset of TB-IRIS non-developers [26]: they are insufficient to drive TB-IRIS manifestation (and do not drive the IL-6 increase associated with TB-IRIS). Furthermore, ARV therapy induces an **increase in CD25/IL-2R⁺CD127/IL-7R^{lo}FoxP3⁺ T_{reg}** [287]. However, **at the time of TB-IRIS**, while the **proportion of FoxP3⁺ T_{reg} are equivalent to those of controls** [26], the **T_{reg}/T_{em} ratio remains higher**, suggesting that expanding T_{em} must have been present at much lower levels. However, these **T_{reg} exhibit decreased suppressive capacity (e.g. IL-10 secretion)** [287], although there appears to be no significant difference in T_{em} activation relative to controls. Ex vivo, there is a higher percentage of Mtb (especially AC1)-responsive activated polyfunctional T_{em} (with no difference from controls in anergy marker – CD71, the transferrin receptor – expression) [26]. Even post-IRIS, the higher T_{em}/T_{cm} ratio persists (including a higher percentage being Mtb-specific), but these T_{em} are mainly IFNγ-monofunctional. Regarding Th1-type responses, we have already seen that **IFNγ, TNFα, and IP-10** [288] [238] [161] **are higher at the time of TB-IRIS (IP-10 levels increase and MIG also peaks** [146] [31]), suggesting induction of type II ISG, enhancement of phagocyte microbicidal activity, and increased recruitment of activated macrophages, DC, NK cells, and T- and B-lymphocytes. The ex vivo IP-10 response to RD1 antigens is also higher [235]. MIP-1 dynamics have not been investigated in this context.

Taken together, the above results suggest reactivation, redistribution, and expansion of Mtb-specific T_{mem}, which mount a Th1-type response characterised by production of TNFα and IFNγ (with the latter inducing expression of ISG such as IP-10), and that the IRIS phenomenon is dependent on heightened production of TNFα. Such T_{mem} - already having lower activation thresholds - may be activated either by a rapid increase in availability/levels of Mtb antigens induced by ATT and ARV therapy ± DAMPs and alarmins from stressed/dying host cells. The alpha-crystallin 1 protein (AC1) is an Mtb hypervirulence protein associated with bacterial proliferation (i.e. highly antigenic), and the DosR regulon member most dramatically induced in response to hypoxia and NO stress [289] [290]. If the ATT interval is short enough to leave some Mtb bacilli viable, inflammation (hypoxia, but not NO)- or drug (ATT, ARV)-induced Mtb stress may induce active expression of immunogenic DosR member AC1. Large, rapid responses to this antigen would support a memory response, despite Mtb-activated CD4⁺ T-cells (i.e. Mtb-focused TCR specificities) being preferentially depleted by HIV (perhaps apart from relatively quiescent T_{mem}).

Simultaneously/alternately, increased PBMC responsiveness to Mtb (relative to non-Mtb) antigens may suggest a larger *pre-existing* pool of Mtb-specific T_{mem} (i.e. more advanced TB at baseline, or – surprisingly – decreased HIV-induced depletion of Mtb-specific T-cells) rather than a memory response

initiated specifically by Mtb antigens. As mentioned, higher baseline levels of IL-7 would support development and maintenance of T_{mem}. Although systemic clinical manifestation (including lymphocyte proliferation in secondary lymphoid organs) is relatively slow (~two weeks), it is possible that sub-clinical memory responses to Mtb antigens (either sequestered antigens being liberated, or antigens produced at higher rates by stressed viable Mtb) already begin much earlier in organ sites previously affected by TB prior to ATT. If we consider the TST response, specifically, it represents delayed-type hypersensitivity (i.e. executed by previously-sensitised T-cells in response to antigen re-exposure), occurring within 24 and waning within 72 hours of re-exposure [291] [292], although this responsiveness persists for months in TB-IRIS developers. Terminology refers to “restoration” of TST responses as HIV-mediated immunosuppression is lifted by ARV therapy (with a tendency towards fever in TB-IRIS patients), but the phenomenon may also represent new TST responses. Overall, TB-IRIS most likely represents a combination of initial recall and subsequent new primary immune responses.

Decreased T_{reg} suppressive ability may be due to inhibition by higher levels of IL-7 [287]. Impaired T_{reg} function may contribute to the inappropriate/non-resolving inflammatory response characterising TB-IRIS. A non-exhaustive list of T_{reg} inhibitory mechanisms includes [293] [294] [295] [296]: direct inhibitory cell-cell contact, production of immunoregulatory cytokines (e.g. IL-10, TGFβ, and IL-35) and other soluble suppressive factors (e.g. the DC- and Th-inhibitor adenosine, and the antigen presentation attenuator deubiquitinase A20), and depletion of various factors required by or stimulatory to T-cells (e.g. IDO depletes tryptophan and generates additional suppressive metabolites such as kynurenine, depletion of IL-2 via IL-2R and via cAMP transfer across gap-junctions to inhibit IL-2 production, depletion of ATP - a DAMP - via CD39 and CD37, reduction of IL-6), and inhibition of synthesis of antioxidants - e.g. glutathione - which support production/proliferation of Th1 and NK cells. It is possible that such mechanisms are more active at baseline and recede at the time of IRIS.

Despite decreased FoxP3⁺ T_{reg} IL-10 secretion, ARV therapy **further increases already-high circulating IL-10 levels** [31], suggesting production by another cellular source (e.g. FoxP3⁺ Tr1 or regulatory myeloid cells). Levels of IL-10 are apparently higher at the time of TB-IRIS (although findings are mixed [103] [41] [281]) and indeed **corticosteroids suppress IL-10** levels concomitantly with symptom reduction [269]. Higher IL-10 levels would impair antigen clearance via inhibiting antigen presentation and Th1-type immunity, in an attempt to resolve inflammation and limit inflammation-mediated tissue damage. Conversely, ARV therapy **decreases TGFβ** levels, which – **at the time of TB-IRIS – trend lower** [41]. Surprisingly (given radiographic findings during TB-IRIS), this suggests a *decreased* fibrotic tendency. Such lower TGFβ levels may be due to reciprocal counter-regulation by higher IL-7: each down-regulates expression of the other, with TGFβ for example inhibiting IL-7-induced pre-B-cell proliferation [297].

Type 2 cytokines (produced by and favouring induction of type 2 leukocytes, e.g. ILC2, mast cells, eosinophils, basophils, M2, and Th2) include IL-4, IL-5, IL-9, IL-13, IL-25, IL-32, IL-33, and TSLP (the latter acting partly via the IL-7R) [298] [299]. Mtb is known to induce IL-32 (in an IFNγ- and COX2-dependent manner) [298], and internalisation of HIV by epithelia is known to induce TSLP production [300]. Furthermore, via modulation of TLR and NOD signalling and NFκB, MAPK-AP-1, and CASP1 activation, IL-32 elicits TSLP, IL-1β, IL-6, TNFα, and IL-8 [298]. This would explain above observations regarding TB-IRIS, but IL-32 has yet to be investigated in this context. Polarisation towards a Th2 phenotype licenses T-cells to produce IL-4, IL-5, and IL-13, although innate cell types are also capable of producing these cytokines. During both HIV [301] [302] infection and TB [303] [301] (including co-infection [301] and **at the time of TB-IRIS manifestation** [150]), circulating **IL-4 becomes elevated** [150], perhaps due to a skewing of responses by Th2-inducers e.g. IgE, TSLP, etc. (or a return to an inflammatory state similar to that present at baseline, provided that TB/HIV-coinfection was more advanced to start with – although IL-4 levels at baseline remain uninvestigated in this context). Circulating levels of IL-5 also increase during TB [304], and when TB is sufficiently advanced, ATT induces a further IL-5 increase (rather than decrease) [136]. At the time of TB-IRIS, ex vivo stimulated PBMC produce higher levels of IL-5 and IL-13 [41] (the latter perhaps due to e.g. NKT cell conditioning by higher levels of IL-4).

Regarding Th17-type responses, as mentioned, a **greater increase in circulating IL-17** occurs on ARV therapy [118], but levels *at the time* of TB-IRIS have not been explicitly determined. An ARV-induced **increase in circulating IL-22** also occurs in TB-IRIS patients [281]. This suggests higher levels of both these cytokines (and likely also IL-21) at the time of TB-IRIS; higher NK cell-mediated production of IL-22 could

be stimulated by higher IL-15 levels, although IL-15 dynamics have not been investigated in this context (recall also that elevated IFN γ levels enhance epithelial IL-22R expression).

Taken together, these findings suggest simultaneous activation of multiple opposing immune response types (Th1, Th2, Th17, and regulatory) in response to ARV therapy initiation and at the time of TB-IRIS. Incompatible biological programmes thus do not function as intended, and can contribute to immunopathology. As mentioned above, one publication suggests that the CD4:CD8 ratio becomes elevated during TB-IRIS pathogenesis.

CD8⁺ T-cells

Although overall CD8 count is roughly maintained during HIV infection, the proportions of IFN γ ⁺ and TGF β ⁺ CD8⁺ T-cells decline and increase, respectively, suggesting higher levels of TGF β production by CD8⁺ T_{reg} in more advanced HIV. In TB-IRIS developers, ARV therapy initiation does not alter CD8⁺ T-cell activation status relative to controls, and this situation persists at the time of TB-IRIS despite a concomitant **increase in the proportion of T_{em} and terminally differentiated cells (with increased terminal effector activation)**. Ex vivo PBMC stimulation also demonstrates diminished responsiveness to non-Mtb antigens. Even **post-IRIS, higher proportions of terminally-differentiated CD8⁺ T-cells persist**.

B-cells and Antibodies

Up to and at the time of TB-IRIS, titres of anti-PGL-Tb1 antibodies remain lower [248], but then increase over months before decreasing again [246]. "The anti-PGL-Tb1 antibody response is independent of the degree of immunosuppression in HIV-TB patients and the clinical presentation of TB" [248]. Similarly, **titres of antibodies against other Mtb antigens (ManLAM, ESAT-6/CFP10) remain equivalent (relative to controls) throughout follow-up** [246], again perhaps suggesting already-maximal titres.

1.6.2.4. Pathogenesis: Conclusions

Though baseline already exhibits both, ARV therapy further enhances neutrophilia (now likely G-CSF-driven) and inflammasome activation (fig. 6). Neutrophils are activated, traffic to inflammatory sites including lymph nodes, and are likely a source (via secretion or necrosis) of elevated MMP-8 and thus collagen turnover. Airway inflammation evident on radiology and histology indeed suggests leukocyte infiltration, matrix destruction, and fibrosis; symptoms preferentially affecting previously TB-affected organs suggests the highest levels of Mtb antigens are present in these locations (whether residual Mtb antigens sequestered within granulomas but now being liberated, or antigens newly-produced by stressed Mtb).

Despite a lesser decrease in GM-CSF, chemokine-mediated monocyte trafficking to inflammatory sites post-ARV therapy initiation, and decreasing monocyte TLR-2 (though only partial) and TLR-4 expression (likely due to lifting of Mtb- and HIV-induced inhibition of TLR signalling), monocyte counts only trend higher [104] (lower ex vivo DC proportions likely also indicate trafficking to disease sites, and ARV therapy stimulates increased mDC TLR-2 expression). Likely monocyte/macrophage-derived *MMP-7 is also elevated and contributes to collagen turnover (although additional targets include pro-TNF α and pro-MMP-1, -2, and -9). Resembling baseline, intermediate monocytes continue to express levels of C5 which trend higher, and exhibit even more pronounced PRR and imbalanced C1q/C1-INH levels than at baseline (as well as dysregulation of hematopoiesis - including B-cell development, primary immunodeficiency, innate-adaptive communication - including T-cell signalling, and tryptophan degradation - including NAD biosynthesis from tryptophan). However, circulating transcripts of Fc γ R1 also increase at the time of TB-IRIS, suggesting increased mediation of phagocytosis (with consequent enhanced myeloid microbicidal activity and Th1 responses relative to uptake via complement receptors), but proteomic evidence conversely suggests relative under-utilisation of leukocyte Fc γ R-mediated phagocytosis.

ARV therapy also induces a further increase in both sCD14 and sCD163, indicating intensified myeloid activation (and possibly increased cellular sensitivity to TLR-2 ligands as well as even more pronounced haptoglobin-complexed Hb uptake). Indeed, blood transcriptomic profiling identifies many members of the TLR/MyD88 signalling and TREM-1 activation of inflammasome pathways, along with elevated AIM2 and NLRP2 transcripts, and elevated levels of circulating CASP-1 and -5, and IL-1 β and IL-18. Inhibition of

MyD88 or group 1 caspases significantly decreases both inflammasome-matured and other pro-inflammatory cytokine production *ex vivo*. Many of these effects may be attributable to modulation of autophagy by Mtb and HIV, and subsequent alteration of this by ATT and ARV therapy. For example, autophagy negatively regulates NLRP3 and non-canonical inflammasome activity, as well as modulating IL-1 β and type I IFN production [305]. While both pathogens induce and oppose autophagy [306], if the former effect predominates, ATT and ARV may indirectly facilitate less-restrained inflammation. Similarly ARV therapy decreases VL, precipitously dropping the stimulus for production of type I IFN, which also normally counter-regulates IL-1.

ARV therapy appears to rapidly initiate gut mucosal integrity recovery (although recovery continues for many years), and – similarly to baseline – bacterial translocation does not appear to contribute to inflammation, while Mtb-derived antigens (e.g. LAM; ARV therapy stimulates increased production of anti-LAM IgG, for example, and thus increased deposition in hepatic macrophages) likely do. It is also possible that ARV-induced host cell stress and damage lead to further production of alarmins/DAMPs, stimulating myeloid cell (including inflammasome) activation. Indeed, in response to ARV therapy, greater increases in *IL-1 β , *IL-18, *IL-6, *TNF α , and IL-8 occur, and both *IL-12 and MCP-1 (and possibly IL-23) also increase, although MCP-1 remains lower than in controls at the time of TB-IRIS (suggesting ongoing deficits in monocyte-mediated antigen clearance). NO levels, too, remain lower (possibly due to iNOS inhibition by IL-6 and TNF α , or due to glutathione depletion), suggesting ongoing deficits in microbicidal activity.

Eicosanoid cascade LOX and COX arms are active, with higher expression of COX-1 and higher levels of linoleic acid metabolites 9-HPODE and 9-HODE (likely rather than LTB₄, although mutations increasing LTA4H activity are associated with more severe TB-IRIS presentations). Via an oxidised fatty acid receptor, 9-HODE may activate PPAR γ , modulating LXR, cholesterol homeostasis, and CD36 expression, and causing secretion of PAI-1/SERPINE1 to inhibit conversion of plasminogen to fibrinolytic plasmin by tPA and uPA/PLAU (thereby favouring thrombosis). Activated myeloid cells express higher levels of PLAUR and associated CR3. ARV therapy indeed further elevates circulating TF and coagulability.

Altogether, the above findings suggest continued higher inflammasome activation at the time of TB-IRIS (when TLR signalling is much more prominent than at baseline), perhaps due to further induction of host cell stress/death and release of alarmins/DAMPs (e.g. ATP, altered K⁺/Ca²⁺ signalling, mtROS, mtDNA, or HMGB1, among others), increased availability of Mtb antigens (e.g. lipomannans signalling via TLR-2/CD14), Mtb or HIV nucleic acids (signalling via intracellular TLR or AIM2/NLRP3), and perhaps in part due to a decrease in counter-regulation by Mtb- and/or HIV-induced autophagy. Indeed, findings appear to support that cytoplasmic nucleic acids (perhaps including host mtDNA) contribute to inflammasome/cGAS/TLR activation (although circulating type I IFN levels are apparently equivalent to those of controls at the time of TB-IRIS [263], which would indeed be possible if TB-IRIS developers experience a more pronounced virologic response to ARV therapy), and that extracellular/endocytosed Mtb antigens (e.g. LAM) rather than LPS contribute to TLR activation of innate myeloid cells (probably enhanced by TREM-1), leading to production of ROS and RNS (but not NO), inflammatory cytokines, IL-10, MMPs, and TF. Indeed, it appears that both Mtb-derived TLR-2/CD14 agonists and TREM-1 ligands are proximal triggers of the ARV-induced inflammation underlying TB-IRIS, although additional proximal triggers (e.g. viral or host antigens) may also exist/contribute. Ultimately, the identities of such proximal post-ARV therapy initiation inflammasome-, TLR-, and cGAS-activating signals remain to be confirmed.

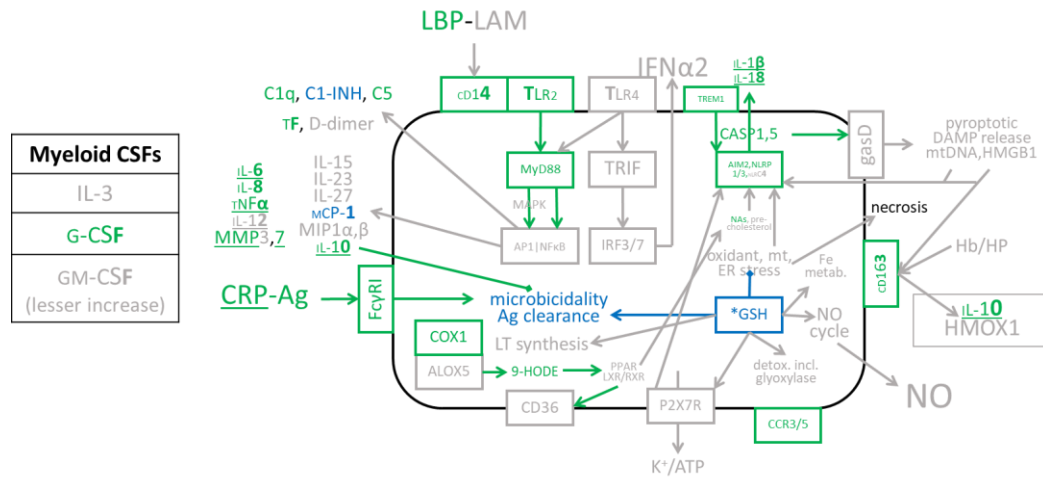


Figure 6 Myeloid Immunometabolic Model of TB-IRIS During Pathogenesis (after ARV therapy initiation and at the time of TB-IRIS). The diagram represents findings regarding monocyte subsets and circulating factors during pathogenesis (i.e. after ARV therapy initiation) and at the time of TB-IRIS manifestation. Green represents elevated/activating, blue represents depressed/inhibiting, grey represents unknown. Factors represented by changing font size increase or decrease during ARV therapy. Underscored factors represent those decreased by exposure to corticosteroids. Although monocyte counts now only trend non-significantly higher (increasing GM-CSF may now assist in elicitation/mobilisation), baseline neutrophilia persists, although now likely due to elevated G-CSF as well as elicitation/mobilisation by C5a and IL-8. These neutrophils are activated (shedding L-selectin) and express CCR2 and 5, covering chemokines such as MCPs (MCP-1 levels are now increasing), MIPs, β -defensins (not investigated but likely elevated given elevated IL-22 and mucosal recovery; pictured in the following figure), and RANTES (the same chemokines which ligate monocyte-expressed CCR3 and 5, pictured above). Further elevating and still higher circulating MMP-8 levels likely derive from these activated neutrophils, and in general higher activated myeloid MMP (as well as PIINP) levels suggest ongoing ECM turnover. At least *ex vivo*, proportions of DC are decreased. Activated myeloid cells continue their elevated expression of CD163 and CD14 and shedding in their soluble forms. Similarly, higher C1q activation (unopposed by C1-INH) and C5 production, as well as higher Fc γ RI and CRP expression levels continue (with proteomic studies continuing to suggest under-utilisation of Fc γ R-mediated uptake pathways, still perhaps due to inhibition of phagosome maturation by IL-10, which would also retard antigen clearance). Levels of LPS and anti-LPS antibody continue being equivalent to CTRLs, though urine LAM has not been investigated in this context. The pattern of complement, TF, and cytokine elevation continues to be consistent with TLR-MyD88-MAPK-AP-1 (and possibly also NF κ B) and inflammasome signalling (with both TLR and inflammasome signalling enhanced by TREM-1 ligation), although iNOS/NO have not been investigated in this context. Since TLR/MyD88 and TREM-1/inflammasome signalling data derive from whole blood transcriptomics, the leukocyte type of origin is unclear, and may even represent lysed myeloid cells (e.g. neutrophils, which express high levels of these components). Since 9-HODE is known to induce SERPINE1 expression, it is possible that this protein inhibits fibrinolytic tPA and uPA/PLAU, further exacerbating the pro-thrombotic state (even though activated myeloid cells express elevated PLAUR in association with C3). Although both partially-treated pathogen- and stressed-host-derived factors may contribute to inflammation, neither LAM (as mentioned) nor circulating mtDNA levels have been investigated in this context. Given baseline host cell metabolic stress is likely further induced by inflammation (including the effects of cell stressors IL-6 and TNF α , for example), it is likely that hypothesised intracellular GSH depletion (and therefore oxidant, mitochondrial, and ER stress, as well as altered Fe metabolism and consequent oxidative stress) continues, though Hb levels have not been investigated in this context. Although type I IFN levels should fall precipitously as antiretroviral therapy suppresses viral load, circulating type I IFN levels are apparently equivalent to those of controls at the time of TB-IRIS (9-HODE will likely continue to impact cholesterol homeostasis).

Regarding T-cell-supporting cytokines, ARV therapy increases IL-2 levels (indicating T-cell activation and support of expansion and terminal differentiation to Th1-type effectors) and IL-7 nevertheless remains higher at the time of TB-IRIS (supporting T-cell – including T_{mem} – maintenance). ARV therapy is known to lower VL, increase CD4 count, and restore the TCR repertoire and the ability of $\delta 2$ to produce IFN γ in response to phosphoantigen challenge (in the SIV/BCG model, restoration of BCG phosphoantigen-induced $\gamma\delta$ T-cell expansion and function is dependent on expansion of competent PPD-specific IFN γ ⁺ Th). Indeed, ARV therapy further increases the proportion of $\delta 2$ lacking inhibitory receptors (which perhaps expand in response to host and/or Mtb isoprenoids), the NK cell count (with a higher proportion expressing IL-18R, but loss of baseline elevation in degranulation marker expression), and the *ex vivo* PBMC proportion of iNKT cells (confirmed to express perforin, and which may contribute to GM-CSF production). In TB-IRIS developers, ARV-induced CD4 expansion (including Mtb-specific IL-2⁺IFN γ ⁺TNF α ⁺ T-cells) is particularly pronounced, with reactivation and conversion of T_{cm} to T_{em} (*ex vivo*, the proportion of Mtb – especially AC1 – specific activated polyfunctional T_{em} is higher) which likely traffic to local sites of inflammation, guided in part by MIG and IP-10. However, expansion of Mtb-specific CD4⁺ T-cells and elevated IFN γ levels only associate with TB-IRIS when IL-6 rises concomitantly, indicating that it is likely not such T-cell responses which drive IL-6 production, that such changes in isolation are insufficient to manifest TB-IRIS, and (unsurprisingly) that IL-6 is involved in TB-IRIS manifestation (furthermore, while the higher T_{em}/T_{cm} ratio persists post-IRIS, only IFN γ – but not TNF α – expression is retained). It is very likely that TNF α is also involved in TB-IRIS pathogenesis, including clinical manifestations (e.g. fever, malaise, weight loss, etc.) and tissue damage.

Typically, TNF α activates NF κ B-mediated pro-inflammatory cytokine and anti-apoptotic molecule expression [307]. However, via TNFR1 TNF α induces CASP-8-mediated apoptosis, though in the absence

of caspases/presence of (e.g. viral) caspase inhibitors, this instead becomes (RIP1/3-, FADD-, TRAF2-, and mitochondrial respiratory chain ROS-dependent) necroptosis (interestingly, IFN γ and dsRNA also induce cytotoxicity when caspases are inhibited) [308] [309] [307]. Depending on the ubiquitination state of RIP1, its function switches between pro-survival or pro-death [307]. Similarly, RIP3 also acts as a molecular switch between RIP1-mediated necroptosis but not apoptosis (RIP3 is only required for the former), through interaction with metabolic enzymes: RIP3-mediated activation of PYGL, GLUL, and GLUD1 favours necroptosis through enhancing energy metabolism and mtROS generation at ubiquinone (furthermore, enzymatic steps during mitochondrial glutamine oxidation are thought to “sensitise mitochondria to TNF-induced perturbation”) [310].

Interestingly, although CD8 count is largely maintained during HIV infection, the proportion of IFN γ + CD8+ T-cells declines. In TB-IRIS developers, ARV therapy does not alter overall CD8+ T-cell activation status relative to controls, despite an increase in the proportion of CD8+ T_{em} and increased activation of such more terminally-differentiated effectors (with diminished ex vivo responsiveness to non-Mtb antigens).

In TB-IRIS developers, the baseline cytokine milieu shifts, influencing leukocyte (including T-cell) polarisation and phenotype (fig. 7). While IL-1 β and IL-18 remain high, IL-6, TNF α , and IL-12 (in the presence of T-cells sensitised to it) become elevated. Dynamics of IL-15 and IL-27 during TB-IRIS pathogenesis remain unstudied, but elevation of both in response to ARV therapy is possible and even likely. Either way, the cytokine milieu suggests continued support/induction of a Th1-type response by IL-1 β , IL-18, IL-12, and possibly IL-15 (including elevated IFN γ - and TNF α -mediated enhancement of phagocyte microbicidal activity and leukocyte cytotoxicity, as well as induction of type II ISG). Indeed, both NK and Th1 IL-18 receptor expression peaks at the time of TB-IRIS, and levels of IFN γ , TNF α , MCP-1, and MIG increase (as well as IFN γ , TNF α , and IP-10 being higher at the time of TB-IRIS; chemokines suggests increased recruitment of activated macrophages, DC, NK cells, and T- and B-lymphocytes).

Simultaneously, however, elevated IL-1 β , IL-6, and possibly IL-23 may support a Th17-type response (recall that mucosal Th17 are rapidly depleted during HIV infection), including production of IL-17 and IL-22 from neutrophils, $\gamma\delta$ T-cells, NKT cells, NK cells, and Th17 (including production of IL-21 by NKT and Th17). Indeed, IL-17 (perhaps from elevated-count neutrophils or NK cells) increases, IL-22 (probably from NK cells, perhaps in response to IL-15) is higher at the time of TB-IRIS, and elevated IFN γ induces epithelial IL-22R expression. Both IL-17 (via enhancing neutrophilic inflammation) and IL-22 (e.g. via enhancing defensin production) enhance mucosal integrity (in agreement with I-FABP findings), while IL-21 supports Th17 induction and B-cell activity (perhaps supporting production of mucosal-protective antibodies). Indeed, it has been shown that effective ARV therapy (especially in patients with comorbid inflammation-relevant diseases) produces neutrophil hyperactivation with loss of the apoptosis/necrosis balance, in association with high circulating levels of IL-17 and IL-22 [311]. Furthermore, during TB/HIV-coinfection and at the time of TB-IRIS, IL-4 too is higher, perhaps due to induction of Th2-type responses by tissue stress/damage-derived alarmins/DAMPs (e.g. IL-33) and/or for example TSLP or IgE (specific and total IgE levels are known to increase during Mtb infection [312]). Typically, IL-4 also induces IL-13 production. TB also increases levels of IL-5, and in advanced cases ATT causes a further increase (instead of decrease) in these levels. At the time of TB-IRIS, ex vivo stimulated PBMC produce higher levels of IL-5 and IL-13. Indeed, both Th2- and Th17-type responses are implicated in regulation of fibrotic healing (e.g. IL-17A enhances neutrophil recruitment, and both IL-4 and IL-13, with or without TGF β , elicit fibroblast collagen production) [313].

Leukocytes capable of cytotoxicity possess granules containing the cytotoxic mediators perforin, granzyme B, and granulysin. Neutrophils, innate lymphocytes, and CD8+ T-cells all express such mediators. Ex vivo stimulation of PBMC from TB-IRIS patients with Mtb antigens leads to elevated perforin and granzyme production, and circulating granzyme B levels are elevated at the time of TB-IRIS. This suggests either a contribution by cytotoxicity (via degranulation) to TB-IRIS pathogenesis, and/or cell death-mediated extracellular release of granzyme B. Sources may include the elevated level of neutrophils (which can have a higher granzyme B production capacity than T-cells) or $\gamma\delta$ T-cells (since their decreased proportion among circulating PBMC may reflect infiltration into inflammatory sites), both CD4+ and CD8+ T-cells from TB-IRIS cases are sources of perforin (including iNKT cells, whose ex vivo proportions among PBMC are elevated at the time of TB-IRIS, though CD8+ T-cell frequency is still orders of magnitude higher). Both neutrophilia and elevated neutrophil (but not T-cell) granzyme B expression have been associated with delayed antigen clearance and higher Mtb bacillary burdens. Neutrophils

Regarding regulatory cytokines (which may be derived from myeloid or lymphoid regulatory leukocytes), in TB-IRIS developers, ARV therapy further elevates *IL-10 (which may be higher at the time of TB-IRIS), but decreases TGFβ (which trends lower at the time of TB-IRIS). In these patients, ARV therapy increases the number of CD25/IL-2R+CD127/IL-7R^{lo}FoxP3⁺ T_{reg} (although these cells exhibit decreased suppressive function, e.g. IL-10 secretion), but at the time of TB-IRIS the proportion of FoxP3⁺ T_{reg} is equivalent to that of controls. This suggests IL-10 provision by another cellular source (e.g. FoxP3⁺ Tr1 or regulatory myeloid cells), and IL-10-mediated impairment of antigen clearance (reminiscent of baseline). Findings regarding lower TGFβ (suggesting a decreased tendency towards collagen deposition and fibrosis) are surprising, given that during HIV infection the proportion of TGFβ⁺ CD8⁺ T_{reg} increases, and given radiographic findings suggestive of fibrotic changes during and after TB-IRIS. Such lower TGFβ levels may be due to reciprocal counter-regulation by higher IL-7. Observed decreased T_{reg} suppressive ability may also be due to inhibition by higher levels of IL-7, and impaired T_{reg} function may contribute to inappropriate/non-resolving inflammation during TB-IRIS.

Finally, findings suggest abnormal simultaneous activation of multiple incompatible immune response types (Th1, Th2, Th17, and regulatory) manifest in response to ARV therapy initiation and at the time of TB-IRIS and contribute to pathogenesis, with regulatory responses attempting to resolve inflammation and promote healing despite ongoing inflammation-triggering stimuli or inflammation-intensifying circuits.

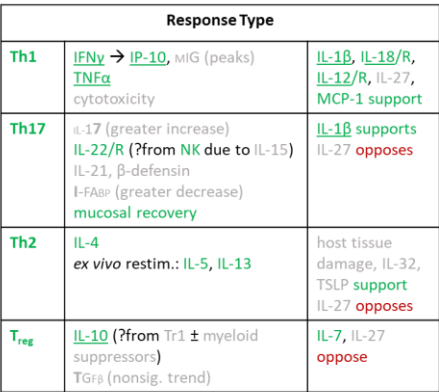


Figure 7 Lymphocyte Immunologic Model of TB-IRIS During Pathogenesis (after ARV therapy initiation and at the time of TB-IRIS). Green represents elevated/activating, blue represents depressed/inhibiting, grey represents unknown. Factors represented by changing font size increase or decrease during ARV therapy. Underscored factors represent those decreased by exposure to corticosteroids. It is noteworthy that the elevated NK cell marker CD56 is also a marker of cytotoxicity (although these cells, while displaying elevated numbers, appear to be less activated and undergoing degranulation levels equivalent to CTLs). At least ex vivo, PBMC restimulation results in higher perforin and granzyme production; although these may originate from increased-count iNKT cells, other producers (CD4⁺ T-cells, CD8⁺ T-cells, and neutrophils) are still present in circulation at far higher frequencies and are more likely sources in vivo (especially given neutrophilia during TB-IRIS, as well as neutrophils possessing larger numbers of granzyme B⁺ granules than T-cells, and large numbers of granzyme B⁺ neutrophils typically being present in granulomas during TB). Steroids lower levels of underscored soluble factors (CRP, IL-1 β , IL-18, IL-6, IL-10, IL12p40, TNFa, IFN γ , IP-10) concomitant with symptom reduction, but (since effects are not due to inhibition of the Mtb-specific T-cell response) mechanisms of steroid benefits during TB-IRIS are incompletely understood [83] [89] [40] [85] [87] and it remains unclear which of these diminishing factors may contribute to symptom reduction. Given that TLR-2, IL-6, and TNFa levels normalise post-IRIS, while higher CD4⁺ IFN γ ⁺ monofunctional T_{em}:T_{cm} ratios and higher activated, terminally-differentiated CD8⁺ T_{em} levels persist post-IRIS, the former PRR and cytokines are more likely to be involved in TB-IRIS pathogenesis than the latter (indeed, IL-6 and TNFa are the two cytokines most consistently elevated during TB-IRIS). It is notable that IL-32 (unstudied in this context, but known to be elicited by IFN γ during Mtb infection, and induced during HIV infection, also favouring IDO production [314] [315]) elicits TSLP (unstudied in this context, but IL-4 is elevated), IL-1 β , IL-6, TNFa, and IL-8, which matches the milieu observed during TB-IRIS.

The vast majority of chronic inflammatory diseases exhibit a myeloid-dominant infiltrate (cells which possess an extensive arsenal of tools for tissue damage), often with activatory support by Th (cells unlikely to execute tissue damage) [316]. Indeed, post-mortem immunohistochemical examination of pulmonary tissue from a patient who died from unmasking TB-IRIS-related lung failure demonstrated infiltration of the parenchyma and alveolar spaces by only few T-cells and NK cells, but an overwhelming number of myeloid cells [10]. The marker (CD68, a lysosomal protein) used in that study to discriminate myeloid cells has classically been used to identify monocytes/macrophages, but is in fact also strongly expressed by endothelia and fibroblasts, and more weakly by lymphocytes [317].

1.7. Discussion: Hypotheses Arising from Integrated Baseline and Pathogenesis-associated Literature

Epidemiological and clinical observations strongly suggest that more advanced TB/HIV-coinfection is what predisposes to the TB-IRIS phenomenon (and could also account for highly-variable incidence rates between studies). This would result in more profound and less reversible immunometabolic equilibrium disturbances, including on therapeutic lifting of dual pathogen-induced immune embargoes (e.g. type I IFN/autophagy-mediated opposition of inflammation, inhibition of TLR signalling), requiring longer treated intervals to re-approach homeostasis.

Such immunometabolic disturbances may include, for example: inhibited TLR signalling, increased type I IFN $\pm$ Th2-type signalling, higher Mtb-induced IL-27/IL-32 levels, abnormally-activated myeloid cells (exhibiting e.g. higher complement production/activity, increased iron sequestration, lower NO production, and possibly elevated IL-10 production), more advanced SLO disruption, a diminished TCR repertoire, an immunological state primed for exaggerated myeloid/memory lymphocyte activation, dyslipidemia, glucose intolerance, cachexia, glutathione depletion, susceptibility to oxidative/mitochondrial/ER stress, and a metabolic state primed for necrosis/necroptosis/ferroptosis rather than apoptosis of stressed cells.

It is very likely that higher Mtb and derived antigen loads are also cleared more slowly in TB-IRIS developers, due to various factors (e.g. CR- rather than FcR-mediated uptake, IL-10 inhibiting phagosome maturation, and possibly-depleted glutathione interfering with microbicidal activity). This would also support the requirement for a longer ATT treatment interval prior to initiating ARV therapy (not justifiable in patients with a CD4 count < 50 cells/ μ L).

Overall, literature supports abnormal baseline myeloid (including complement) activity preceding TB-IRIS, at which time disturbed immune homeostasis results in a relatively-unrestrained further increase in myeloid (including complement) activation, simultaneous induction of incompatible lymphoid response programmes, and transition to inflammation chronicity (either due to persistence of the inciting stimuli, or failure of immune regulatory mechanisms). Corticosteroids may support myeloid-mediated antigen clearance as well as affecting energy metabolism, but partial prevention and alleviation of TB-IRIS symptoms may be largely due to suppression of downstream mediators (e.g. IL-6, TNFa) of clinical manifestation, rather than acting on proximal triggers (the nature of which remain unclear; fig. 8).

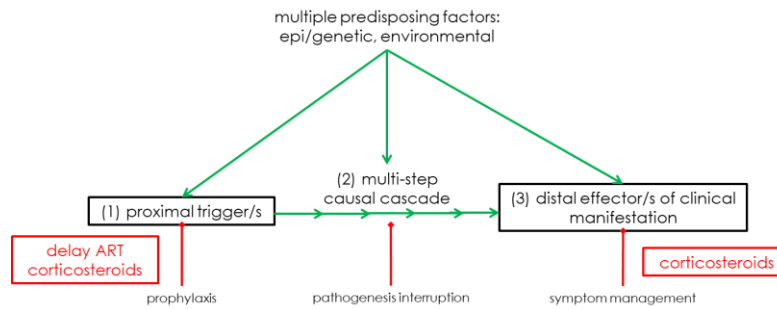


Figure 8 TB-IRIS as a Complex Disorder. Although delaying ARV therapy (ART) or providing pre-ART corticosteroids can prevent TB-IRIS, and corticosteroids can help alleviate TB-IRIS symptoms, much remains unknown about pathogenic chain mechanisms leading to TB-IRIS.

Indeed, we propose a hypothesis most parsimoniously explaining all above observations: in patients experiencing more advanced TB/HIV-coinfection, viable and non-viable Mtb (and derived antigens) are sequestered within tissues (including inside myeloid phagocytes such as neutrophils and macrophages, including within granulomas in competent patients) at sites of TB disease (and ATT stress may induce production of additional antigens by viable Mtb/increase availability of additional antigens as bacilli are degraded). Such granuloma-resident myeloid cells are already metabolically stressed at baseline, and are especially predisposed to inflammatory forms of cell death (necrosis, necroptosis, ferroptosis) rather than apoptosis on exposure to further (e.g. xenobiotic, inflammatory) stress, due to various possible factors (e.g. higher pathogen load, iron sequestration, glutathione depletion worsening oxidative/mitochondrial/ER/ferric stress). We propose that the addition of ARV therapy and rapid drop in HIV replication on this background triggers massive co-ordinated death of such granuloma-resident myeloid cells, leading to release of pro-inflammatory molecules in a manner mimicking new infection (i.e. release of host cell alarmins and DAMPs as well as previously-sequestered Mtb-derived PAMPs and antigens). Due to disturbed immune homeostasis (including a relative lack of normal immunoregulatory mechanisms), this initiates overzealous myeloid activation as well as multiple incompatible lymphoid response programmes (likely initially memory responses), leading to more host cell stress, further-delayed antigen clearance, and chronic inflammation (including the hepatic acute-phase response, hypothalamically-triggered fever, hypercoagulability, SLO lymphocyte expansion, and airway inflammatory infiltrates producing pulmonary symptoms and tissue damage). Tissue damage likely results from a combination of proteolytic enzymes released by dying myeloid cells, from the effects of complement and TNF α , and from the actions of cytotoxic cell types (including neutrophils) and their granule contents.

Indeed, this idea is supported by older observations that ARV therapy re-establishes a vigorous host granulomatous response (with or without caseation in *Mycobacterium avium* complex-associated IRD, and with or without lymph node suppuration due to extreme necrotising inflammation in mycobacterial IRD); restoration of the granulomatous response is also supported by reports of elevated serum 1,25-dihydroxycholecalciferol in functional granulomas and hypercalcaemia (due to production of the former by functional granulomas) during mycobacterial IRD [39].

1.8. Research Rationale, Aims, and Objectives

A typical primary immune response is initiated by the innate system (including myeloid cells), leading to eventual co-ordinated activity of bi-directionally-communicating innate and adaptive systems (although a secondary response does feature earlier involvement of memory lymphocytes). Myeloid cells are heavily implicated in both active TB (particularly, a neutrophil-derived type I IFN signature) and in TB-IRIS pathogenesis. For example, neutrophilia (including elevations in IL-8 and MMP-8) precedes and is present at the time of TB-IRIS. In TB, neutrophilia and neutrophil- (but not T-cell-)derived granzyme B is known to associate independently with decreased Mtb clearance and an increased pulmonary bacillary burden. Given the production of type I IFN by neutrophils during active TB, these cells may also contribute to a potentially-elevated type I IFN level at baseline in TB-IRIS developers. Myeloid cell types (including neutrophils) are over-represented in granulomas at sites of TB disease, and it is known that ARV therapy results in hyperactivated and necrosis-prone neutrophils, including increased levels of IL-17

and IL-22. Thus far, general TB-IRIS research has focused mainly on CD4⁺ T-cells and monocytes; neutrophils have not been adequately studied in this context and – especially given our pathogenic hypothesis above – may provide key insights into early TB-IRIS pathogenesis.

We aim to further elucidate the pathogenesis of TB-IRIS through differential proteomic profiling of patient (IRIS) versus control (CTRL) neutrophils, and in so doing identify candidate biomarkers (risk stratification, diagnostic, prognostic, treatment monitoring) as well as more disease-specific putative treatment targets (for repurposing of existing safe and effective drugs). Objectives are as follows (fig. 9):

- (1) determine appropriate sample size to achieve desired power
- (2) assemble appropriate patient cohort
- (3) obtain patient data (demographic, clinical, laboratory) and venous blood
- (4) isolate and flash-freeze neutrophils using optimised protocols
- (5) lyse neutrophils in a manner maximally preserving proteins
- (6) tryptically digest proteins
- (7) remove detergents and salts from resulting peptides
- (8) identify and quantitate proteins via peptide liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS)
- (9) using biological statistics, bioinformatics, and computational biology, determine how ARV therapy differentially impacts neutrophil proteomes between patients and controls
- (10) validate inferred hypotheses via orthogonal research methods.

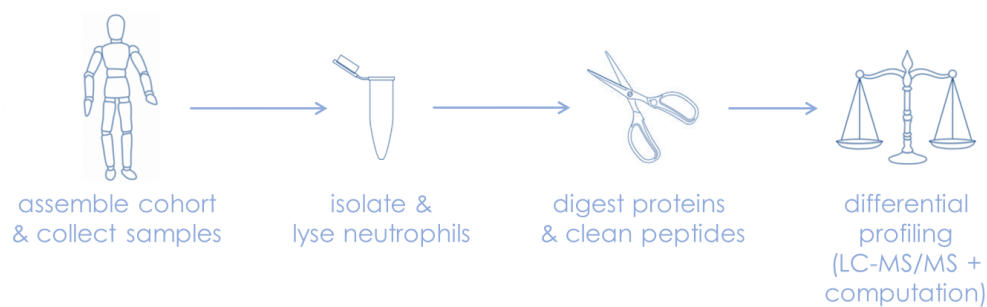


Figure 9 Project Workflow

Appendix A TB-IRIS Clinical Literature

Table 1 Independent Review of Prospective and Retrospective Paradoxical TB-IRIS Studies

Study Type	Study Year	Study Authors	Study Location	Size (HIV/TB-Coinfected Participants)	Incidence (%)	*Median ARV-IRIS Interval (days)	***Most Frequent Symptoms	****Most Frequent Risk Factors				
								EPTB	Low BL CD4	High BL VL	Short ATT-ARV Interval	Pronounced Immune/Virological Response
Prospective	1998	Narita et al. [319]	USA	33	36	12	constitutional lymphadenopathy pulmonary infiltrates	NR	N	N	Y	Y (HIV RNA)
	2004	Patel et al. [320]	India	126	9	within 30	NR	NR	Y (trend)	NR	NR	Y (CD4)
	2007	Burman et al. [111]	USA	137	18	34	constitutional lymphadenopathy pulmonary infiltrates	Y	N	N	Y	N
	2009	van Tieu et al. [321]	Thailand	126	18	14	lymphadenopathy constitutional respiratory	Y	N	N	N	Y (VL)
	2009	Pepper et al. [322]	SA	N/A	N/A	14	N/A	NR	NR	NR	NR	NR
	2009	Meintjes et al. [323]	SA	N/A	N/A	14	constitutional lymphadenopathy pulmonary infiltrates respiratory	NR	NR	NR	NR	NR
	2010	Meintjes et al. [324]	SA	287	38	10 (placebo arm) 14 (prednisone arm)	lymphadenopathy pulmonary infiltrates recurring TB symptoms	NR	NR	NR	NR	NR
	2011	Dibyendu et al. [325]	India	96	19	57 (mean)	constitutional lymphadenopathy respiratory	Y	N	N	N	Y
	2011	Karmakar et al. [326]	India	123	4	73	lymphadenopathy constitutional respiratory pulmonary infiltrates	N	N	N	Y (trend)	Y (VL; CD4 trends)
	2012	Conesa-Botella et al. [327]	Uganda	81	48	12	NR	NR	NR	NR	NR	NR
	2012	Worodria et al. [328]	Uganda	254	21	14	constitutional lymphadenopathy respiratory	NR	Y	NR	NR	NR
	2013	van der Plas et al. [329]	SA	112	42	12	NR	NR	NR	NR	NR	NR
	2013	Narendran et al. [330]	India	57	54	11	constitutional lymphadenopathy pulmonary infiltrates	Y	Y	Y	Y	NR
	2015	Goovaerts et al. [331]	Uganda	254	24	15 (early) 98 (late)	NR	NR	NR	NR	NR	NR
	2015	Ravimohan et al. [332]	Botswana	170	19	28	NR, supplementary table available	N	N	NR	N	NR
	2016	Musselwhite et al. [333] (incl. unmasking)	multicentre	267	19 (paradoxical)	13 (incl. unmasking)	NR	NR	N	N	NR	NR
	2017	Walker et al. [334]	SA	49	59	6 (onset) 14 (presentation)	constitutional pulmonary infiltrates	NR	NR	Y	NR	Y
	2018	Meintjes et al. [335]	SA	240	40	8 (placebo arm) 10 (prednisone arm)	lymphadenopathy pulmonary infiltrates	NR	NR	NR	NR	NR

							of 11 reporting studies: constitutional : 10 (91%) lymphadenopathy: 10 (91%) infiltrates: 8 (73%) respiratory: 5 (45%)	4 of 6 reporting studies: 67%	2 of 10 reporting studies: 20%	2 of 8 reporting studies: 25%	3 of 7 reporting studies: 43%	6 of 7 reporting studies: 86%
		**Totals (N = 18 studies)		151 ± 84 (ave±std ev)	29 ± 16 (ave±std ev)	14 (median)						
Retrospective	2000	Fishman et al. [336] (CXR-based)	USA	31	45	2-32 (interval)	pulmonary infiltrates lymphadenopathy constitutional	NR	Y (trend)	NR	NR	NR
	2004	Kumarasamy et al. [337]	India	144	8	47	constitutional lymphadenopathy	NR	N	NR	Y (trend)	N
	2005	Michailidis et al. [338]	UK	55	26	~18	lymphadenopathy constitutional	Y	Y	N	NR	Y (VL, CD4)
	2005	Shelburne et al. [114] (3 types of OI-IRIS)	multi-centre	86	30	NR	lymphadenopathy pulmonary infiltrates constitutional	N	N	N	Y	Y (HIV RNA)
	2006	Manosuthi et al. [339]	Thailand	167	13	32	constitutional lymphadenopathy	Y	N	N	N	Y (CD4)
	2007	Tansuphasawadikul et al. [340]	Thailand	101	15	14	NR	N	NR	NR	Y	NR
	2007	Serra et al. [341] (some ARV-experienced)	Brazil	84	12	61.7 (mean)	lymphadenopathy constitutional	Y	Y	NR	NR	NR
	2010	Sharma et al. [342]	India	237	8	22	constitutional lymphadenopathy respiratory	N	Y	N	Y	Y (CD4)
		**Totals (N = 8 studies)		113 ± 67	20 ± 13	22 (median)	of 7 reporting studies: lymphadenopathy: 7 (100%) constitutional : 7 (100%) infiltrates: 2 (29%) respiratory: 1 (14%)	3 of 6 reporting studies: 50%	3 of 7 reporting studies: 43%	0 of 4 reporting studies: 0%	3 of 5 reporting studies: 60%	4 of 5 reporting studies: 80%

*It is notable that a difference exists between the time of symptom onset and presentation to the clinic, as exemplified in Walker et al. (2017). Additionally, it is possible that early and late TB-IRIS may represent slightly different phenomena. **Values excluded from calculations include unmasking, onset, intervals, and means, as they are non-comparable with paradoxical, presentation, and median values. ***Constitutional symptoms typically include fever ± rigor/chills (sometimes weight-loss, night-sweats) and respiratory symptoms typically include cough/dyspnea. Less common symptoms (not mentioned above) include lymph node suppuration, serositis (e.g. pleural/pericardial effusion/ascites), abdominal manifestations (e.g. pain, diarrhea, hepato- or splenomegaly), CNS manifestations (e.g. meningitic, space-occupying, seizures), cutaneous manifestations (e.g. abscess), etc. ****Univariate and multivariate findings were included, non-significant trends were not. Three of the above studies (one of which also includes unmasking TB-IRIS) report low baseline haemoglobin as a risk factor [328] [329] [330]. Two of the above studies report high baseline CRP as a risk factor [329] [330]. Although two additional studies [110] [113] not included above report low baseline BMI as a risk factor, Ravimohan et al. (2015) reports increased baseline BMI as a risk factor. Additional potential risk factors reported by single studies: gender (female [333], male [114]), ethnicity (black [111]), and elevated baseline heart rate and respiratory rate [334].

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Chapter 2

Clinical Cohort Assembly, Characterisation, and Sample Processing

“Research is formalised curiosity.” - Zora Neale Hurston

Abstract

Background As described in Chapter 1, neutrophils may contribute to TB-IRIS pathogenesis. **Methods** Based on an *a priori* power calculation and relevant inclusion/exclusion criteria, an appropriate clinical cohort of control (CTRL) and case (IRIS) participants - deriving from two parent studies - was assembled. Binary and continuous demographic, clinical, and biochemical characteristics were analysed, including between-group summary statistics, inspection of value distributions, t-test-based comparisons, and relative risk as well as 95% confidence interval calculation. Peripheral blood neutrophils were isolated and flash-frozen to preserve native proteomes for subsequent analysis (described in Chapter 3). **Results** A sample size of > 42 participants per comparison class provides > 95% power to detect a two-fold change with 99% confidence. Case and control groups are roughly matched for baseline characteristics (e.g. age, gender, and alcohol use). Cases exhibit known TB-IRIS risk factors (e.g. lower baseline CD4 count and shorter ATT-ARV interval), though trends are non-significant. While there is no significant difference in baseline WCC and neutrophil percentage between cases and controls, minimum neutrophil percentage is much lower in controls. During ATT, WCC and neutrophil percentage decrease in both groups. However, during ATT, controls and cases exhibit (non-significantly) differing and opposite BMI trends, with the latter favouring weight gain. At initiation of ARV therapy, CRP levels surprisingly trend higher in controls. During ARV therapy, WCC, neutrophil percentage, and CRP increase in both groups, but this is significantly more pronounced in cases. Also during ARV therapy, controls and cases again exhibit significantly differing and opposite BMI trends, with the latter now favouring weight loss. After two weeks of ARV therapy, cases exhibit significantly higher WCC, neutrophil percentage, and CRP values. Various binary factors (e.g. ARV-induced increase in WCC or decrease in BMI) appear to increase TB-IRIS risk, while prednisone exposure prior to ARV initiation trends towards decreasing TB-IRIS risk, but only smoking status achieves significance: smoking apparently decreases TB-IRIS risk by 60% (an effect not better accounted for by participant anti-fungal or antihistamine exposure). The majority of cohort participants received the ARV regimen which was first-line at the time of the study (NNRTI EFV-based), but three received an EFV-lacking alternate regimen (3TC, LPVr, NVP), and one received a (NRTI) TDF-lacking alternate regimen (EFV, ABC, 3TC). No participants on alternate regimens developed TB-IRIS. Apart from ATT and ARV therapy, various other pharmaceutical drugs (e.g. antihistamines, NSAIDs and other analgesics, antibiotics, anti-fungal agents, statins, ACE inhibitors, etc.) were incidentally prescribed in this cohort. Of 12 and 15 participants exposed to antihistamines or anti-fungal agents (respectively) prior to ARV therapy initiation, only 41.7% and 26.7% went on to develop TB-IRIS. Regarding sample processing, average times from sample collection to both PBMC and neutrophil isolation were within the recommended four-hour limit. Neutrophil isolation yield exceeds 15×10^6 /sample in the control group, and 25×10^6 /sample in the TB-IRIS group, likely reflecting higher circulating neutrophil numbers in the latter. Isolated neutrophil purity exceeds 80% in both groups; the few low-purity samples are excluded from subsequent proteomic analysis. **Discussion & Conclusions** Samples are of sufficient quantity and quality for downstream proteomic analysis. Case and control groups are comparable. Cohort characteristics conform to expected risk factors and changes during TB-IRIS manifestation (e.g. increased WCC, neutrophil percentage, CRP). An ATT-induced decrease in circulating leukocytes in both groups suggests some resolution of inflammation. ATT-induced weight gain in cases suggests more advanced TB at baseline, which may explain the more pronounced response to ATT known to occur in this group. Higher CRP levels in controls prior to ARV therapy initiation suggests differing inflammatory dynamics between the groups, perhaps with higher levels of inflammation in controls at ARV initiation. The ARV-induced increase in circulating leukocytes and CRP in both groups (albeit more pronounced in cases, including after two weeks of ARV therapy) indicates that ARV therapy increases inflammation levels, including an acute-phase response, and that this effect is more pronounced in cases. This is in keeping with prior knowledge regarding the association between increased inflammation, neutrophilia, and manifestation of TB-IRIS. ARV-induced weight loss in cases suggests increased metabolic demands of this more activated immune system, perhaps partially recapitulating the TB metabolic state. The ARV-induced increase in WCC or decrease in BMI may predict TB-IRIS, while prednisone may partially prevent TB-IRIS, but only smoking apparently significantly decreases TB-IRIS risk, perhaps via mechanisms similar to those which account for the protective effect of smoking against other complex immunological disorders: ulcerative colitis, or more severe manifestations of COVID-19. Perhaps in some support of later proteomic-driven and microscopy-supported hypotheses, EFV-lacking alternate regimens may be less likely to induce TB-IRIS (although too few data-points exist to draw formal conclusions). Although low numbers again preclude formal conclusions, the low percentage of anti-fungal agent-exposed participants who go on to develop TB-IRIS suggests that exposure to certain fungi or pharmaceutical drugs with immunomodulatory properties prior to ARV therapy initiation may impact TB-IRIS risk. Indeed, long-term exposure to cotrimoxazole prior to ARV therapy initiation is known to mitigate TB-IRIS risk, and it has been hypothesised that the NSAID aspirin may be a safe and effective alternative to the corticosteroid prednisone for treatment of mild TB-IRIS. A commonality between smoking and fungal infection is enhanced Th17-type responses prior to ARV initiation, which may thus be protective against TB-IRIS. However, many genetic or lifestyle factors beyond those recorded and analysed here may participate in immunomodulation to contribute to differential TB-IRIS risk.

2.1. Background, Study Design, and Power

Based on prior knowledge (Chapter 1), we hypothesised that neutrophils contribute to TB-IRIS pathogenesis.

Our hypothesis-generating prospective case-control study aims to identify protein expression profile differences between neutrophils derived from TB-IRIS case (IRIS) and matched TB-HIV co-treatment control (CTRL) participants at two time-points: immediately prior to ARV initiation (week 0), and at the typical time of IRIS onset two weeks post-ARV initiation (week 2). Proteomic differences reflect pre-existing phenotypic/functional differences, as well as differences in phenotypic/functional changes in response to ARV therapy. The primary purpose of the study is inference of neutrophil functional differences between control and case participants in order to facilitate hypothesis generation regarding the molecular and cellular mechanisms contributing to TB-IRIS predisposition and immunopathogenesis. Secondly, we also aim to identify candidate clinical biomarkers for various purposes, as well as novel prophylactic/therapeutic targets. Experimental and data analytical methods are detailed in Chapter 3.

At the time of study conception, no consensus method yet existed to calculate desired power-based sample sizes for discovery mass spectrometry. We therefore based our calculations on established

methods within the analogous field of gene expression microarrays [1], adopting the following equation (which assumes relatively normal distribution of expression measurements among samples within comparison classes):

$$N = \frac{4 \left(\frac{z_{\alpha}}{2} + z_{\beta} \right)^2}{\left(\frac{\delta}{\sigma_T} \right)^2}$$

Where: **N** = total sample size ($\frac{N}{2}$ = n is the number of samples per comparison class, and z_{α} and z_{β} are normal distribution percentiles with n-2 degrees of freedom; $\left(\frac{z_{\alpha}}{2}\right) = -3.29$ and $z_{\beta} = -1.645$ when employing the recommended $\beta = 0.05$)

δ = minimum detectable effect size ($\delta = 1$ represents $\log_2$ of the typically-sought minimum two-fold expression change)

σ_T = predicted total variance, expressed in terms of the co-efficient of variation (CV).

Total variance comprises biological and technical variance ($\sigma_T = \sigma_b + \sigma_t$). In clinical discovery mass spectrometry experiments, biological variance (between-patient proteome variability) is by far the greater contributor, whereas technical variance is relatively minor. A proteomic profiling study of human sera - representative of human biological fluids and tissues - estimates biological diversity of 19 healthy individuals at a median value of 22.3%, while diversity contributed by sample preparation and LC-MS/MS analysis were estimated at 20.4% and 15.1% respectively [2]. However, in-house estimates of variance contributed by sample preparation and LC-MS/MS analysis were negligible (data not shown).

We thus calculated *a priori* that a sample size of $N = 42$ (i.e. $n = 21$ each of cases and controls, at each time-point) would provide 95% power to detect at least a two-fold change in protein expression (two-sided $\alpha = 0.01$). More recently, Skates et al. (2013) estimated the probability of biomarkers successfully passing the discovery stage (taking into account many of the factors discussed above, as well as what proportion of potential candidates will be taken forward for verification), and outcomes confirm the estimates provided by our own calculation: when aiming to verify the top 20 biomarker candidates, $N = 50$ will provide 83.2-100% power to detect a two- to five-fold change, provided the marker of interest is detectable in at least 80% of participants [3].

2.2. Participant Recruitment and Sample Collection

The study described herein is entitled *Differential Proteomic Profiling Towards Elucidation of TB-IRIS Pathogenesis*, and was approved annually by the University of Cape Town (UCT) Human Research Ethics Committee (HREC; ref. no. 231/2014). Clinical samples utilised by the current study were derived from the longitudinal arms of two prospective parent studies:

(a) *Defining Mechanisms of Immunopathology in TB-HIV Co-infection and TB-IRIS* (TDTB2, HREC ref. no. 516/2011) [4]

(b) *Preventing tuberculosis-associated immune reconstitution inflammatory syndrome in high-risk patients: a randomized placebo-controlled trial of prednisone* (PredART Trial, HREC ref. no. 136/2013, clinicaltrials.gov identifier NCT01924286) [5].

ARV-naïve patients at risk for paradoxical TB-IRIS, meeting eligibility criteria (Appendix B) and willing to provide informed consent were recruited from Ubuntu Clinic (Khayelitsha Site B) for enrolment in the parent studies. Briefly, eligibility criteria included: TB/HIV-coinfection responding to ATT, eligibility for ARV

therapy initiation (but current ARV-naivety), preferable presence of TB-IRIS risk factors, and ability to provide informed consent. Regarding study site and population, Khayelitsha is an urban township community made up largely of previously-disadvantaged South Africans of low socioeconomic status. Ubuntu Clinic receives a high number of patients presenting with TB/HIV-coinfection and who are eligible for co-treatment (i.e. at risk for paradoxical TB-IRIS). Such a study location and population is relatively unique to Sub-Saharan Africa, and particularly to Cape Town, given ready access to ATT and ARV therapy. In addition, Ubuntu Clinic is situated relatively close to state-of-the-art laboratory facilities required for sample isolation and high-throughput proteomic studies, available at the University of Cape Town (UCT)'s Institute for Infectious Disease and Molecular Medicine (IDM).

For the current study, the biological sample of interest (peripheral venous blood) was obtained from participants at weeks 0 and 2. Venous blood was collected in 9ml sodium-heparin Vacuettes (Greiner bio-one, GmbH) pre-tested for LPS contamination (LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific Pierce). At baseline and at each visit, participants were fully clinically characterised (including relevant laboratory investigations: Appendix C), and were longitudinally assessed for emergence of paradoxical TB-IRIS (according to the INSHI consensus clinical case definition: Appendix D) over a 12-week period post-ARV initiation (to facilitate classification of each participant as IRIS or CTRL). Study data were captured into on-site electronic databases, and additional data of interest to the current study were manually extracted from clinical folders. During the PredART trial, participants were additionally randomised to two possible treatment arms – prednisone or placebo – within which treatment was provided from weeks 0 through 4 (in line with the main aim of that study: determining whether prednisone prevents emergence of IRIS). For the current study, cohort demographic, clinical, and biochemical factors (including as relating to ATT and ARV therapy) were characterised through calculation of summary statistics, and relevant factors (both binary and continuous) were compared between groups, including analysis of their impact on/association with TB-IRIS development. For baseline binary factors, relative risk and 95% confidence interval were calculated, while for continuous factors (at all time-points) value distributions were initially inspected, followed by t-tests to assess statistical significance of between-class differences.

For the current study, sample collection proceeded from August of 2012 through October of 2016 (with TDTB2 recruitment concluding in December 2013, PredART recruitment initiating in 2014, and PredART contributions to the current study initiating in October 2014), a period significantly exceeding initial estimates due to low rates of participant eligibility, asymmetric uptake of cases versus controls at different time-points, and additional non-clinical responsibilities (such as assay optimisation and conference/internship attendance). During the TDTB2 parent study, sample collection involved spending mornings at the study site in order to receive and characterise scheduled participants and update study folder information, followed by sample transport for processing and storage. During the PredART parent study, sample collection involved only receiving samples for processing and storage. Blood samples were transported at room temperature to a biosafety level two (BSL2) facility at the IDM for processing - peripheral blood mononuclear cell (PBMC) and neutrophil isolation and storage, as described below – within four hours of collection. Isolation of PBMC required between two and four hours, depending on the number of samples, and neutrophil isolation required a further two to four hours. Great care was taken to maximise standardisation of sample collection, transport, and processing.

In addition, neutrophils and PBMC from ten age- and gender-matched healthy volunteers were isolated at the IDM. Healthy volunteers were defined as being TB- and HIV-uninfected, free from respiratory or systemic symptoms at the time of phlebotomy, and free from serious comorbidity. They were additionally required to be non-pregnant and competent to provide informed consent.

2.3. Leukocyte Isolation and Storage

All isolation steps were performed in a BSL2 facility, and all open-tube or potentially aerosol-generating steps took place inside a sterile class II biosafety cabinet, employing aseptic technique throughout.

Standard density gradient centrifugation was used to isolate PBMC. Briefly, 48ml whole blood was mixed 1:1 with Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich), layered 2:1 over Ficoll-paque (GE Healthcare Life Sciences), and centrifuged at 700g for 20min (room temperature, brake 1). During centrifugation, PBMC migrate to the plasma-Ficoll interface, while red cells and granulocytes pellet below the Ficoll layer. PBMC were harvested into 10ml RPMI-1640 medium with L-glutamine and sodium bicarbonate (RPMI; Sigma-Aldrich). After washing by centrifugation at 600g for 10min (room temperature, brake 3), PBMC were re-suspended in a further 10ml RPMI and a 20ul sample was taken for counting via Trypan blue exclusion [6]. After a second identical washing step, PBMC were re-suspended to 4×10^6 per ml in a standard cryoprotective medium consisting of RPMI containing 50% foetal calf serum (FCS; Sigma-Aldrich) and 10% dimethylsulfoxide (DMSO; Sigma Aldrich) at 4°C. Overnight, PBMC were slowly frozen ($\sim 1^\circ\text{C}/\text{min}$) to -80°C in a Mr. Frosty (Nalgene) with isopropyl alcohol insulation, before transfer to liquid nitrogen vapour (below -130°C) for long-term storage. Depending on PBMC yield, up to 11 x 1ml cryovials (Corning) were stored per participant, of which at least 1 vial (TDTB2) and 2-6 vials (PredART) are available for potential follow-up investigations based on results of the current study.

Untouched neutrophils were isolated using a red cell sedimentation and hypotonic lysis method optimised in-house (Appendix E). Briefly, the Ficoll layer remaining after PBMC isolation was removed by pipetting, the red cell/granulocyte fraction was diluted to a total volume of 45ml using PBS at room temperature, and gently inverted 10x to ensure adequate re-suspension. To this was added 5ml of 0.2um-filtered 6% dextran (MW 35-40 000; Sigma Aldrich) in sterile normal saline (at room temperature) to induce red cell sedimentation, and the mixture was gently inverted 30x to ensure adequate mixing. After allowing red cells to sediment for no more than 20min, the neutrophil-rich upper layer was transferred to a fresh 50ml Falcon tube. Neutrophils were pelleted by centrifugation (10min, 500g, brake 1), and the supernatant discarded by pouring (stopping immediately when remaining red cells approach the tube edge). Pellets were re-suspended in 50ml each 1x PharmLyse buffer (BD Biosciences) at room temperature, consisting of 5ml 10x PharmLyse made up to 1x with 45ml 0.2um-filtered sterile deionised water (per sample) by initial addition of 1ml buffer with gentle trituration, before addition of the remaining buffer volume and gently inverting 20x to ensure adequate mixing. After incubating for 10min to allow red cell lysis, neutrophils were again pelleted (5min, 500g, brake 1), the supernatant discarded as before, and the neutrophil pellet re-suspended in 50ml PBS, as before. Neutrophils were again washed (5min, 500g, brake 1), the supernatant discarded as before, and the neutrophil pellet re-suspended in 2ml RPMI at room temperature by gentle trituration to achieve a single-cell suspension (RPMI resuspension was required for collaborator neutrophil protocols, and therefore had to be included as a step in this protocol). A 100ul aliquot was taken for automated differential counting (ABX Pentra automated haemocytometer; HORIBA), and a volume representing 5×10^6 cells was transferred to each of a maximum number of 1.5ml sterile microcentrifuge tubes for pelleting (5min, 500g, brake 1). The supernatant was carefully removed by pipetting, and the neutrophil pellets re-suspended in 50ul PBS at room temperature, for transfer to a 1.5ml cryovial (Corning). A further 50ul PBS at room temperature was used to wash the original microcentrifuge tube into the corresponding cryovial, prior to flash-freezing in liquid nitrogen (to disrupt neutrophils while preserving the native proteome) and storage in liquid nitrogen vapour. Flash-freezing facilitated direct ex vivo spectrometry, such that proteomes reflected *in vivo* conditions and functions as closely as possible.

Furthermore, plasma and urine collected and stored as part of the parent studies (including by myself as part of the TDTB2 study) are also available for follow-up investigations based on results of the current study, pending primary investigator confirmation and ethics amendment approval.

2.4. Results and Discussion: Cohort Assembly

2.4.1. Sample Size Achieves Sufficient Power

Neutrophils were isolated from a total of 98 participants (28 TDTB2 participants and 70 PredART participants; satisfactory neutrophil isolation optimisation was only achieved in April 2013, partway

through the TDTB2 study, resulting in the TDTB2 study contributing relatively fewer neutrophil samples than the PredART study to the current study). After *post hoc* exclusion of three TDTB2 and two PredART participant/s for clinical and/or laboratory reasons (e.g. uncertain diagnoses, low isolated neutrophil purity), stored neutrophils from a total of 93 participants were available for preparation and analysis (including 49 CTRL and 44 IRIS participants, for 27 of whom neutrophils from both time-points were available). Thus, a total of 121 stored high-quality neutrophil samples were available for analysis (Table 1). Given *a priori* power calculations, sample sizes of $n > 21$ for any comparison would provide $> 95\%$ power to detect a two-fold change in protein expression ($p < 0.01$). Given that a maximum of 900 LFQ-quantifiable neutrophil proteins (of over 2500 detectable neutrophil proteins) are included per comparison, this represents a potential maximum of only 9 false differential expression identification events.

Table 1 Stored Neutrophil Samples Available for Analysis

	CTRL	IRIS	Totals
week 0	33	30	63
week 2	31	27	58
Totals	64	57	121

The CTRL group includes 20 participants classified as 'other' (e.g. pneumonia).

The IRIS group includes six participants classified as 'non-INSII IRIS'.

Regarding sub-group analyses, 22 and 24 prednisone-exposed samples derived from week 0 and week 2, respectively, while 41 and 34 prednisone non-exposed samples derived from week 0 and week 2, respectively (Tables 2 and 3). At the very least, this is sufficient for a pilot comparison of prednisone-exposed versus non-exposed neutrophil proteomes.

Table 2 Week 0 Prednisone Status

	CTRL	IRIS	Totals
prednisone exposed	12	10	22
prednisone non-exposed	21	20	41
Totals	33	30	63

Table 3 Week 2 Prednisone Status

	CTRL	IRIS	Totals
prednisone exposed	13	11	24
prednisone non-exposed	18	16	34
Totals	31	27	58

2.4.2. Comparison Class Participants Are Roughly Matched for Age and Gender

Participants are partially described by demographic data (Tables 4 and 5).

Table 4 Participant Age by Comparison Class and Time-point

	CTRL (median $\pm$ stdev)	IRIS (median $\pm$ stdev)
week 0	39.7 $\pm$ 8.8	34.9 $\pm$ 9.1
week 2	36.7 $\pm$ 8.5	33.9 $\pm$ 7.6

Age data were available for 100% of participants ($n_{CTRL} = 49$, $n_{IRIS} = 44$).

Between-group ages appear similar by inspection (fig. 1), but t-testing reveals a statistically significant difference in overall mean age between CTRL (38.57 ± 9.27) and IRIS (35.18 ± 8.76) participants ($p = 0.037$), which also holds for within-time-point mean age. Nevertheless, comparison classes are roughly comparable by age.

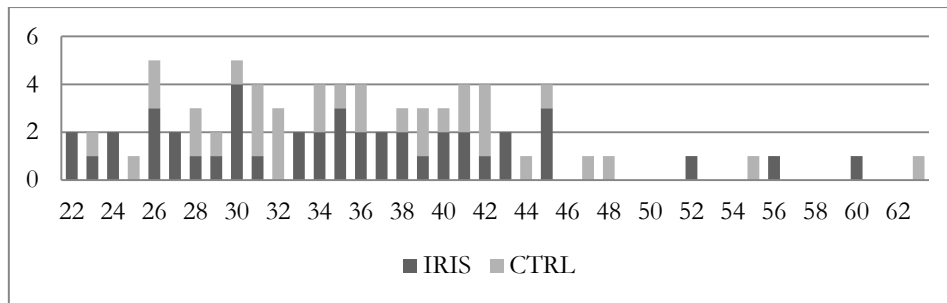


Figure 1 Contribution of Within-class Participants to Cohort by Age

Table 5 Participant Gender by Comparison Class and Time-point

		CTRL (frequency)	IRIS (frequency)
week 0	M	20	17
	F	13	13
week 2	M	17	16
	F	13	10

Gender data were available for 100% of participants ($n_{CTRL} = 49$, $n_{IRIS} = 44$).

While there is an overall preponderance of males, gender contributions are comparable between comparison classes.

2.4.3. Comparison Class Participants Are Matched for Alcohol Use, but Not Smoking Status

Participants are partially described by alcohol use and cigarette smoking status (Tables 6 and 7).

Table 6 Participant Alcohol Use Status by Comparison Class

	CTRL (frequency)	IRIS (frequency)	totals
current drinker	15	14	29
ex/non-drinker	22	24	46
totals	37	38	75

Alcohol use data were available for 81% of participants ($n_{CTRL} = 37$, $n_{IRIS} = 38$).

There is no significant difference between the ratio of drinkers to non/ex-drinkers between the CTRL and IRIS groups. This is desirable in light of alcohol's complex and sometimes apparently-paradoxical effects on innate and adaptive immune function [7]. For example, acute alcohol exposure impacts immune homeostasis in ways that slow recovery from physical trauma, and chronic exposure increases vulnerability to infection (e.g. pneumonia) and systemic inflammation [7]. Chronic drinkers exhibit impaired lower respiratory tract and gut immunity [7]. Indeed, alcohol use is associated with various pulmonary infections, including TB [7]. It interferes with upper airway ciliary function, impairs alveolar macrophage and neutrophil function, and disrupts lower airway epithelial barrier function [7]. During HIV infection, mucosal barrier function and immunity are disrupted, and alcohol enhances this dysfunction, favouring HIV progression [7]. One might therefore predict that pre-existing alcohol use may influence immunity in a way that impacts IRIS (although it apparently does not: Table 11). However, many participants who are current drinkers reported drinking significantly fewer alcohol units and/or less frequently since illness onset.

Table 7 Participant Smoking Status by Comparison Class

	CTRL (frequency)	IRIS (frequency)	totals
current smoker	13	4	17
non/ex-smoker	23	35	58
totals	36	39	75

Smoking data were available for 81% of participants (nCTRL = 36, nIRIS = 39).

There appears to be a trend towards more non/ex-smokers in the IRIS relative to the CTRL group. This is interesting in light of smoking generally being conceptualised as having negative health consequences, including in infectious settings. Active smokers are at increased risk of infectious and inflammatory pulmonary diseases (e.g. asthma, interstitial lung disease, COPD) [8]. Smoking increases the risk of respiratory infection by multiple pathogens, including *M. tuberculosis* [8]. However, smoking is actually known to have a protective effect against certain complex immunological disorders, such as ulcerative colitis and the severe form of COVID-19.

An estimated third of the world's adult population are smokers [9]. Cigarette smoke contains >4000 chemicals [8] (including but not limited to nicotine, polycyclic aromatic hydrocarbons, carbon monoxide, and cadmium), and acts as a source of reactive oxygen and nitrogen species (ROS, RNS) [9]. The effects of smoking on immune function are pleiotropic: it can induce both pro- and anti-inflammatory effects in specific immune circuits and impacts chronic inflammation and autoimmunity [9]. Acutely, smoking impacts many signalling pathways, for example activating the MAPK cascade, signalling via TLR-4 (including promoting TLR-2 and -4 transcription), and favours inflammation mediated by the H1R-TLR2/4-COX2 axis [9]. Nicotine, for example, acts on nicotinic acetylcholine (nACh) receptors – present in the central nervous system, neuromuscular junction, and on leukocytes – to counteract leukocyte (e.g. macrophage, T-cell, and B-cell)-mediated inflammation, including enhancement of Ca²⁺ channel activity and suppression of NO signalling (smoking – perhaps via NOS inhibition – also decreases exhaled NO, which normally contributes to respiratory tract defence and opposes bronchoconstriction, vasoconstriction, and platelet aggregation [10]) [9]. Signal transduction via this receptor leads to JAK2-induced STAT3 phosphorylation and inhibition of NFκB nuclear translocation (both inhibiting inflammatory cytokine transcription), or activation of IRAK-M to dampen TLR signalling [9]. *Thus, smokers experience constant suppression of TLR-NFκB signalling, to which their physiology has likely adjusted.*

Smoking exhibits specific effects on innate immunity, including enhanced epithelial IL-33 production, coupled with increased macrophage and NK cell IL-33R expression [9]. Similarly, acute exposure to cigarette smoke activates neutrophil- and monocyte-mediated inflammation [9]. Although passive smoking leads to pulmonary infiltration by DC and their activation by IL-1α, cigarette smoke also suppresses DC maturation and T-cell-stimulatory function, and decreases pDC transcription of TLR-7 and expression of IFNα [9]. Regarding NK cells, cigarette smoke primes enhanced IL-17 expression, but decreases NK cell activation and cytotoxicity, and suppresses IFNγ and TNFα expression by CD56⁺ NK cells [9]. Regarding macrophages, smoking and nicotine increase IL-8 expression, but cigarette smoke inhibits phagocytosis and intracellular bacterial killing (favouring M2 polarisation of THP-1 macrophages), and cigarette smoke extract *inhibits TLR-initiated expression of TNFα, IL-6, IL-10, and RANTES (but not IL-8, suggesting selective recruitment of neutrophils) in association with decreased MAPK p38, ERK, and p65 (NFκB RELA) activation* [9] [11]. In fact, the bronchoalveolar lavage (BAL) fluid macrophages of smokers produce a neutrophil-recruiting chemotactic factor, induced by smoke exposure [12].

Indeed, specifically regarding neutrophils, “the chemotactic response to IL-8 and partly towards LTB4 is increased in smokers, positively associated with serum TNFα levels” [13]. However, neutrophils of active smokers exhibit suppressed overall peripheral chemotaxis [14] but increased peripheral ROS generation [15]. Nevertheless, active smokers exhibit pulmonary neutrophil accumulation; at least in smoking-induced COPD, this is accounted for by enhanced recruitment in combination with prolonged survival and delayed efferocytosis [8]. Indeed, both cigarette smoke and isolated nicotine suppress apoptosis (by inhibiting diaphosphoinositol pentakisphosphate generation [16], thereby preventing Akt

deactivation) [8]. Cigarette smoke inhibits phagocytosis of bacteria, but cigarette smoke extract induces secondary granule exocytosis (likely favouring pulmonary tissue damage), strongly inhibits caspase 3 activity and thus spontaneous apoptosis (thereby leading to an atypical form of cell death exhibiting features of both apoptosis and necrosis), and induces autophagy [8]. Such dying neutrophils undergo efferocytosis by monocyte-derived macrophages (likely via recognition of oxidised LDL-like structures displayed by neutrophils after smoke exposure) [8]. While apoptosis and efferocytosis contribute to resolution of inflammation, and autophagy is a key mechanism involved in pathogen control and immune regulation, tobacco-induced necrosis facilitates release of neutrophil proteases (e.g. elastase, MMP-8, and MMP-9) implicated in pulmonary damage [8]. There is some uncertainty as to whether neutrophil- or macrophage-derived proteases are more responsible for acute cigarette smoke-induced connective tissue degradation: while neutrophil elastase is responsible for elastin and collagen breakdown (preventable by α 1-antitrypsin), macrophage-derived metalloproteinases may be key factors in smoke-induced emphysema [17]. "Compromised neutrophil" function is implicated as a major mechanism whereby smoking increases vulnerability to infectious disease [8].

Smoking also exhibits specific effects on adaptive immunity, including activated CD4⁺ T-cell pulmonary infiltration in response to prolonged cigarette smoke exposure [9]. Passive smoking is associated with a higher circulating frequency of naïve CD3⁺ T-cells [9]. At least in COPD, the BAL of smokers exhibits a lower CD4⁺ but higher CD8⁺ T-cell percentage (chronic cigarette smoke exposure fails to induce emphysema in CD8 KO mice, perhaps because CD8⁺ T-cell-derived IP-10 elicits macrophage elastase) [9]. Pulmonary CD8⁺ T-cells from cigarette smoke condensate-exposed humans exhibit higher TLR-4 and -9 expression levels [9]. After 6mos of cigarette smoke exposure, murine BAL contains higher numbers of Th1 and Th17, smoke-induced murine emphysema co-occurs with an increased proportion of circulating IL-21⁺ (Th17 and CD8⁺) T-cells (with increased expression of IL-17 and -21 leading to increased perforin and granzyme expression), and psoriatic smokers exhibit higher levels of circulating Th17 than psoriatic non-smokers [9]. It has been suggested that smoking induces rheumatoid arthritis by – acting via T-cell aryl hydrocarbon receptors (AhR) – promoting Th17 development [9]. Cigarette smoke is protective in a murine induced-colitis model (decreasing colonic levels of Th1/Th17 cytokines "including TNF α , IFN γ , and IL-17") and passive smoking decreases effector and memory T-cell infiltration into the lungs and spleens of Mtb-infected mice [9]. The Th1-type cytokine IL-12 suppresses while Th2-type cytokine IL-4 enhances CD4⁺ T-cell expression of anti-inflammatory α 7 nACh receptors [9]. However, in allergen-sensitised rats, nicotine suppresses pulmonary Th2-type chemokine and cytokine expression, slowing eosinophil migration [9]. Smoking is also associated with a loss of T_{reg} homeostasis [9].

Nicotine receptors are also present on B-cells and, at least epidemiologically, smoking is associated with an increased frequency of peripheral B_{mem} and pulmonary IgG⁺ B_{mem}, along with higher levels of circulating IgE [9]. *H. pylori*-infected smokers (relative to infected non-smokers) exhibit fewer and less functional B_{reg}, and uninfected smokers produce lower IgA, IgM, and IgG levels [9]. "Smoke exposure down-regulates murine marrow production of B-cells" [9].

Smoke-derived polycyclic aromatic hydrocarbons (PAH) act as AhR ligands, inducing expression of CYP450, including CYP1A and B1 (which convert promutagenic PAH into carcinogens) [18]. Smoke condensate, also via AhR, induces CYP1 enzyme transcription (and CYP1B1 protein expression) in human primary macrophages infected with HIV [19]. In a monocytic cell line, it also enhances HIV replication [19] (smoking is known to be associated with enhanced HIV progression); CYP450 may play a role in smoking-induced viral replication [18]. Interestingly, CYP1A1 and 2 protect against oxidative stress-mediated lung injury, while CYP1B1 worsens such injury [20] (recall that smoking itself also induces oxidative stress). Inflammatory cytokines such as TNF α potentiate AhR ligand-mediated induction of CYP1B1 expression via mechanisms involving p38 MAPK-MSK1 signalling, likely enhancing promutagen bioactivation [21]. Interestingly, CYP1B1 also regulates metabolism of endogenous "steroid hormones, fatty acids, melatonin, and vitamins", and modulating CYP1B1 expression/activity has beneficial metabolic effects [22]. In the context of TB-IRIS, it has been demonstrated that CYP1B1 transcription is modulated at two weeks following ARV therapy initiation in TB-IRIS developers [23].

Thus, one might therefore predict that pre-existing cigarette use may influence immunity (and/or metabolism) in a way that impacts IRIS risk, and indeed it apparently does (Table 11). It is possible that pre-existing chronic smoking induces adaptive stress responses (e.g. involving increased robustness of the mitochondrial and ER networks), and/or combats the molecular and cellular mechanisms underlying TB-IRIS pathogenesis, and/or in key ways mimics the immunological state of TB-IRIS itself; any or all of these may subsequently protect against development of TB-IRIS.

Given that these data rely on self-reporting, and that it is sometimes difficult to elicit an accurate smoking history, this finding must be interpreted cautiously and should be confirmed across a larger independent cohort. Additionally, given only the current data, it cannot be excluded that another confounding factor more common in smokers (e.g. co-treatment with an additional medication, or another, unknown, factor) better explains the protective association in this cohort. While the hypothesis of a smoking/IRIS association – if confirmed – obviously cannot lead us to recommend smoking as a preventive strategy against TB-IRIS, it can perhaps help us better understand the mechanism of TB-IRIS pathogenesis (especially in light of what is already known regarding the mechanisms by which smoking protects against ulcerative colitis), or facilitate manipulation of nACh receptors in a prophylactic/therapeutic manner.

2.4.4. Impact/Predictive Value of Immunomodulatory/Immune-induced Clinical and Biochemical Characteristics

As mentioned in Chapter 1, major TB-IRIS risk factors are: extrapulmonary TB, lower baseline CD4 count, shorter ATT-ARV therapy interval, and more robust immunological/virologic response to ARV therapy. Patients in the current cohort are partially described by clinical and biochemical characteristics (Tables 8 and 9). Additionally, notable changes in such characteristics over time are interrogated (Table 10).

Table 8 IRIS-Relevant Participant Clinical/Biochemical Characteristics by Comparison Class

	CTRL				IRIS			
	Min	Ave	Max	Stdev	Min	Ave	Max	Stdev
Rif – ARV interval (days)	10	23.79	147	23.46	9	18.98	56	8.72
CD4 (cells/ml)	3	79.87	233	54.98	3	68.34	220	61.57
VL (copies/ml)	5746	659515	3759449	913452	7714	681689	7349940	1174481

Treatment interval, CD4 count, and VL data were available for 99% (n_{CTRL} = 48, n_{IRIS} = 44), 100% (n_{CTRL} = 49, n_{IRIS} = 44), and 98% (n_{CTRL} = 48, n_{IRIS} = 43) of participants, respectively. It must be noted that for TDTB2, pre-ARV CD4 nadir and peak VL are recorded, whereas for PredART, pre-ARV CD4 and VL as measured at the baseline visit are recorded. Since measurement types are inconsistent, care must be taken in interpreting comparisons. **Rif** rifampicin; **CD4** CD4⁺ T-cell count; **VL** viral load.

As mentioned, a shorter ATT-ARV interval increases TB-IRIS risk. While the average interval here is indeed shorter in the IRIS group (Table 8), a t-test demonstrates no statistically significant difference in mean interval between CTRL (23.79 ± 23.46) and IRIS (18.98 ± 8.72) participants (p > 0.05), likely due to the large standard deviation in the CTRL group. However, comparing means in this way also obscures trends more evident from distributions (fig. 2), such as the broader spread of intervals in the CTRL group. Indeed, histograms also suggest non-different means, but the IRIS group does display a tendency towards a shorter interval.

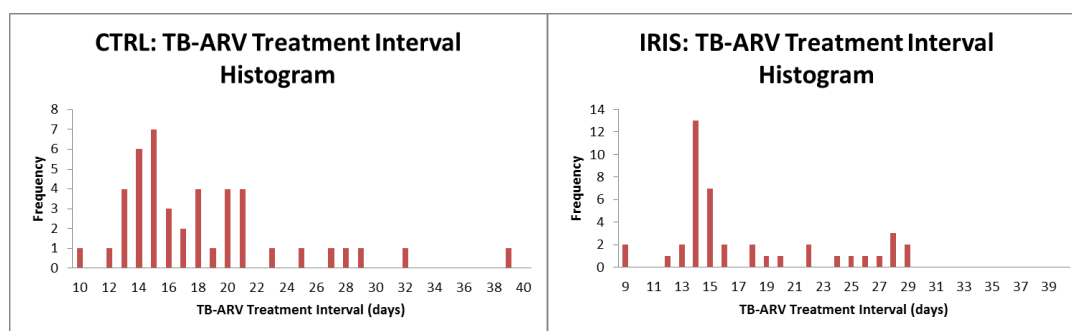


Figure 2 Histograms of ATT-ARV Therapy Initiation Interval by Comparison Class.

Two and three extreme outliers, respectively, have been excluded from the CTRL and IRIS groups, without changing overall data impressions.

Similarly, a lower CD4 count or higher VL at ARV therapy initiation increases TB-IRIS risk. While the mean CD4 count here is indeed lower in the IRIS group (Table 8), a t-test demonstrates no statistically significant difference in mean CD4 count between CTRL (81.88 ± 56.13) and IRIS (68.34 ± 61.57) participants ($p > 0.05$), likely due to large standard deviations in both groups. This result may also be due to the TDTB2 and PredART studies recording CD4 nadir and baseline (not readily comparable), respectively. Similarly, and likely for similar reasons, while the mean VL appears higher in the IRIS group, a t-test also demonstrates no statistically significant difference in mean VL between CTRL (645482.6 ± 908576.2) and IRIS (681689.2 ± 1174481) participants ($p > 0.05$). A separate analysis per parent study may be more prudent. Too few participants presented with extrapulmonary TB to warrant an analysis assessing this known TB-IRIS risk factor.

Table 9 PredART Participant Clinical/Biochemical Characteristics by Comparison Class and Time-point

		CTRL				IRIS			
		Min	Ave	Max	Stdev	Min	Ave	Max	Stdev
BMI	BL	15.1	21.6	37.5	4.61	16.5	21.8	29.6	3.4
	week 0	15	21.3	38.1	4.5	16.4	21.4	28.9	1.0
	week 2	14.1	22.1	39.7	14.1	16.5	21.1	28.4	3.1
CRP	week 0	0.9	26.4	190.2	35.5	0.9	18.4	80.2	20.0
	week 2	1.8	44.5	216.6	42.6	0.9	97.7	274.1	66.6
	BL	2.2	4.8	10.3	2.0	1.5	4.4	13.0	2.4
WCC	week 0	1.8	4.4	8.4	1.6	1.5	3.8	7.9	1.6
	week 2	2.0	5.6	14.1	2.2	2.2	8.6	16.7	3.6
	BL	22.7	62.4	87.0	14.4	46.1	64.9	85.9	10.2
Neutrophil percentage of WCC	week 0	22.0	56.8	82.0	15.0	30.2	60.2	80.0	13.0
	week 2	31.0	66.6	90.2	15.6	55.3	77.9	97.0	10.7

BL, week 0, and week 2 BMI data were available for 72% ($n_{CTRL} = 35$, $n_{IRIS} = 32$), 66% ($n_{CTRL} = 31$, $n_{IRIS} = 30$), and 84% ($n_{CTRL} = 40$, $n_{IRIS} = 38$) of participants, respectively. CRP values were not analysed at BL. Week 0 and 2 CRP data were available for 97% ($n_{CTRL} = 47$, $n_{IRIS} = 43$) and 96% ($n_{CTRL} = 46$, $n_{IRIS} = 43$) of participants, respectively. BL, week 0, and week 2 WCC data were available for 69% ($n_{CTRL} = 35$, $n_{IRIS} = 29$), 92% ($n_{CTRL} = 43$, $n_{IRIS} = 43$), and 92% ($n_{CTRL} = 43$, $n_{IRIS} = 43$) of participants, respectively. BL, week 0, and week 2 NE% data were available for 69% ($n_{CTRL} = 35$, $n_{IRIS} = 29$), 95% ($n_{CTRL} = 45$, $n_{IRIS} = 43$), and 91% ($n_{CTRL} = 43$, $n_{IRIS} = 42$) of participants, respectively. **BL** baseline; **BMI** body mass index; **CRP** C-reactive protein; **WCC** white cell count.

Table 10 Changes in Clinical/Biochemical Characteristics over Time, by Comparison Class

		CTRL				IRIS			
		Min	Ave	Max	Stdev	Min	Ave	Max	Stdev
Δ BMI	BL to wk0	-3.2	-0.02	1.1	0.8	-3.5	0.03	2.3	1
Δ BMI	wk0 to wk2	-1.3	0.5	16.6	2.7	-2.5	-0.5	2.5	1
Δ CRP	wk0 to wk2	-66.6	17.9	202.4	42.8	-9.1	79.4	263.4	70.3
Δ WCC	BL to wk0	-5.9	-0.5	3	1.8	-9.5	-0.9	1.4	2.2
Δ WCC	wk0 to wk2	-1.6	1.2	7.2	1.8	-1.3	4.8	13.2	4.1
Δ NE%	BL to wk0	-43	-5.9	8.1	11.5	-39.4	-7.5	8.7	12.6
Δ NE%	wk0 to wk2	-23.8	9.5	46.9	15.3	-10.2	17.4	44.2	14.1

Δ delta, representing change; **BMI** body mass index; **BL** baseline; **CRP** C-reactive protein; **WCC** white cell count; **NE%** neutrophil percentage.

Lower BMI is a potential risk factor for TB-IRIS. Here, there appears to be little difference in BMI between the CTRL and IRIS group, apart from a marginally larger range of BMI values at each time-point in the former. Specifically, there appears to be little difference between CTRL and IRIS participant baseline BMI. Indeed, by t-test, there is no statistically significant difference in baseline mean BMI between CTRL (21.79 ± 4.92) and IRIS (21.87 ± 3.37) participants ($p > 0.05$). In the interval (not equivalent for all

participants) between baseline and week 0, IRIS participants display a marginal trend towards increasing BMI, whereas CTRL participants exhibit decreasing BMI. Indeed, distributions (fig. 3) also suggest that a higher number of IRIS participants experience larger BMI increases relative to CTRL participants. These trends suggest that the IRIS group experiences a slightly more pronounced weight gain in response to ATT (perhaps suggesting that this group had more advanced TB disease, with a more cachectic state, at presentation). However, by t-test there is no statistically significant difference in mean BMI change from baseline to week 0 between CTRL (0 ± 0.74) and IRIS (0.04 ± 1.06) participants ($p > 0.05$). This may be due to the margin of error during clinical weight and height measurement being quite large (relative to other clinical/biochemical quantitative measurements), which can result in more noise and may thus obscure a true signal.

At week 0, there again appears to be little difference between CTRL and IRIS participant BMI, again supported by a t-test determining no statistically significant difference in mean week 0 BMI between CTRL (21.45 ± 4.73) and IRIS (21.78 ± 3.18) participants ($p > 0.05$). During the interval between weeks 0 and 2, IRIS (relative to CTRL) participants appear more prone to decreasing BMI (weight loss), which may represent a recapitulation of TB symptomatology due to increased immune system metabolic requirements. Distributions (fig. 4) suggest that a greater number of IRIS participants exhibit a more pronounced decrease in BMI, and that fewer IRIS than CTRL participants achieve a BMI increase (including to a lesser degree). Indeed, by t-test, there is a statistically significant difference in mean BMI change from week 0 to 2 between CTRL (0.54 ± 2.69) and IRIS (-0.55 ± 1.04) participants ($p = 0.008$). However, there is no statistically significant difference in week 2 mean BMI between CTRL (22.21 ± 5.53) and IRIS (21.2 ± 3.06) participants ($p > 0.05$).

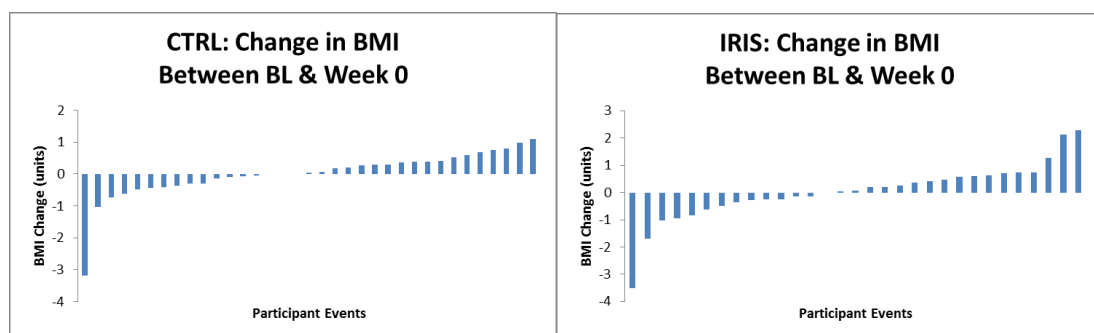


Figure 3 Distribution of BMI Change Values During ATT by Comparison Class

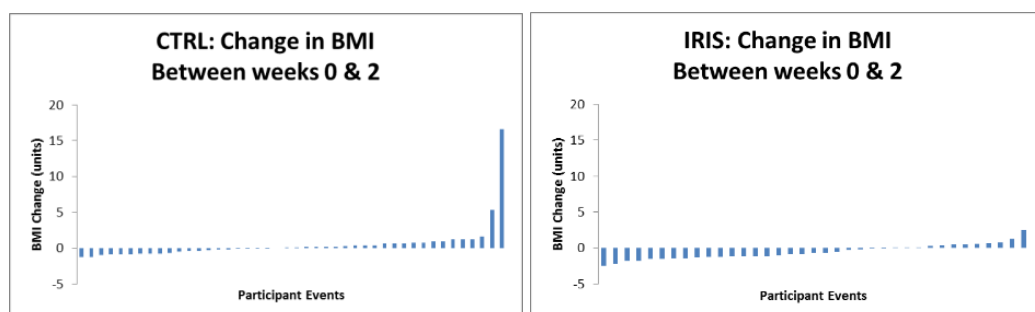


Figure 4 Distribution of BMI Change Values During ARV Therapy by Comparison Class

Higher CRP is a potential risk factor for TB-IRIS. Here, however, it appears that week 0 CTRL group CRP levels may be higher overall (relative to week 0 IRIS group CRP levels), perhaps suggesting that CTRL (but not IRIS) participants have already experienced peak ATT-triggered inflammation. However, by t-

test there is no statistically significant difference in mean CRP at week 0 between CTRL (25.82 ± 35.28) and IRIS (18.49 ± 20.19) participants ($p > 0.05$), although this could be due to high data variability. As expected, however, the increase in CRP from week 0 to week 2 is much larger in the IRIS than in the CTRL group, and - at week 2 - IRIS participants display overall higher CRP values (with 39 of 42 experiencing a CRP increase) than do CTRL participants (while 36 of 45 experiencing a CRP increase, but to a lesser degree). Distributions (fig. 5) indicate that the majority of IRIS and CTRL patients experience an upward trend in CRP level between weeks 0 and 2, that greater numbers of IRIS participants experience more extreme CRP increases, and that greater numbers of CTRL participants experience more extreme CRP decreases. By t-test, there is a statistically significant difference in mean CRP change from week 0 to 2 between CTRL (17.68 ± 42.46) and IRIS (78.51 ± 70.91) participants ($p = 0.00004$), despite high data variability. A statistically significant difference in week 2 mean CRP also exists between CTRL (48.17 ± 51.72) and IRIS (97 ± 67.17) participants ($p = 0.0001$), again despite high data variability. These results suggest that in response to ARV therapy initiation, both CTRL and IRIS participants undergo induction of an inflammatory acute phase response - during which increased hepatic CRP synthesis is usually elicited by IL-6 - but that this is more controlled in the CTRL group and/or more extreme in the IRIS group. While diagnostically supportive, CRP is a poor IRIS-specific biomarker, given that its levels rise during any type of inflammation.

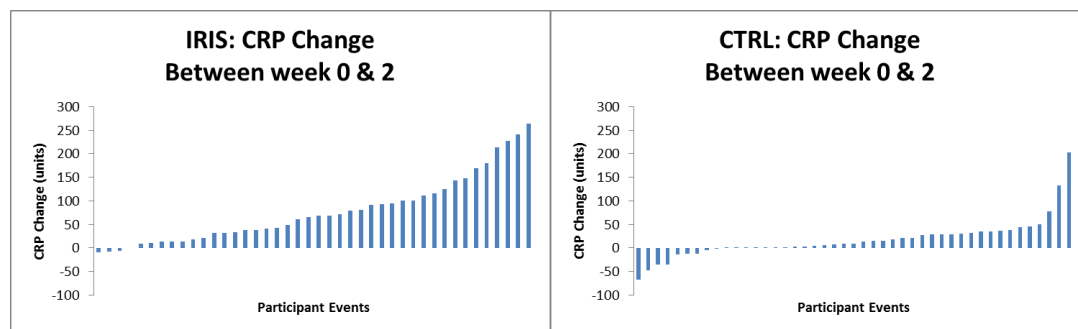


Figure 5 Distribution of CRP Change Values During ARV Therapy by Comparison Class.

While we might expect lower lymphocyte and higher monocyte and neutrophil counts, summary statistics reveal little difference between CTRL and IRIS participant baseline WCC, apart from a marginally broader range in the IRIS group. Indeed, by t-test, there is no statistically significant difference in mean baseline WCC between CTRL (4.84 ± 1.93) and IRIS (4.38 ± 2.46) participants ($p > 0.05$). In the interval between baseline and week 0, both CTRL and IRIS participants display a marginal trend towards decreasing WCC levels (19 of 35 CTRL participants exhibit a decreasing WCC, whereas 17 of 28 IRIS participants display a decreasing WCC). Distributions (fig. 6) further suggest greater WCC decreases in the IRIS group, and greater increases in the CTRL group. Indeed, IRIS patients are known to exhibit a more pronounced response to ATT prior to ARV therapy initiation. However, by t-test, there is no statistically significant difference in mean WCC change from baseline to week 0 between CTRL (-0.48 ± 1.69) and IRIS (-0.89 ± 2.3) participants ($p > 0.05$).

At week 0, there again appears to be little difference between CTRL and IRIS participant WCC, again supported by a t-test determining no statistically significant difference in mean week 0 WCC between CTRL (4.43 ± 1.57) and IRIS (3.85 ± 1.58) participants ($p > 0.05$). During the interval between weeks 0 and 2, both CTRL and IRIS participants display a trend towards increasing WCC levels (31 of 43 CTRL participants, and 38 of 41 IRIS participants for whom data are available), although the degree is more pronounced in the IRIS group. Distributions (fig. 7) clearly demonstrate that a greater number of IRIS participants exhibit an increase in WCC, and to a greater degree. Indeed, by t-test, there is a statistically significant difference in mean WCC change from week 0 to 2 between CTRL (1.31 ± 1.97) and IRIS (4.74 ± 4.12) participants ($p = 0.00004$). This is in keeping with prior knowledge regarding WCC elevation during the onset of TB-IRIS. However, the CTRL group thus also displays at least biochemically- if not clinically-apparent signs of increasing inflammation during early ARV therapy. At week 2, IRIS

group overall WCC values appear larger than CTRL group WCC values. Indeed, by t-test, there is a statistically significant difference in mean week 2 WCC between CTRL (5.77 ± 2.24) and IRIS (8.6 ± 3.63) participants ($p = 0.0002$). This is not unexpected, given that leukocyte counts typically increase during inflammation and are known to rise during the onset of TB-IRIS. We have not specifically examined which leukocyte subsets account for WCC elevation, but do examine neutrophil count changes below.

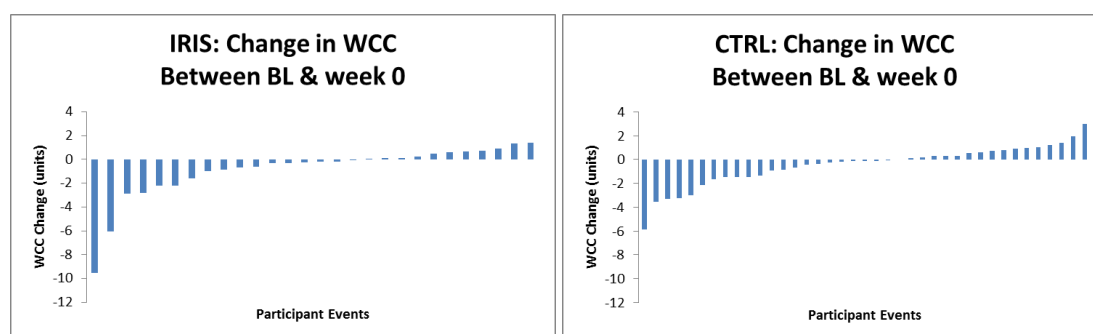


Figure 6 Distribution of Baseline to Week 0 WCC Change Values During ARV Therapy by Comparison Class.

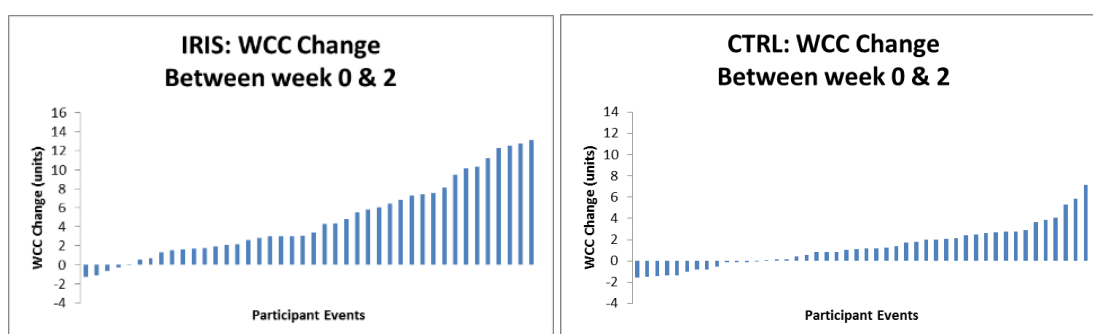


Figure 7 Distribution of Week 0 to 2 WCC Change Values During ARV Therapy by Comparison Class.

Summary statistics reveal little difference between CTRL and IRIS participant baseline neutrophil (NE) %, apart from a marginally broader range in the CTRL group, and the minimum IRIS group value being double that of the minimum CTRL group value (this may suggest that IRIS participants are also more inflamed or primed for inflammation, even at baseline). Indeed, by t-test, there is no statistically significant difference in mean baseline NE% between CTRL (60.96 ± 14.16) and IRIS (65.15 ± 10.3) participants ($p > 0.05$). In the interval between baseline and week 0, both CTRL and IRIS participants display a marginal trend towards decreasing NE% (21 of 33 CTRL participants exhibit a decreasing NE%, whereas 18 of 28 IRIS participants for whom these data are available display a decreasing NE%), mirroring the overall decrease in WCC during this interval. Distributions (fig. 8) suggest little difference in NE% change from baseline to week 0 between CTRL and IRIS participants. Indeed, by t-test there is no statistically significant difference in mean NE% change from baseline to week 0 between CTRL (-5.42 ± 11.35) and IRIS (-7.35 ± 12.78) participants ($p > 0.05$), despite IRIS participants being known to exhibit a more pronounced response to ATT prior to ARV initiation.

At week 0, there again appears to be little difference between CTRL and IRIS participant NE%, again supported by a t-test determining no statistically significant difference in mean week 0 NE% between CTRL (55.89 ± 14.52) and IRIS (60.48 ± 12.96) participants ($p > 0.05$). During the interval between weeks 0 and 2, both CTRL and IRIS participants display a trend towards increasing NE% (34 of 42 CTRL participants, and 34 of 40 IRIS participants for whom data are available), mirroring the WCC increase during this interval. Distributions (fig. 9) suggest that a greater number of IRIS participants exhibit an increase in NE%, and to a greater degree (while those participants experiencing an NE% decrease exhibited a more severe decrease in CTRL relative to IRIS participants). Indeed, by t-test, there is a statistically significant difference in mean NE% change from week 0 to 2 between CTRL (9.67 ± 15.43)

and IRIS (16.8 ± 13.72) participants ($p = 0.013$), indicating that neutrophils contribute towards the significant WCC change observed during this interval. This is in keeping with prior knowledge regarding neutrophilia during the onset of TB-IRIS. Again, the CTRL group thus also displays at least biochemically- if not clinically-apparent signs of increasing inflammation during early ARV therapy. At week 2, IRIS group overall NE% values appear marginally larger than CTRL group NE% values. Indeed, by t-test, there is a statistically significant difference in mean week 2 NE% between CTRL (65.84 ± 16.19) and IRIS (77.64 ± 10.67) participants ($p = 0.0006$). This suggests a relatively larger circulating neutrophil population in the IRIS relative to the CTRL group at week 2.

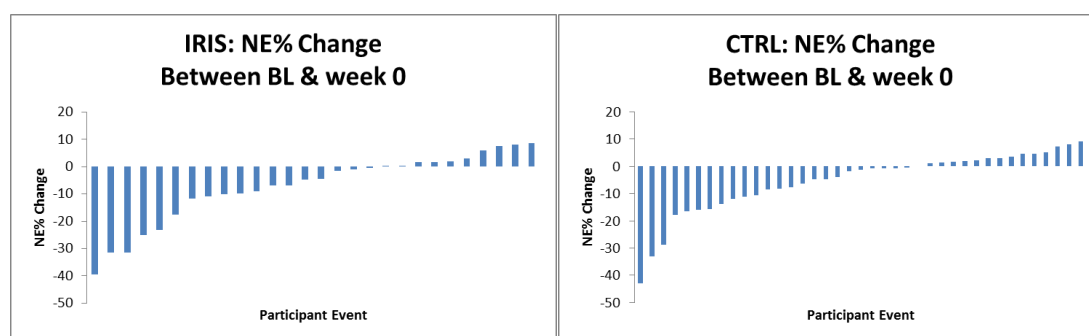


Figure 8 Distribution of baseline to Week 0 NE% Change Values During ARV Therapy by Comparison Class.

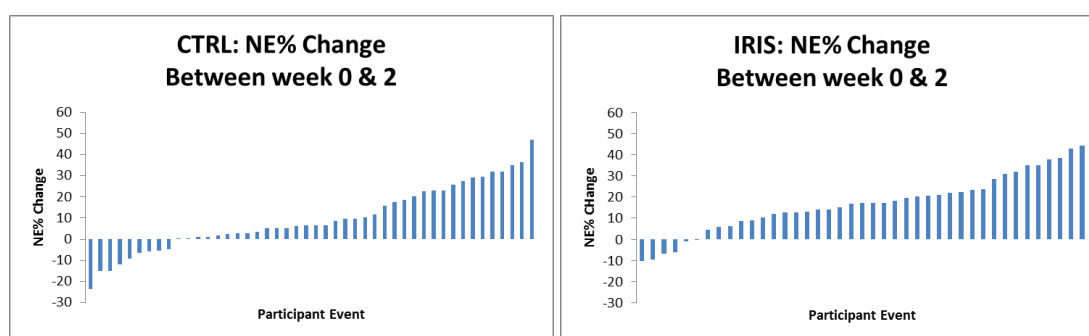


Figure 9 Distribution of Week 0 to 2 NE% Change Values During ARV Therapy by Comparison Class.

2.4.5. Do Associations Exist Between Binary Factors and TB-IRIS Risk?

Results of prospective association analyses (Appendix G) between binary demographic/clinical/ biochemical factors and outcome (development of TB-IRIS) are reported (Table 11). Only baseline smoking status appears to be associated with IRIS risk: given smoking-mediated immunometabolic modulation, TB-IRIS may be a disease of non-smokers, or this association may be better accounted for by unknown confounding factors. This finding should ideally be replicated in a larger independent at-risk cohort longitudinally followed for TB-IRIS development and explicitly characterised for smoking status (indeed, the full parent studies of this work may represent such existing datasets). *In vitro* and *ex vivo* studies to clarify mechanisms by which tobacco smoke components may decrease TB-IRIS risk would be complicated by the absence of certain relevant immune cells and soluble factors, as well as of other key biological systems. Furthermore, each animal model only recapitulates certain facets of human TB-IRIS. Thus, non-clinical mechanistic studies may result in inferences less likely to be clinically translatable. However, a preliminary future experiment may, for example, examine the impact of nicotine (nACh) receptor ligation and blockade on the molecular pathways identified in this thesis (discussed in Chapter 7) to be implicated in TB-IRIS predisposition and pathogenesis, e.g. MAPK-AP-1 signalling, mitochondrial, cell death, and autophagy pathways.

Table 11 Evaluating Potential Binary Demographic/Clinical/Biochemical Risk Factors for IRIS

Binary Factor		Cumulative IRIS Incidence (%)	Relative Risk (RR)	95% Confidence Interval (CI)	Statistically significant?	Interpretation
gender	M	45.9	0.92	0.55, 1.55	N	being female appears to ↑ IRIS risk by 8% but this effect is statistically insignificant
	F	50	1.08			
BL smoking status	smoker	24	0.4	0.16, 0.97	Y	being a non-smoker ↑ IRIS risk by 60% and this effect is statistically significant
	non-smoker	60	1.6			
BL alcohol use status	drinker	48	0.92	0.57, 1.48	N	being a non-drinker appears to ↑ IRIS risk by 8%, but this effect is statistically insignificant
	non-drinker	52	1.08			
BMI response to ATT (BL to wk0)	decrease	48	0.96	0.57, 1.6	N	a BMI ↑ in response to ATT (pre-ARV therapy) appears to ↑ IRIS risk by 4% but this effect is statistically insignificant
	increase	50	1.04			
BMI response to ARV (wk0 to wk2)	increase	36	0.62	0.37, 1.03	N	a BMI ↓ in response to ARV therapy addition appears to ↑ IRIS risk by 38% but this effect is statistically insignificant
	decrease	58	1.38			
CRP response to ARV (wk0 to wk2)	decrease	25	0.48	0.18, 1.32	N	a CRP ↑ in response to ARV therapy addition appears to ↑ IRIS by 52% but this effect is statistically insignificant
	increase	52	1.52			
WCC response to ATT (BL to wk0)	increase	41	0.87	0.5, 1.51	N	a WCC ↓ in response to ATT (pre-ARV therapy) appears to ↑ IRIS risk by 13% but this effect is statistically insignificant
	decrease	47	1.13			
WCC response to ARV (wk0 to wk2)	decrease	20	0.36	0.13, 1.02	N	a WCC ↑ in response to ARV therapy addition appears to ↑ IRIS risk by 64% but this effect is statistically insignificant
	increase	55	1.64			
NE% response to ATT (BL to wk0)	increase	45	0.98	0.57, 1.7	N	an NE% ↓ in response to ATT (pre-ARV therapy) appears to ↑ IRIS risk by 2% but this effect is statistically insignificant
	decrease	46	1.02			
NE% response to ARV (wk0 to wk2)	decrease	43	0.86	0.45, 1.65	N	an NE% ↑ in response to ARV therapy addition appears to ↑ IRIS risk by 14% but this effect is statistically insignificant
	increase	50	1.14			

2.4.6. Immunomodulatory Pharmaceutical Drugs May Influence TB-IRIS

In this cohort, prednisone exposure prior to ARV initiation appears to decrease the risk of developing TB-IRIS during the study period by 33% (Appendix G), but this effect is not statistically significant in this particular cohort (95% CI 0.4, 1.12). However, this finding is in agreement with findings of a statistically significant prednisone-induced 30% decrease in TB-IRIS risk published after conclusion of the full PredART trial [5]. Notwithstanding ATT and ARV therapy regimens (including cotrimoxazole (Bactrim) and pyridoxine prophylaxis) or prednisone, the commonest systemically-administered drug classes prescribed within this cohort were: antihistamines (cetirizine, promethazine, chlorpheniramine), analgesics (NSAIDs, paracetamol, tramadol, and amitriptyline, the latter of which also displays H1-receptor inhibitor activity), antimicrobial agents (amoxicillin ± clavulanic acid, benzathene penicillin, ciprofloxacin, doxycycline, metronidazole, ceftriaxone, azithromycin, nystatin, fluconazole, aciclovir), anti-emetics (metoclopramide), antacids (ranitidine, an H2-receptor antagonist), anticoagulants (warfarin, heparin), diuretics (furosemide, hydrochlorothiazide), and stool-softening/-bulking/diarrheal or anti-spasmodic preparations (senekot, lactulose, loperamide).

Certain systemically-administered pharmaceutical drugs (e.g. anti-histamines, NSAIDs, antifungals, doxycycline, and simvastatin; Table 12) commonly prescribed for IRIS-independent or -related conditions may impact IRIS risk through pleiotropic immunometabolic modulatory actions. Indeed, cotrimoxazole has immunomodulatory (including anti-inflammatory) properties, and long-term cotrimoxazole therapy prior to ARV therapy (relative to cotrimoxazole initiated concurrently with ARV therapy) is known to decrease IRIS incidence [24]. Cotrimoxazole consists of the antibiotics trimethoprim and sulphamethoxazole combined to exploit their antibacterial synergy, although it also has antifungal effects. As per national treatment guidelines, all participants in this cohort initiated cotrimoxazole along with ARV therapy, with the exception of two participants (one who went on to develop IRIS, and one who did not).

Table 12 Known Immunomodulatory Effects of Drugs Commonly-prescribed to Study Cohort

Drug Class	Mechanism of Action	Example(s)	Immunomodulatory Actions	References
anti-histamines	H1 receptor antagonism (four histamine receptors are known); most also have off-target effects	1st-generation H1 receptor antagonists: chlorpheniramine, promethazine	modulate bradykinin activity anti-vasodilatory modulate ICAM expression decrease neutrophil LTB4 production	[25] [26] [27] [28]

		**2nd-generation: cetirizine note: ranitidine is an H2 receptor antagonist	enhance neutrophil oxidative burst (intracellular ROS generation), but inhibit extracellular ROS release oppose NFκB activation (incl. IL-6, TNFα production) alter epithelial, leukocyte immunological polarisation inhibit NK cell cytotoxicity	[29] [30] [31] [32] [33] [34] [35] [36] [37]
NSAIDs	inhibition of COX-mediated prostaglandin and thromboxane synthesis	aspirin ibuprofen	decrease neutrophil adhesion (anti-L-selectin effect is associated with NADPH oxidase-dependent ROS generation at the plasma membrane) inhibit neutrophil recruitment by IL-8 and C5a no effect on degranulation or phagocytic uptake though ibuprofen decreases IL-8-induced CD11b expression aspirin attenuates NO production aspirin enhances TNFα release from LPS-activated RAW264.7 cells aspirin attenuates Th proliferation and IL-2-induced Tcyt proliferation induce mitochondrial intrinsic pathway (involving caspase 9 activation) induce death-receptor mediated extrinsic pathway (involving caspase 8 activation)	[38] [39] [40] [41] [42] [43] [44] [45] [46]
antifungal agents	various	nystatin fluconazole cotrimoxazole	fluconazole alters neutrophil ROS production fluconazole increases macrophage phagocytosis, ROS production, NO production, and microbicidal activity (lower doses decrease adherence, phagocytosis) during infection, fluconazole induces a Th1 response featuring IL-12, IFNγ, and IL-10 levels nystatin alters TNFα, IFNγ, and IL-10 levels	[47] [48] [49]
tetracycline antibiotics	protein synthesis (translation initiation) inhibition	doxycycline	inhibits mast cell activation (and thus histamine release) inhibits neutrophil motility and activation inhibits IL-8 and MMP-1 scavenges ROS inhibits T-cell proliferation and granuloma formation	[50]
nitroimidazole antibiotics	selective nucleic acid synthesis inhibition in anaerobic bacteria and protozoa	metronidazole	anti-inflammatory immunosuppressive inhibits neutrophil ROS generation	[50]
statins	inhibition of HMG-CoA to lower endogenous cholesterol production	simvastatin	anti-inflammatory effects via small GTPase (e.g. Rho, Ras) inhibition inhibit adhesion inhibit NFκB activation activate anti-inflammatory PPARs inhibit IL-8, IL-6, IL-1β, TNFα, and MMP1, 3, and 9 production inhibit MCP-1-mediated monocyte and T-cell recruitment impacts monocyte cytokine production to inhibit Th17 differentiation prevents NADPH oxidase-mediated respiratory burst and ROS formation enhance eNOS and suppress iNOS expression	[51] [52]

While it would be interesting to look at the prospective impact of common drugs on the risk of IRIS development, in this cohort too few participants received such drugs to draw statistically meaningful conclusions. It is nevertheless interesting to note certain observations and trends:

(1) Of participants exposed to H1 receptor-inhibiting antihistamines just prior to or at the time of ARV therapy initiation (n = 12), only 41.7% went on to develop IRIS, while 58.3% did not. Of course, atopy/hypersensitivity themselves alter immune status and thus perhaps IRIS risk: the allergic immune response relies more on mechanisms not typically employed during the response to intracellular pathogens, including a relatively increased reliance on eosinophils (rather than neutrophils and monocytes), Th2 (rather than Th1)-type inflammation (including IL-4), and IgE (rather than IgG). Thus, a predilection to allergy (possibly including drug-induced hypersensitivity reactions) may co-exist with an altered risk of developing IRIS. A single participant exhibiting untreated rhinitis and conjunctivitis did go on to develop (non-INSHI) IRIS, a single participant who exhibited baseline cotrimoxazole-induced hypersensitivity did not go on to develop IRIS, and a single participant who developed drug-induced hypersensitivity at week 2 did develop IRIS. Importantly, since only one smoker and three non-smokers were exposed to antihistamines, this is likely not a confounding factor explaining the apparent increased IRIS risk noted in non-smokers. It would further be interesting to determine whether ATT-induced pruritis (possibly representing mast cell activation) at potential participant screening is able to predict IRIS.

(2) Of only two participants exposed to NSAIDs just prior to or at the time of ARV therapy initiation, neither went on to develop IRIS. As discussed in Chapter 1, NSAIDs may represent an alternative to prednisone for the treatment of TB-IRIS, perhaps through modulation of the eicosanoid cascade, or through their ability to inhibit C5a- and IL-8-mediated neutrophil recruitment.

(3) Of participants exposed to anti-fungal agents just prior to or at the time of ARV therapy initiation (n = 15), only 26.7% went on to develop IRIS, while 73.3% did not. Fungal infections themselves can modulate (e.g. suppress) immunity, which itself may impact TB-IRIS risk. Indeed, the anti-fungal immune response differs from that against intracellular bacteria and viruses, relying to a relatively greater extent on myeloid cell C-type lectin receptors, phagocytosis, IL-23 (instead of IL-12)-conditioned T-cells, and Th17 (rather than Th1)-type inflammation. Alternatively, susceptibility to fungal infections may represent a watershed change that has occurred in the immune status of certain co-infected individuals. Consistent with this hypothesis, four participants exhibiting pre-existing (baseline or prior to week 0) fungal infections (two untreated fungal skin infections and two fluconazole-treated cryptococcal infections) did not develop TB-IRIS. Although anti-fungal agents (or fungal infections) may help protect against IRIS, seven non-smokers and three smokers were pre-exposed to anti-fungal agents, so this is likely not a confounding factor explaining the apparent differential IRIS risk observed in smokers. It is worth noting that azole antifungals inhibit CYP3A4, which mediates a minor efavirenz (EFV) metabolism pathway (CYP2B6 and CYP2A6 are largely responsible for EFV metabolism, but their diminished activity – e.g. due to polymorphisms or drugs – may drive more EFV metabolism along the CYP3A4 pathway) [53] [54] [55]. Certain ARV (e.g. EFV) metabolising enzymes (e.g. CYP2B6 and CYP2A6) are indeed highly polymorphic [54], and resulting differential impact of ARV therapy on leukocyte function may contribute to TB-IRIS risk. Previously, EFV metabolites have been implicated as mediators of EFV-induced neurotoxicity, with ketoconazole-mediated CYP3A4 inhibition ameliorating this effect [56]. It would be interesting to determine whether TB-IRIS is associated with a higher rate of (or more severe) adverse reactions to ARV therapy.

(4) The single participant exposed to doxycycline just prior to or at the time of ARV therapy initiation went on to develop TB-IRIS, despite the hypothesis that doxycycline – via inhibition of MMPs - may help mitigate TB-IRIS [57] [4].

(5) The single participant chronically exposed to simvastatin did not go on to develop IRIS, but was also receiving aspirin and the ACE inhibitor enalapril. In terms of the latter's effects on immune responses, there is some evidence that enalapril weakly modulates macrophage cytokine production [58]. Furthermore, ACE is responsible for production of angiotensin II, which is potentially pro-inflammatory and induces cellular stress responses via binding to the AT1R receptor [59].

(6) It is unclear whether two participant with pre-existing diagnoses of diabetes mellitus were receiving metformin (an AMPK agonist), but both went on to develop TB-IRIS. *If* these participants had indeed been receiving metformin, it argues against the hypothesis that AMPK agonists may help protect against TB-IRIS.

Of course, participants are exposed to many other potent immunomodulatory factors beyond pharmaceutical drugs, including genetic (e.g. HLA or CYP450 polymorphisms), lifestyle (e.g. diet, exercise), co-morbidities (immunosuppressive malnutrition, diabetes, malignancy, etc.; inflammatory rheumatological disorders), and so on. It is clearly impractical to collect data on every potential immunomodulatory factor in a single study. Furthermore, we may be unaware of pharmaceutical drugs with long-term effects taken prior to screening. The single participant exhibiting pre-existing comorbid malignancy (cervical cancer) did not go on to develop TB-IRIS, but also received antifungal agents from week 0.

More specifically concerning ARV therapy, it is possible that different ARV regimens carry differential TB-IRIS risks. Within this cohort, only three participants (PA146, PA204, and PA263) received a non-EFV-based regimen, consisting of lamivudine (3TC), lopinavir (LPVr), and nevirapine (NVP). None developed TB-IRIS. It is possible within this cohort that adverse neurological reactions to EFV (perhaps indicating decreased EFV/metabolite processing rates, or an increased sensitivity to EFV adverse effects) predict

TB-IRIS, although this remains to be formally assessed. The single participant not exposed to tenofovir (TDF; i.e. receiving an alternate regimen consisting of EFV, ABC, and 3TC) also did not develop TB-IRIS.

2.5. Results and Discussion: Neutrophil Processing

Average times to both PBMC isolation (2.3hrs) and neutrophil isolation (~3.5hrs) are well within the recommended 4hr limit (Table 13). Neutrophil isolation yield exceeds 15×10^6 /sample in the CTRL group, and 25×10^6 /sample in the IRIS group (Table 13), likely reflecting higher circulating neutrophil numbers in the latter. Isolated neutrophil purity exceeds 80% in both groups (Table 13), with lymphocytes representing the majority of contaminating cells. This value is skewed by the presence of few very low-purity samples, subsequently excluded from downstream proteomic analysis.

Table 13 Neutrophil Isolation and Processing Results

	CTRL				IRIS			
	Min	Ave	Max	Stdev	Min	Ave	Max	Stdev
phlebotomy-processing interval (hours)	0.83	2.3	5.25	0.85	1	2.31	3.92	0.76
phlebotomy-neutrophil isolation interval (hours)	1.83	3.52	6.83	1.02	2	3.49	5.08	0.8
total neutrophils recovered ($\times 10^6$ /ml)	1.67	15.13	109.47	17.6	1.01	25.22	33.4	7.05
recovered cell purity (neutrophil %)	24.11	83.35	95	13.09	63.2	86.53	95.7	6.8

2.6. Conclusions

Initial sample size targets were exceeded to facilitate *post hoc* exclusion of samples not meeting clinical or laboratory quality standards (e.g. diagnostic certainty, isolated neutrophil purity), as well as to facilitate eventual sub-group analyses (e.g. impact of prednisone on neutrophil proteomes). Thus, following cohort assembly, sample acquisition, isolation, and storage, and sample preparation/assay optimisations (which occurred in parallel), available high-quality stored samples were of sufficient quantity and quality for downstream optimised proteomic analysis: neutrophil lysis, tryptic protein digestion, peptide purification, and subsequent LC-MS/MS (Chapter 3). Furthermore, IRIS and CTRL groups were roughly matched for age, gender, BMI, alcohol use, baseline WCC and neutrophil percentage, sample processing time, and neutrophil purity. Comparisons therefore likely – at least in part – measure the impact of the variable of interest (TB-IRIS status) rather than of any other (confounding) variable known to the study. In various ways, cohort characteristics conform to known risk factors (e.g. lower baseline CD4 count and shorter ATT-ARV interval) and expected changes during TB-IRIS manifestation (e.g. increased CRP, WCC, and NE%).

The ATT-induced decrease in circulating leukocytes in both groups suggests some resolution of inflammation as Mtb load decreases. ATT-induced weight gain in cases suggests more advanced TB at baseline, which may explain the more pronounced response to ATT known to occur in this group. The ARV-induced increase in circulating leukocytes and CRP in both groups (albeit more pronounced in cases, including after two weeks of ARV therapy) indicates that ARV therapy increases subclinical inflammation in *both* groups, including an acute-phase response, and that this effect is more pronounced in cases. This is consistent with prior knowledge regarding the association between increased inflammation, neutrophilia, and manifestation of TB-IRIS. The apparent inflammatory increase even in the control group is also in agreement with a trial reporting that a shorter ATT-ARV interval in HIV-associated TB meningitis increases the incidence of grade four adverse events (not including TB-IRIS) [60]. ARV-induced weight loss in cases suggests increased metabolic demands of this more activated immune system, perhaps to some degree recapitulating the TB metabolic state.

The ARV-induced increase in WCC or decrease in BMI may predict TB-IRIS, and prednisone exposure prior to ARV initiation likely mitigates TB-IRIS risk, but only smoking apparently significantly decreases the

risk of IRIS, perhaps via mechanisms similar to those which account for the protective effect of smoking against other complex immunological disorders. Such mechanisms may include pre-adaptation to immunometabolic changes induced by smoking, and which are similar to the changes that would otherwise occur during TB-IRIS pathogenesis. Pre-existing binary factors other than smoking may fail to achieve significance as risk factors due to relatively small sample sizes (cohort sizes are typically larger when calculating relative risk in order to determine prospective factor association with risk). Notably, the apparent association between non-smoking status and IRIS represents a novel risk factor supported by reasonable mechanistic hypotheses.

Perhaps in some support of later proteomic-driven and microscopy-supported hypotheses, EFV-lacking alternate regimens may be less likely to induce TB-IRIS (although too few data-points exist to draw formal conclusions). Although low numbers again preclude formal conclusions, the low percentage of anti-fungal agent-exposed participants who go on to develop TB-IRIS suggests that exposure to certain immunomodulatory microbes or pharmaceutical drugs prior to ARV therapy initiation may impact TB-IRIS risk. Indeed, long-term exposure to cotrimoxazole prior to ARV therapy initiation is known to mitigate TB-IRIS risk, and it has been hypothesised that the NSAID aspirin may be a safe and effective alternative to the corticosteroid prednisone for treatment of mild TB-IRIS. Regarding drug indications, the allergic immune response relies more on eosinophils (rather than neutrophils and monocytes), Th2 (rather than Th1)-type inflammation and IgE (rather than IgG), while the anti-fungal immune response relies on IL-23 (rather than IL-12)-conditioned T-cells, and Th17 (rather than Th1)-type inflammation. Indeed, as mentioned in Chapter 1, management of non HIV-associated IRIS – apart from corticosteroids, NSAIDs, and intravenous immunoglobulins - may include statin-mediated Th1/Th17-type response inhibition. However, many genetic or lifestyle factors beyond those recorded and analysed here may participate in immunomodulation and contribute to differential TB-IRIS risk.

Appendix B *Eligibility (Inclusion and Exclusion) Criteria and Ethical Considerations*

(1) Inclusion Criteria

Eligible patients must fulfil all of the following:

have history and symptoms highly-suggestive of pulmonary TB or laboratory-confirmed TB

have initiated anti-tuberculous medication

be HIV positive as confirmed by rapid testing

have CD4 < 200

be ARV-naïve

be eligible to start HAART on the basis of the S.A. 2010 ARV guidelines

preferably have risk factors for the development of TB-IRIS at baseline (e.g. low CD4 count, high VL, extra-pulmonary TB, recent onset of TB infection, short interval between initiation of anti-TB agents and HAART)

(2) Exclusion Criteria

Eligible participants must not fulfil any of the following:

age <18 years

asthma or chronic obstructive pulmonary disease (COPD)

pneumothorax

pregnancy

unknown HIV status, unwilling to test

unable to give informed consent (cognitive deficit), language barrier

mental illness

prisoners

on anti-retroviral therapy (ARVs)

previous pulmonary TB (although some cases may be permitted)

suspected drug resistant TB (as identified by GeneXpert)

withheld informed consent

(3) Ethical Considerations

Ethical approval for all three studies was obtained from UCT's Human Research Ethics Committee (HREC). All work was performed in compliance with:

- the World Medical Association Declaration of Helsinki 2008, amended in 2013: <http://www.wma.net/en/20activities/10ethics/10helsinki/DoH-Oct2013-JAMA.pdf>
- the South African Department of Health Ethics in Health Research Principles, Processes and Structures, 2nd Edition, 2015: <http://www.nhrec.org.za/docs/Documents/EthicsHealthResearchFinalAused.pdf>
- good clinical practice guidelines: <http://www.crc.uct.ac.za/gcp>

- good laboratory practice guidelines: <https://www.who.int/tdr/publications/documents/glp-handbook.pdf>

Study participation did not alter routine care of patients by Ubuntu clinical staff as per national and international guidelines, and any additional required investigation or management noted at study visits were referred to treating clinicians. No social, economic, medical, or ethical implications of study participation were considered unusual or unacceptable within the framework of the above guidelines. Strategies were implemented to minimise potential physical, psychological, social, and economic risks to participants and study personnel. For example, parent study procedures (e.g. chest x-ray, phlebotomy) were minimally-invasive, required a relatively small time investment, and carried no risk of serious adverse effects. Participants were compensated a nominal amount (~R30) for their time, inconvenience, and travel expenses. Study personnel were exposed to no additional risk beyond that normally incurred in a clinical setting in this region.

Prospective participants were informed of study protocols and risks using simple written and verbal explanations in English or Xhosa, providing sufficient time for consideration and questions. Participants were enrolled only after informed consent was provided (via signature or thumb-print, without coercion) and counter-signed by study personnel. Enrolled participants retained the right to withdraw from the study at any time without negative impact on their medical care.

Study participant and data confidentiality were ensured through assignment of anonymous study identifiers separated from identifying data to both data and clinical samples, as well as password-protected electronic records only accessible by selected study personnel. Data resulting from laboratory investigation of samples would not produce clinically-significant information, or information with further social, economic, or ethical implications for individual participants. Consent was sought for sample storage, with the caveat that any future investigations not already specified in the existing study protocol would be subject to comprehensive ethical review prior to approval. However, "investigations for mutations associated with inherited genetic diseases" may never be performed on such stored samples.

No conflicts of interest were recognised by the study teams. Research staff involved in design, conduct, and analysis had no proprietary interests relating to the studies.

Appendix C Parent Study Aims & Routine Clinical/Laboratory Characterisation

(1) TDTB2 Study

<i>Study title</i>	Defining mechanisms of immunopathology in TB-HIV co-infection and TB Immune reconstitution inflammatory syndrome (TB-IRIS; HREC reference number 516/2011).
<i>Recruitment period</i>	2012 – 2013 (current study: August 2012 through December 2013)
<i>Research questions</i>	Which mechanisms drive tissue destruction in TB/HIV-coinfection and TB-IRIS? Can therapeutic immunomodulatory agents ameliorate immunopathology?
<i>Study hypothesis</i>	Tissue destruction in TB/HIV-coinfection results from excessive MMP activity, amplified by activated NKT cell cytokine release and perforin- as well as granzyme B-mediated cytotoxicity (based on pilot study data).

Study aims

(1) Cross-sectional study (part I): MMP activity and NKT cell profiling in control (TB-HIV⁻), HIV⁻ respiratory symptomatic, HIV-TB⁺, and TB⁺HIV⁺ participants.

(2) Longitudinal study (part II, from which current project samples derive): analysis of MMP and NKT cell dynamics in TB/HIV-coinfected participants at risk for TB-IRIS.

(3) Laboratory study: investigating MMP-regulating communication networks between NKT cells and macrophages. Sputum and plasma MMP-1, -3, -7, -9, IL-1 β , TNF α , IFN γ , and IL-10 were examined by RT-PCR and Luminex array; MMP activity was examined by gelatin and casein zymography; tissue inhibitor of metalloproteinase (TIMP) concentration was examined by ELISA; PBMC surface expression of MMPs was examined by flow cytometry; and baseline $\pm$ stimulated PBMC MMP and cytokine gene expression was examined by RT-PCR. PBMC cytokine responses to stimulation (using recombinant matrix-related and MMP-degradation of collagen/elastin-derived peptides and proteins) were examined using by ELISpot and Luminex; reactive PBMC also underwent flow cytometric assessment and proliferation assays (NKT cell enumeration and subset determination using CD3, CD4, CD8, CD19, CD56, V α 23, V β 11, NKp30, NKp46, and alphaGal/Cer tetramer). Finally, plasma antibodies to components of the lung extra-cellular matrix were quantified by ELISA.

(2) PredART Trial

<i>Study title</i>	Preventing tuberculosis-associated immune reconstitution inflammatory syndrome in high-risk patients: a randomized placebo-controlled trial of prednisone (PredART Trial, HREC ref. no. 136/2013, clinicaltrials.gov identifier NCT01924286).
<i>Recruitment period</i>	2014 – 2016 (current study: October 2014 through October 2016)
<i>Research question</i>	Does prednisone exposure prior to ARV initiation mitigate TB-IRIS risk?
<i>Study hypothesis</i>	Prednisone suppresses molecular mechanisms responsible for TB-IRIS immunopathogenesis, is safe and effective for amelioration of TB-IRIS-related morbidity and mortality, and may contribute to TB-IRIS prevention.

Study aims

(1) Enrol TB/HIV-coinfected patients at risk for TB-IRIS (i.e. CD4 count $\leq$ 100), randomise to placebo or prednisone (40 mg/day for two weeks, followed by 20 mg/day for two weeks) treatment arms, and follow for TB-IRIS manifestation.

(2) Assess the impact of prophylactic prednisone on paradoxical TB-IRIS incidence in at-risk patients (primary end-point: manifestation of TB-IRIS within 12 weeks of ARV therapy initiation)

(3) Routinely-performed Clinical Assessments and Laboratory Investigations

Participants were assessed at baseline, at ARV initiation (week 0), at weeks 2, 4, and 12 post-ARV initiation, and at any required interim visits. At every visit, a medical history was taken and a clinical examination performed (including updating height and weight measurements).

Baseline visit

sputum GeneXpert, and microscopy, culture, and sensitivity

Follow-up visits

- full blood count and differential
- CD4 count and viral load
- urea, creatinine and electrolytes
- C-reactive protein level
- chest radiograph
- any other clinically-relevant investigations, as required

Appendix D Paradoxical TB-IRIS Consensus Case Definition [61]

Please note that due to the need for case definition accuracy, much of the text in this appendix (indicated by quotation marks) is reported from the source near-verbatim.

(1) Antecedent requirements (both must be met)

Diagnosis of TB "made before starting ART and fulfilling WHO criteria for diagnosis of smear-positive or -negative PTB, or extrapulmonary TB.

Initial response to TB treatment: stabilized or improving condition on appropriate TB treatment before ART initiation (e.g. cessation of night sweats, fevers, cough, weight loss). This *does not* apply to patients starting ART within 2 weeks of starting TB treatment since insufficient time may have elapsed for a clinical response to manifest".

(2) Clinical criteria

"Onset of TB-IRIS manifestations should be within 3 months of ART initiation, reinitiation, or regimen change because of treatment failure.

At least one major criterion or two minor clinical criteria must be present:

Major Criteria

New or enlarging lymph nodes, cold abscesses, or other focal tissue involvement (e.g. TB arthritis)
New or worsening radiological features of tuberculosis (on CXR, abdominal USS, CT, or MRI)
New or worsening CNS TB (meningitis or focal neurological deficit)
New or worsening serositis (pleural effusion, ascites, or pericardial effusion)

Minor Criteria

New or worsening constitutional symptoms (e.g. fever, night sweats, or weight loss)
New or worsening respiratory symptoms (e.g. cough, dyspnoea, or stridor)
New or worsening abdominal pain with peritonitis, hepato/splenomegaly, or abdominal adenopathy"

(3) Alternative explanations for clinical deterioration which must be excluded if possible

"Failure of tuberculosis treatment because of tuberculosis drug resistance
Poor adherence to tuberculosis treatment

Another opportunistic infection or neoplasm (it is particularly important to exclude an alternative diagnosis in patients with smear-negative pulmonary tuberculosis and extrapulmonary tuberculosis where the initial tuberculosis diagnosis has not been microbiologically confirmed)

Drug toxicity or reaction"

Appendix E Neutrophil Isolation Optimisation

(1) PBMC and Neutrophil Losses During Isolation

This analysis included peripheral venous blood from four healthy volunteers. Whole blood differential counts were performed prior to Ficoll density gradient centrifugation-based PBMC isolation, followed by differential counts of each phase during Ficoll isolation, in order to facilitate fractional PBMC and neutrophil tracking throughout the procedure. Whole blood differential cell counts (i.e. prior to isolation) are provided in Table 1. Separation phase-specific differential counts during isolation are provided in Table 2.

Table 1 Differential Cell Count Prior to Ficoll Isolation

Cell type	Count	Percentage	Reference Range
lymphocytes	$76.09 \pm 4.17 \times 10^6$	34.76	20-40%
monocytes	$7.47 \pm 1.56 \times 10^6$	3.41	2-10%
neutrophils	$135.36 \pm 37.59 \times 10^6$	61.83	40-80%
total	$218.92 \pm 43.32 \times 10^6$	100	100%

Table 2 Phase-specific Differential Counts During Isolation

Phase	Cell type	% Cells Lost to Phase
plasma	lymphocytes	0
	monocytes	5.35 ± 0.22
	neutrophils	0.17 ± 0.05
PBMC layer	lymphocytes	63.33
	monocytes	63.97
	neutrophils	0
Ficoll	lymphocytes	26.73 ± 0.09
	monocytes	11.25 ± 1.28
	neutrophils	9.83 ± 0.02
red cell/granulocyte pellet	lymphocytes	4.59 ± 1
	monocytes	24.78 ± 5.17
	neutrophils	~90

These data indicate that a few monocytes are unintentionally lost in the plasma layer; as expected, the majority of PBMC (and no neutrophils) reside in the PBMC layer; a significant proportion of each cell type (especially lymphocytes) is lost in the Ficoll layer; and a significant proportion of monocytes is unintentionally lost in the red cell/granulocyte pellet, which otherwise contains the majority of neutrophils, as expected. Thus, the volume of whole blood available from the parent studies, in conjunction with the Ficoll isolation method (Appendix F), is able to provide sufficient PBMC and neutrophil numbers for the purposes of the current study. Post-isolation total PBMC viability is always >95% (fig. 1).

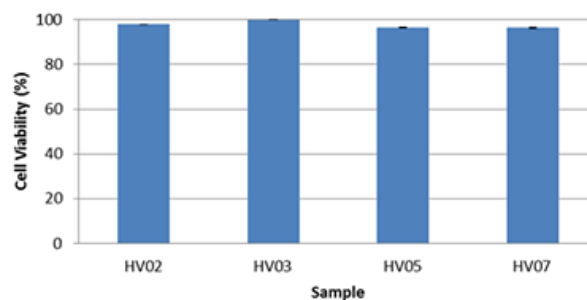


Figure 1 Post-isolation Viability of Total PBMC

(2) Neutrophil Isolation Protocol Optimisation

A number of attempts using an original erythrocyte sedimentation and lysis-based neutrophil isolation protocol [62] resulted in poor neutrophil yields and purity (fig. 2), as well as total isolated cell viability of only $53.33 \pm 11.24\%$ as measured by Trypan blue exclusion.

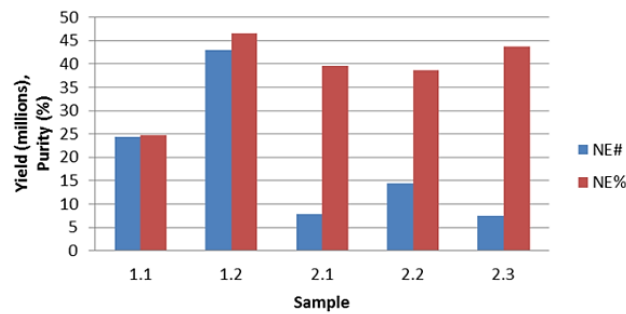


Figure 2 Neutrophil Yield and Purity Achieved Using Original Protocol

Multiple rounds of optimisation based on information extracted from literature led to the following protocol adaptations: minimising the time interval from phlebotomy to blood processing, performing all leukocyte isolation steps at room temperature instead of on ice (in order to avoid temperature fluctuations), optimising Ficoll-based PBMC isolation (i.e. blood dilution volumes and Ficoll:diluted blood ratio), ensuring maximal removal of PBMC above the Ficoll layer prior to neutrophil isolation, preventing exposure to glass or unnecessary agitation during neutrophil isolation (including performing all neutrophil wash steps at centrifuge brake 1) in order to minimise neutrophil activation, minimising the delay prior to Ficoll layer removal, increasing the initial volume for neutrophil pellet resuspension from 25 to 45ml, replacing Hank's balanced salt solution (HBSS) lacking Ca^{2+} and Mg^{2+} with more readily available phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+} , increasing dextran concentration from 3% to 6% while reducing added dextran volume from 25 to 5ml, allowing erythrocyte sedimentation to proceed for no more than 20min (so as to sediment the maximum number of erythrocytes while avoiding sedimentation and loss of neutrophils), replacing H_2O -based erythrocyte lysis with PharmLyse-based lysis, minimising neutrophil contact time with erythrocyte lysis buffer, implementing numerous high-volume wash steps to remove residual lysis buffer, and minimising neutrophil contact time with RPMI (which contains divalent ions) in order to minimise neutrophil activation.

This resulted in an optimised protocol (Appendix F) consistently providing high yield, and purity $\geq 85\%$ (fig. 3), although individual participant sample characteristics (e.g. degree of inflammation, lymphocyte:neutrophil count) occasionally result in lower purity, as for TDTB2_196 (week 2 sample).

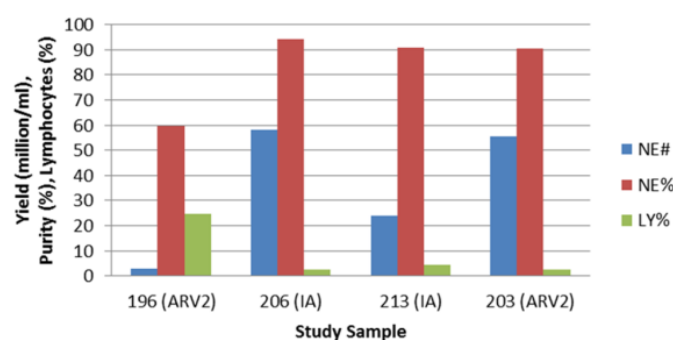


Figure 3 Neutrophil Yield and Purity with Optimised Protocol

Although we noted a tendency towards lower neutrophil purity (higher monocyte contamination) in samples from more clinically-unwell patients, this has yet to be formally confirmed (e.g. via CRP level-monocyte contamination percentage association analyses). It is possible that activated cells are molecularly stickier (e.g. more prone to clumping due to the presence of extracellular activated fibrin) or that they undergo shape/density changes leading to higher numbers of PBMC migrating into the erythrocyte/granulocyte layer during density centrifugation.

(1) PBMC Isolation Description and Method

PBMC are isolated using a validated density gradient centrifugation method. Briefly, whole blood is mixed in a 1:1 ratio with Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich), is layered over Ficoll-paque (GE Healthcare Life Sciences) in a ratio of 2:1 diluted blood to Ficoll, and is centrifuged at 700g for 20min (room temperature, brake 1). During centrifugation, PBMC migrate to the plasma-Ficoll interface, while red cells and granulocytes pellet below the Ficoll layer. PBMC are harvested into 10ml RPMI-1640 medium with L-glutamine and sodium bicarbonate (RPMI; Sigma-Aldrich). After washing by centrifugation at 600g for 10min (room temperature, brake 3), PBMC are resuspended in a further 10ml RPMI and a 20ul sample is taken for counting by the Trypan blue exclusion method. After a second identical wash step, PBMC are resuspended to 5-10 x 10⁶ PBMC per ml in a validated cryopreservation solution (consisting of RPMI containing a final concentration of 50% FCS and 10% DMSO) at 4°C. PBMC are then slowly frozen (1°C/min) to -80°C overnight in a Mr. Frosty (Nalgene) with isopropyl alcohol insulation before being transferred to liquid nitrogen vapour (below -130°C) for long-term storage.

- (1) label Falcons: blood + PBS, Ficoll + blood, RPMI + PBMC (also label cryovials per patient)
- (2) dilute patient blood 1:1 with PBS in 50ml Falcon, rinsing each blood tube with some of the PBS
- (3) pipette 15ml Ficoll into fresh Falcon and layer 30ml diluted blood slowly over it
- (4) centrifuge 700g, break 1 in stable centrifuge for 20min
- (5) pipette 15ml RPMI into fresh Falcon
- (6) from centrifuged Falcon, remove half of plasma supernatant by careful pipetting (do not disturb PBMC layer)
- (7) use Pasteur pipette to vacuum up PBMC layer (multiple passes) into RPMI tube; remove as much of PBMC layer as possible, until no visible cells still resting on layer beneath (otherwise neutrophil purity will be poor)
- (8) centrifuge RPMI + PBMC 600g, break 3, 10min (meanwhile, begin neutrophil isolation – see below)
- (9) gently pour off supernatant, close tube, flick to disperse pellet
- (10) add another 10ml RPMI into same tube, washing down sides, and gently triturate to resuspend well
- (11) count: mix 20ul Trypan blue + 20ul PBMC + RPMI mix in a 96-well plate well, then triturate & place 10ul into a counting chamber and count 16 squares (then multiply total by 2 x 10 x 10⁴ = number of PBMC per 10ml)
- (12) repeat centrifugation as for step (8)
- (13) pour off supernatant
- (14) resuspend in 50% RPMI + 10% FCS (add first!) and FCS + 20% DMSO (add second, slowly and drop-wise, while swirling tube to mix) such that final PBMC concentration is 5-10 million PBMC per ml
- (15) aliquot 1ml into each cryovial, add cryovials to Mr. Frosty, and freeze at -80°C
- (16) move to liquid N₂ vapour the following morning.

(2) Neutrophil Isolation Description and Method

Neutrophils are isolated from the red cell/granulocyte pellet remaining after Ficoll-based PBMC isolation, using an in-house-optimised method (based on Nauseef's original protocol) performed – in its entirety – at room temperature 14. Briefly: The remaining plasma-Ficoll supernatant is aspirated from above the red cell-granulocyte pellet resulting from 30ml of diluted blood, which is then resuspended to a volume of 45ml with PBS and mixed by inversion. 5ml of 6% dextran Mr 450,000-650,000 (Sigma-Aldrich) in normal saline is added and the tube is mixed well by inversion. Red cell sedimentation is allowed to proceed for no more than 20min, and the straw-coloured supernatant (containing suspended granulocytes) is aspirated and transferred to a sterile 50ml conical tube before centrifugation at 500g for 10min (room temperature, brake 1) to pellet granulocytes. The supernatant is discarded and the pellet resuspended in 50ml 1x PharmLyse buffer (BD Biosciences) for 10min at room temperature to lyse remaining red cells. Centrifugation at 500g for 5min (room temperature, brake 1) pellets granulocytes, and the supernatant is discarded before resuspending the pellet in 50ml PBS and washing by centrifugation as before. The supernatant is discarded and the pellet resuspended in 2ml RPMI for automated differential counting.

The volume of sample corresponding to 5×10^6 neutrophils is transferred to the maximum number of sterile 1.5ml flip-top microcentrifuge tubes, and these are centrifuged at 1750rpm for 5min (room temperature, brake 1) to pellet neutrophils. The supernatants are aspirated and each neutrophil pellet resuspended in 50ul PBS for transfer to a 1ml cryogenic vial (Corning). Each 1.5ml microcentrifuge tube is rinsed out with a further 50ul PBS into the corresponding cryogenic vial. All cryogenic vials are then flash-frozen in liquid nitrogen (<196°C) before being transferred to liquid N₂ vapour for long-term storage.

(1) make normal (0.9%) saline: mix 0.9g saline into 100ml sterile water and filter through 0.22um into sterile container; store at 5°C

(2) make 6% dextran in normal saline (warm 100ml saline to 37°C, then make 6g dextran up to 100ml, swirl to dissolve – can leave at 37°C for longer, if required - and filter through 0.22um into sterile container; store at 5°C

(3) per patient, make fresh: 2x 50ml 1x Pharmlyse from 10x stock in sterile water in Falcons

(4) after PBMC vacuuming, pipette as much of remaining Ficoll off of red cell layer as possible (vigorously suck top parts of layer to remove remaining PBMC, but do not disturb red cell layer)

(5) resuspend red cell layer to 45ml total using PBS

(6) add 5ml 6% dextran and mix (30x gentle inversion)

(7) allow to sediment for ~20min (not longer) and aspirate supernatant (down to ~15ml mark) into fresh Falcons

(8) centrifuge 500g, 10min, break 2 (discard supernatant)

(9) resuspend each pellet in 50ml 1x Pharmlyse for 10min (not longer)

(10) centrifuge 500g, 5min, break 3 (discard supernatant by tipping until red cells approach edge)

(11) resuspend in 2ml RPMI

(12) differential cell count (100ul sample)

(13) centrifuge 500g, 5min, break 3

(14) resuspend to desired concentration in minimal PBS volumes, aliquot, and flash-freeze in liquid N₂.

Appendix G Relative Risk Calculations for Binary Demographic/Clinical/Biochemical Factors

Certain continuous factors have been dichotomised for the purposes of this analysis. While this is not considered best practice when an arbitrary cut-off point is chosen (due to loss of information, such as magnitude of change, or time-to-event), here it is only applied to factors which change in opposing directions (i.e. dichotomous groups exist *a priori*, meaning that dichotomisation makes logical sense) [63].

- Does gender impact relative risk of developing IRIS?

	cases	controls	totals
exposure:	A = 17	B = 20	A+B = 37
male	x1	n1-x1	n1
exposure:	C = 13	D = 13	C+D = 26
female	x2	n2-x2	n2
totals	A+C = 30	B+D = 33	sum(all) = 63

The cumulative incidence of IRIS in the male group was $17/37 = 0.459$, while that in the female group was $13/26 = 0.5$.

$$\begin{aligned}
 RR &= \left(\frac{A}{A+B} \right) / \left(\frac{C}{C+D} \right) \\
 &= (17/37) / (13/26) \\
 &= 0.459/0.5 \\
 &= 0.918
 \end{aligned}$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when gender is male is 0.92. A relative risk of ~1 indicates no risk difference between genders; <1 means less risk in the exposed group (male) vs the unexposed group (female); >1 means higher risk in the exposed group relative to the unexposed group. Therefore, in this cohort, men appear to have an 8% lower risk of developing IRIS relative to women in this cohort (i.e. they had 0.92x the risk of developing IRIS during the study period compared to women). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2} \right)}$$

$$\ln(0.92) \pm 1.96\sqrt{((20/17)/37 + (13/13)/26)}$$

$$\ln(0.92) \pm 1.96\sqrt{(0.032 + 0.038)}$$

$$\ln(0.92) \pm 1.96\sqrt{(0.07)}$$

$$-0.083 \pm 1.96(0.264)$$

$$-0.083 \pm 0.518$$

$$-0.601, 0.436 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.548, 1.546$$

We are 95% confident that the RR of IRIS in males compared to females is between 0.55 and 1.55, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does smoking status impact TB-IRIS risk?

	cases	controls	totals
exposure: smoking	A = 4 x1	B = 13 n1-x1	A+B = 17 n1
exposure: non/ex-smoking	C = 35 x2	D = 23 n2-x2	C+D = 58 n2
totals	A+C = 39	B+D = 36	sum(all) = 75

The cumulative incidence of IRIS in the currently smoking group was $4/17 = 0.24$, while that in the non/ex-smoking group was $35/58 = 0.6$.

$$\begin{aligned}
 RR &= \frac{(A/A+B)}{(C/C+D)} \\
 &= (4/17)/(35/58) \\
 &= 0.24/0.6 \\
 &= 0.4
 \end{aligned}$$

Therefore, the relative risk of acquiring TB-IRIS in the at-risk population subgroup of current smokers is 0.4 compared to the subgroups of non/ex-smokers (stopped for at least 3mos). Therefore, in this cohort, current smokers appear to have a 60% lower risk of developing IRIS relative to non/ex-smokers (i.e. they had 0.4x the risk of developing IRIS during the study period compared to non/ex-smokers). 95% CI formula:

$$\begin{aligned}
 &Ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2}\right)} \\
 &Ln(0.4) \pm 1.96\sqrt{((13/4)/17 + (23/35)/58)} \\
 &Ln(0.4) \pm 1.96\sqrt{(0.19 + 0.01)} \\
 &Ln(0.4) \pm 1.96\sqrt{(0.2)} \\
 &-0.92 \pm 1.96(0.45) \\
 &-0.92 \pm 0.89 \\
 &-1.81, -0.03 \text{ (this is CI for Ln(RR))}
 \end{aligned}$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.16, 0.97$$

We are 95% confident that the RR of IRIS in current smokers (compared to non/ex-smokers) is between 0.16 and 0.97, and since the 95% CI does not include the null value (1), this finding is statistically significant: current smokers appear to have a lower risk of developing IRIS.

- Does alcohol use impact TB-IRIS risk?

	cases	controls	totals
exposure:	A = 14	B = 15	A+B = 29

drinker	x1	n1-x1	n1
exposure: non/ex-drinker	C = 24 x2	D = 22 n2-x2	C+D = 46 n2
totals	A+C = 38	B+D = 37	sum(all) = 75

The cumulative incidence of IRIS in the currently drinking group was $14/29 = 0.48$, while that in the non/ex-drinking group was $24/46 = 0.52$.

$$\begin{aligned}
 RR &= \left(\frac{A}{A+B} \right) / \left(\frac{C}{C+D} \right) \\
 &= (14/29) / (24/46) \\
 &= 0.48/0.52 \\
 &= 0.92
 \end{aligned}$$

Therefore, the relative risk of acquiring TB-IRIS in the at-risk population subgroup of current drinkers is 0.92 compared to the subgroups of non/ex-drinkers (stopped for at least 3mos). Therefore, in this cohort, current drinkers appear to have an 8% lower risk of developing IRIS relative to non/ex-drinkers (i.e. they had 0.92x the risk of developing IRIS during the study period compared to non/ex-drinkers). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2} \right)}$$

$$\ln(0.92) \pm 1.96\sqrt{((15/14)/29 + (22/24)/46)}$$

$$\ln(0.92) \pm 1.96\sqrt{(0.04 + 0.02)}$$

$$\ln(0.92) \pm 1.96\sqrt{(0.06)}$$

$$-0.08 \pm 1.96(0.24)$$

$$-0.08 \pm 0.47$$

$$-0.55, 0.39 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.57, 1.48$$

We are 95% confident that the RR of IRIS in current smokers (compared to non/ex-smokers) is between 0.16 and 0.97, and since the 95% CI does not include the null value (1), this finding is statistically significant: current smokers appear to have a lower risk of developing IRIS.

- Does ATT-induced BMI change (BL to wk0) predict TB-IRIS?

	cases	controls	totals
exposure: BMI decrease	A = 13 x1	B = 14 n1-x1	A+B = 27 n1
exposure: BMI increase	C = 17 x2	D = 17 n2-x2	C+D = 34 n2
totals	A+C = 30	B+D = 31	sum(all) = 61

The cumulative incidence of IRIS in the BMI pre-decrease group was $13/27 = 0.48$, while that in the BMI pre-increase group was $17/34 = 0.5$

$$\begin{aligned}
 RR &= (A/A+B) / (C/C+D) \\
 &= (13/27) / (17/34) \\
 &= 0.48/0.5 \\
 &= 0.96
 \end{aligned}$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when pre-ARV BMI decreases is 0.96 compared to when pre-ARV BMI increases. Therefore, in this cohort, when pre-ARV BMI decreases, there is a 0.04% lower risk of developing IRIS relative to when pre-ARV BMI increases (i.e. they had 0.96x the risk of developing IRIS during the study period compared to when pre-ARV BMI increased). 95% CI formula:

$$\begin{aligned}
 &Ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2}\right)} \\
 &Ln(0.96) \pm 1.96\sqrt{((17/17)/34 + (14/13)/27)} \\
 &Ln(0.96) \pm 1.96\sqrt{(0.03 + 0.04)} \\
 &Ln(0.96) \pm 1.96\sqrt{(0.07)} \\
 &-0.04 \pm 1.96(0.26) \\
 &-0.04 \pm 0.51 \\
 &-0.55, 0.47 \text{ (this is CI for Ln(RR))}
 \end{aligned}$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.57, 1.6$$

We are 95% confident that the RR of IRIS when pre-ARV WCC increases (compared to when it decreases) is between 0.84 and 1.10, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does ARV-induced BMI change (wk0 to wk2) predict TB-IRIS?

	cases	controls	totals
exposure: BMI increase	A = 12 x1	B = 21 n1-x1	A+B = 33 n1
exposure: BMI decrease	C = 26 x2	D = 19 n2-x2	C+D = 45 n2
totals	A+C = 38	B+D = 40	sum(all) = 78

The cumulative incidence of IRIS in the BMI post-ARV increase group was 12/33 = 0.36, while that in the BMI post-ARV decrease group was 26/45 = 0.58.

$$\begin{aligned}
 RR &= (A/A+B) / (C/C+D) \\
 &= (12/33) / (26/45)
 \end{aligned}$$

$$= 0.36/0.58$$

$$= 0.62$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when post-ARV BMI increases is 0.62 compared to when post-ARV BMI decreases. Therefore, in this cohort, when post-ARV BMI increases, there is a 38% lower risk of developing IRIS relative to when post-ARV BMI decreases (i.e. they had 0.62x the risk of developing IRIS during the study period compared to when post-ARV BMI decreased). 95% CI formula:

$$\text{Ln}(\text{RR}) \pm z\sqrt{\left(\frac{(n1 - x1)/x1}{n1} + \frac{(n2 - x2)/x2}{n2}\right)}$$

$$\text{Ln}(0.62) \pm 1.96\sqrt{((21/12)/33 + (19/26)/45)}$$

$$\text{Ln}(0.62) \pm 1.96\sqrt{(0.05 + 0.02)}$$

$$\text{Ln}(0.62) \pm 1.96\sqrt{(0.07)}$$

$$-0.48 \pm 1.96(0.26)$$

$$-0.48 \pm 0.51$$

$$-0.99, 0.03 \text{ (this is CI for Ln(RR))}$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.37, 1.03$$

We are 95% confident that the RR of IRIS when pre-ARV WCC increases (compared to when it decreases) is between 0.84 and 1.10, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does ATT-induced WCC change predict TB-IRIS?

	cases	controls	totals
exposure: WCC increase	A = 11 x1	B = 16 n1-x1	A+B = 27 n1
exposure: WCC decrease	C = 17 x2	D = 19 n2-x2	C+D = 36 n2
totals	A+C = 28	B+D = 35	sum(all) = 62

The cumulative incidence of IRIS in the WCC pre-increase group was $11/27 = 0.41$, while that in the WCC pre-decrease group was $17/36 = 0.47$.

$$\text{RR} = \frac{(A/A + B)}{(C/C + D)}$$

$$= (11/27)/(17/36)$$

$$= 0.41/0.47$$

$$= 0.87$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when pre-ARV WCC increases is 0.87 compared to when pre-ARV WCC decreases. Therefore, in this cohort, when pre-ARV WCC increases, there is an 13% lower risk of developing IRIS relative to when pre-ARV WCC decreases (i.e.

they had 0.87x the risk of developing IRIS during the study period compared to when pre-ARV WCC decreased). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1 - x1)/x1}{n1} + \frac{(n2 - x2)/x2}{n2}\right)}$$

$$\ln(0.87) \pm 1.96\sqrt{((16/11)/27 + (19/17)/36)}$$

$$\ln(0.87) \pm 1.96\sqrt{(0.05 + 0.03)}$$

$$\ln(0.87) \pm 1.96\sqrt{(0.08)}$$

$$-0.14 \pm 1.96(0.28)$$

$$-0.14 \pm 0.55$$

$$-0.69, 0.41 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.5, 1.51$$

We are 95% confident that the RR of IRIS when pre-ARV WCC decreases (compared to when it increases) is between 0.5 and 1.51, but since the 95% CI includes the null value (1), this finding is not statistically significant; there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does ARV-induced WCC change predict TB-IRIS?

	cases	controls	totals
exposure:	A = 3	B = 12	A+B = 15
WCC decrease	x1	n1-x1	n1
exposure:	C = 38	D = 31	C+D = 69
WCC increase	x2	n2-x2	n2
totals	A+C = 41	B+D = 43	sum(all) = 84

The cumulative incidence of IRIS in the WCC post-decrease group was 3/15 = 0.2, while that in the WCC post-increase group was 38/69 = 0.55.

$$RR = \frac{(A/A + B)}{(C/C + D)}$$

$$= (3/15)/(38/69)$$

$$= 0.2/0.55$$

$$= 0.36$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when post-ARV WCC decreases is 0.36 compared to when post-ARV WCC increases. Therefore, in this cohort, when post-ARV WCC decreases, there is a 64% lower risk of developing IRIS relative to when post-ARV WCC increases (i.e. they had 0.36x the risk of developing IRIS during the study period compared to when post-ARV WCC increased). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1 - x1)/x1}{n1} + \frac{(n2 - x2)/x2}{n2}\right)}$$

$$\ln(0.36) \pm 1.96\sqrt{((12/3)/15 + (31/38)/69)}$$

$$\ln(0.36) \pm 1.96\sqrt{(0.27 + 0.01)}$$

$$\ln(0.36) \pm 1.96\sqrt{(0.28)}$$

$$-1.02 \pm 1.96(0.53)$$

$$-1.02 \pm 1.04$$

$$-2.06, 0.02 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.13, 1.02$$

We are 95% confident that the RR of IRIS when post-ARV WCC decreases (compared to when it increases) is between 0.13 and 1.02, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does ARV-induced CRP change predict TB-IRIS?

	cases	controls	totals
exposure:	A = 3	B = 9	A+B = 12
WCC decrease	x1	n1-x1	n1
exposure:	C = 39	D = 36	C+D = 75
WCC increase	x2	n2-x2	n2
totals	A+C = 42	B+D = 45	sum(all) = 87

The cumulative incidence of IRIS in the CRP post-decrease group was $3/12 = 0.25$, while that in the CRP post-increase group was $39/75 = 0.52$.

$$RR = \frac{(A/A + B)}{(C/C + D)}$$

$$= (3/12)/(39/75)$$

$$= 0.25/0.52$$

$$= 0.48$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when post-ARV CRP decreases is 0.48 compared to when post-ARV CRP increases. Therefore, in this cohort, when post-ARV CRP decreases, there is a 52% lower risk of developing IRIS relative to when post-ARV CRP increases (i.e. they had 0.48x the risk of developing IRIS during the study period compared to when post-ARV CRP increased). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2}\right)}$$

$$\ln(0.48) \pm 1.96\sqrt{((9/3)/12 + (36/39)/75)}$$

$$\ln(0.48) \pm 1.96\sqrt{(0.25 + 0.01)}$$

$$\ln(0.48) \pm 1.96\sqrt{(0.26)}$$

$$-0.73 \pm 1.96(0.51)$$

$$-0.73 \pm 1$$

$$-1.73, 0.27 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.18, 1.32$$

We are 95% confident that the RR of IRIS when post-ARV WCC decreases (compared to when it increases) is between 0.13 and 1.02, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does ATT-induced neutrophil % of WCC change predict TB-IRIS?

	cases	controls	totals
exposure: NE% increase	A = 10 x1	B = 12 n1-x1	A+B = 22 n1
exposure: NE% decrease	C = 18 x2	D = 21 n2-x2	C+D = 39 n2
totals	A+C = 28	B+D = 33	sum(all) = 51

The cumulative incidence of IRIS in the NE% pre-increase group was $10/22 = 0.45$, while that in the pre-decrease group was $18/39 = 0.46$.

$$RR = \frac{(A/A + B)}{(C/C + D)}$$

$$= (10/22)/(18/39)$$

$$= 0.45/0.46$$

$$= 0.98$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when pre-ARV NE% increases is 0.98 compared to when it decreases. Therefore, in this cohort, when pre-ARV NE% increases, there is a 0.02% lower risk of developing IRIS relative to when it decreases (i.e. they had 0.98x the risk of developing IRIS during the study period compared to when pre-ARV NE% decreased). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2}\right)}$$

$$\ln(0.98) \pm 1.96\sqrt{((12/10)/22 + (21/18)/39)}$$

$$\ln(0.98) \pm 1.96\sqrt{(0.05 + 0.03)}$$

$$\ln(0.98) \pm 1.96\sqrt{(0.08)}$$

$$-0.02 \pm 1.96(0.28)$$

$$-0.02 \pm 0.55$$

$$-0.57, 0.53 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

0.57, 1.7

We are 95% confident that the RR of IRIS when pre-ARV NE% decreases (compared to when it increases) is between 0.57 and 1.7, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does ARV-induced neutrophil % of WCC change predict TB-IRIS?

	cases	controls	totals
exposure:	A = 6	B = 8	A+B = 14
NE% decrease	x1	n1-x1	n1
exposure:	C = 34	D = 34	C+D = 68
NE% increase	x2	n2-x2	n2
totals	A+C = 40	B+D = 42	sum(all) = 82

The cumulative incidence of IRIS in the NE% post-decrease group was $6/14 = 0.43$, while that in the post-increase group was $34/68 = 0.5$.

$$\begin{aligned}
 RR &= \frac{(A/A+B)}{(C/C+D)} \\
 &= (6/14)/(34/68) \\
 &= 0.43/0.5 \\
 &= 0.86
 \end{aligned}$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population when post-ARV NE% decreases is 0.86 compared to when it increases. Therefore, in this cohort, when post-ARV NE% decreases, there is a 14% lower risk of developing IRIS relative to when it increases (i.e. they had 0.86x the risk of developing IRIS during the study period compared to when post-ARV NE% increased). 95% CI formula:

$$\begin{aligned}
 &Ln(RR) \pm z\sqrt{\left(\frac{(n1-x1)/x1}{n1} + \frac{(n2-x2)/x2}{n2}\right)} \\
 &Ln(0.86) \pm 1.96\sqrt{((8/6)/14 + (34/34)/68)} \\
 &Ln(0.86) \pm 1.96\sqrt{(0.1 + 0.01)} \\
 &Ln(0.86) \pm 1.96\sqrt{(0.11)} \\
 &-0.15 \pm 1.96(0.33) \\
 &-0.15 \pm 0.65 \\
 &-0.8, 0.5 \text{ (this is CI for Ln(RR))}
 \end{aligned}$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

0.45, 1.65

We are 95% confident that the RR of IRIS when pre-ARV NE% decreases (compared to when it increases) is between 0.45 and 1.65, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

- Does prednisone exposure prior to ARV initiation (wk0) alter TB-IRIS risk?

	cases	controls	totals
exposure: prednisone	A = 13 x1	B = 22 n1-x1	A+B = 35 n1
exposure: no prednisone	C = 29 x2	D = 24 n2-x2	C+D = 53 n2
totals	A+C = 42	B+D = 46	sum(all) = 88

The cumulative incidence of IRIS in the prednisone pre-exposure group was $13/35 = 0.37$, while that in the no prednisone group was $29/53 = 0.55$.

$$RR = \frac{(A/A + B)}{(C/C + D)}$$

$$= (13/35)/(29/53)$$

$$= 0.37/0.55$$

$$= 0.67$$

Therefore, the relative risk of acquiring TB-IRIS in an at-risk population with pre-ARV prednisone exposure is 0.67 compared to no prednisone. Therefore, in this cohort, with pre-ARV prednisone, there is a 33% lower risk of developing IRIS relative to no prednisone (i.e. they had 0.67x the risk of developing IRIS during the study period compared to no prednisone). 95% CI formula:

$$\ln(RR) \pm z\sqrt{\left(\frac{(n1 - x1)/x1}{n1}\right) + \left(\frac{(n2 - x2)/x2}{n2}\right)}$$

$$\ln(0.67) \pm 1.96\sqrt{((22/13)/35 + (24/29)/53)}$$

$$\ln(0.67) \pm 1.96\sqrt{(0.05 + 0.02)}$$

$$\ln(0.67) \pm 1.96\sqrt{(0.07)}$$

$$-0.4 \pm 1.96(0.26)$$

$$-0.4 \pm 0.51$$

$$-0.91, 0.11 \text{ (this is CI for } \ln(RR))$$

thus, CI for RR: antilog (i.e. exponent) of the upper and lower limits:

$$0.4, 1.12$$

We are 95% confident that the RR of IRIS with pre-ARV prednisone exposure (compared to no prednisone) is between 0.4 and 1.12, but since the 95% CI includes the null value (1), this finding is not statistically significant: there is insufficient evidence to conclude the groups have statistically significantly different risk of developing IRIS.

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Chapter 3

Materials & Methods:

Study Design, Neutrophil Discovery Proteomics, Output Data Quality, & Analysis Pipeline

"There is a tremendous amount of information regarding evolutionary history and biochemical function implicit in each sequence and the number of known sequences is growing explosively. We feel it is important to collect this significant information, correlate it into a unified whole, and interpret it."

– M. Dayhoff, 1967

Abstract

Background Unravelling the pathogenesis of complex disorders such as TB-IRIS requires analytical techniques capable of interrogating complex biological systems. Proteins represent the most functionally proximal layer of biomolecules within a cell, and are the most commonly pharmacologically-targeted biomolecules. Q-Exactive-based label-free HPLC-MS/MS is well-suited to differentially profiling the neutrophil proteome (identifying and quantitating proteins) between TB-IRIS cases and controls in an unbiased manner. **Methods** Neutrophils were obtained from healthy volunteers (HV), as well as from TB/HIV-coinfected patients who do or do not go on to develop TB-IRIS (IRIS and CTRL, respectively). Patient sampling occurred at two time-points: just prior to ARV initiation (week 0) and at the typical time of IRIS manifestation (week 2). Neutrophils underwent lysis, lysate protein quantitation, on-filter protein trypsinisation, and removal of salts and detergents from resulting peptides prior to neutrophil-optimised HPLC-MS/MS analysis and submission of raw spectra to MaxQuant for identification and quantitation. Although available classes - HV, CTRL0, IRIS0, CTRL2, and IRIS2 - facilitate multiple possible comparisons (all of which were performed), we focus on comparing: (a) both CTRL0 and IRIS0 to HV (and resultant differences) to identify differential effects of partially ATT-treated co-infection (i.e. IRIS-specific predisposition) and (b) CTRL2 to CTRL0 as well as IRIS2 to IRIS0 (and resultant differences) to identify differential effects of ARV therapy (i.e. IRIS-specific pathogenesis). After employing PCA to visualise the existence of class-discriminating differences, comparisons began with protein differential expression analysis, including detectable/undetectable analysis (based on raw intensity) and moderated t-testing (based on log₂LFQ values) to identify significantly differentially expressed proteins. Results informed neutrophil differential functional profiling via GO-ORA (employing FunRich and STRING) and pathway activation state analysis (employing SPIA and a novel deep manual analysis approach focusing on key inference-friendly proteins). The novel approach aims to address a number of shortcomings of current automated functional inference tools and associated knowledgebases. We additionally attempted to detect Mtb (as well as human) proteins, and performed a preliminary analysis of variable protein post-translational modifications (PTMs) of interest to determine whether they agree with functional inferences from differential expression profiling. As an initial step to potential clinical translation, we identified and evaluated candidate biomarkers able to discriminate between classes, via RandomForest and ROC analysis. Hypotheses arising from differential functional analyses also suggested various pathways as potential prophylactic/therapeutic targets. **Results** Regarding neutrophil lysate peptide preparation and analysis, 5x10⁶ neutrophils routinely yielded over 100µg total protein, tryptic digestion was efficient (on average <96% missed cleavages), and – when instrument performance was stable – equivalent peptide injection volumes yielded comparable TIC profiles and intensities. An average 23% spectral identification rate resulted in a total of 2532 protein group identifications (the deepest current neutrophil proteome coverage achieved to date without pre-fractionation, representing ~12% of human protein coding genes, and ~25% of the detectable human proteome). Instrument availability necessitated HPLC-MS/MS analysis in two large (randomised) batches: February 2018 (N = 37, Dataset A) and June 2018 (N = 74, Dataset B). Altered HPLC performance between these time-periods produced retention time drift, precluding combined dataset analysis. Instead, independent analysis of each dataset allowed Dataset A to serve as an independent validation dataset for conclusions drawn from Dataset B. Regarding protein identities and quantities, technical replicates within each dataset exhibit excellent agreement, and between-dataset protein identities and functional inferences also exhibit excellent agreement. We identify a number of proteins apparently never before shown (inferred by transcriptomic or directly demonstrated by proteomic analyses) to be expressed by human neutrophils, as well as one predicted human protein never before observed empirically. Due to complexity, remaining results - of PCA, detectable/undetectable analysis, differential expression analysis, differential functional profiling, inferred predisposing/pathogenic mechanisms, and biomarker candidate performance - are reported separately, in Chapters 4 and 5. It is notable that the novel deep manual analysis approach identifies nine rational, neutrophil-relevant, and causally-understandable functional modules, some of which are not considered in traditional functional module knowledge bases, and all of which are differentially perturbed between comparison classes. **Conclusions** Quantities were stringently controlled at the cellular, protein, and peptide levels, and generated proteomic data are of a high quality, which should – when used as input data for differential expression and functional profiling analysis – lead to high-quality biological inferences and preliminary hypotheses for eventual clinical translation (Chapters 4 and 5), pending validation. Results of the preliminary PTM analysis do not contradict neutrophil functional findings.

3.1. Background

3.1.1. Mass Spectrometry-based Proteomics is Well-suited to Understanding Challenging Complex Disorders Such as TB-IRIS

As mentioned in Chapter 1, the high global TB-HIV co-infection rate produces a high incidence of TB-IRIS. The pathogenesis of this complex disorder remains incompletely understood, and clinical strategies (risk stratification, prophylaxis, diagnosis, prognostication, and therapeutics) remain non-optimal.

Since neutrophils are implicated in TB-IRIS pathogenesis, an improved understanding of their phenotype and function (including within broader context of the immune system) may help elucidate the biology underlying this condition. Proteins are the biomolecule most-proximally responsible for cell function (as

well as the most commonly pharmacologically-targeted biomolecule), but tens of thousands of proteins are expressed by any given cell at any given time-point, and measuring only a handful (e.g. by traditional immunophenotyping or proteomic methods) is insufficient to achieve global cellular understanding. In addition, the sub-cellular functional protein network represents a complex adaptive biological system (which interacts bi-directionally with complex adaptive systems comprising all other categories of cellular biomolecule), as does the immune system itself. Understanding the causal behaviours of such complex biological systems over time is a non-trivial task.

High-performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) facilitates separation and analysis (mass, charge, and intensity) of many peptide ions deriving from enzymatic digestion of proteins in complex biological mixtures (e.g. cell lysates). Protein identities and quantities are then inferred by computationally comparing peptide data to an *in silico* digest of a protein reference database (the human proteome). Discovery proteomics aims to identify proteins present in complex biological mixtures (concurrently with label-free or label-based quantitation) in order to generate novel testable mechanistic hypotheses regarding pathogenesis of the condition under study. These may also be compared to *a priori* hypotheses derived from literature, although *a priori* hypotheses are not strictly required to derive biological meaning via discovery proteomics.

Advances in sample preparation, LC peak capacity and separation speed, high-resolution MS sensitivity, bioinformatic databases, and computational analysis have made LC-MS/MS the proteomic analytical method of choice for deep proteome coverage [1]. The Q-Exactive (quadrupole-orbitrap hybrid) mass spectrometer is especially capable of excellent protein identification and quantitation [2] [3]. Such an HPLC-MS/MS-based neutrophil differential proteomic profiling discovery approach is thus well-suited to studying the molecular and cellular pathogenesis of a complex disorder such as TB-IRIS. Herein, differential proteomic profiling of neutrophils from controls and TB-IRIS cases informs biologically plausible hypotheses regarding predisposing and pathogenic mechanisms, thereby also facilitating improved risk stratification, prophylaxis, diagnostics, prognostication, therapeutics, and treatment monitoring.

3.2. Healthy Volunteer Neutrophils

As detailed in Chapter 2, neutrophils were isolated from clinical samples. In an identical manner, neutrophils were isolated from a small group (n = 10) of healthy volunteers (Table 1) to facilitate pilot comparisons with patient-derived neutrophils.

Table 1 *Healthy Volunteer Demographic Data

Volunteer	Gender	Date of Birth	Ethnicity
A	M	11/04/88	Caucasian
B	M	18/06/78	Caucasian
C	F	25/11/86	Caucasian
D	F	12/04/85	Caucasian
E	M	20/11/89	Indian
F	M	13/08/80	Indian
G	F	05/12/89	Malay
H	M	18/10/90	Coloured
I	M	24/08/83	Caucasian
J	F	06/06/81	Coloured

*Free of TB, HIV-uninfected, and exhibiting no signs or symptoms of illness at the time of phlebotomy.

3.3. Study Design

The number of distinct comparison classes facilitates various possible comparisons (fig. 1), each representing a different research question.

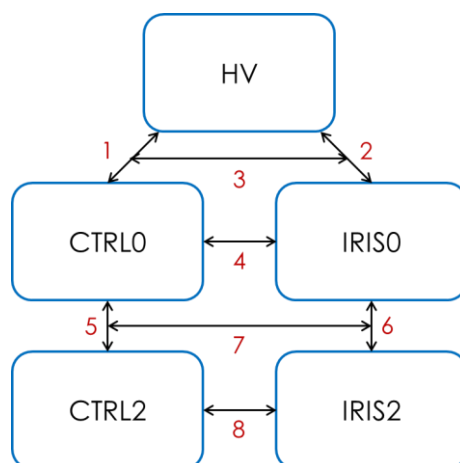


Figure 1 All Possible Class Comparisons. **HV** healthy volunteer group. **CTRL** control group: partially ATT-treated TB/HIV co-infected participants who do not develop TB-IRIS, phlebotomised just prior to initiation (**CTRL0**, baseline) and after two weeks of ARV therapy (**CTRL2**). **IRIS** experimental group: partially ATT-treated TB/HIV co-infected participants who develop TB-IRIS, phlebotomised just prior to initiation (**IRIS0**, baseline) and after two weeks of ARV therapy (**IRIS2**). Red numbers indicate comparisons addressing different research questions (detailed below).

Comparisons facilitate the following research questions regarding the neutrophil proteome and functions:

(1) and (2) How do partially ATT-treated TB/HIV-coinfected participants (CTRL0, IRIS0) differ from healthy volunteers (HV)?

(3) Can differential findings between those who do and do not go on to develop TB-IRIS (predisposing differences) account for differential susceptibility and/or facilitate identification of discriminatory risk stratification/prognostic/treatment response biomarkers?

(4) The above major comparison may be supported by a head-to-head comparison between CTRL0 and IRIS0, further facilitating identification of discriminatory risk stratification/prognostic/treatment response biomarkers.

(5) and (6) Which changes are induced by two weeks' ARV therapy in the control (CTRL) and experimental (IRIS) groups?

(7) Can differential responses to ARV therapy between the CTRL and IRIS groups account for TB-IRIS pathogenesis and/or facilitate identification of discriminatory risk stratification/prognostic/treatment response biomarkers?

(8) The above major comparison may be supported by a head-to-head comparison between CTRL2 and IRIS2, further facilitating identification of discriminatory diagnostic biomarkers.

Thus, we aim to identify IRIS-predisposing as well as pathogenesis-relevant neutrophil proteomic changes. The above comparison sequence - comparing CTRL0/IRIS0 to HV and then CTRL2/IRIS2 to CTRL0/IRIS0 – facilitates identification of a greater number of significantly differentially-expressed (SDE) proteins between classes, as well as providing a broader overview of predisposition and pathogenesis than relying only on head-to-head comparisons prior to and following ARV initiation. It is notable that we will examine not only neutrophil proteins (identities, relative quantities) and inferred biological functions, but also aim to determine: (a) whether any differential protein post-translational modifications (PTMs) of interest are detectable between comparison classes, and (b) whether any *Mycobacterium tuberculosis* proteins (present at much lower abundances) are detectable within these human samples.

3.4. Sample Preparation

Neutrophils underwent physicochemical lysis, on-filter tryptic digestion, stagetip-based desalting, and lyophilisation.

3.4.1. Lysis and Estimation of Protein Content

For downstream sample preparation, stored neutrophils were randomised to batches to minimise the potential confounding effects of technical variability. Snap-frozen single-cell neutrophil suspensions in PBS were defrosted for the minimum time necessary for solid to liquid transition (to minimise protease activity). Each 100µl suspension was chemically disrupted via addition of 100µl 2x Tris/Cl RIPA buffer pH 7.6 (adapted from [4] buffer 4) containing 2x Complete Mini EDTA-free protease inhibitor (Roche) to a final concentration of 2% SDS (1x RIPA, 1x protease inhibitor), prior to brief but thorough vortexing and shaking down. Lysates (in closed cryovials) were then physically disrupted via three cycles of: boiling at 95°C for 3min, sonicating for 3min, and thoroughly vortexing for 20sec. Finally, lysates were cooled to room temperature prior to addition of 0.5ul Benzonase nuclease 25kU (Sigma-Aldrich) and overnight incubation at -20°C to allow full nucleic acid denaturation.

Protein quantitation was carried out using the bicinchoninic acid (BCA) microplate assay (BCA Protein Assay Kit, Thermo Pierce) as per manufacturer instructions. Briefly, a series of five bovine serum albumin (BSA, Thermo Pierce) standards (62.5 to 1000µg/ml) was made up in 1x RIPA buffer for standard curve generation. Each of the following were plated at 25µl, in triplicate: blank (1x RIPA buffer), standards, and neutrophil lysates. To each well, 200µl of BSA working reagent was added prior to agitation and incubation at 37°C for 30min. After cooling to room temperature, absorbance at 560nm was measured using a microplate reader (Biorad iMark™) in conjunction with relevant software (Microplate Manager v. 6.1). This facilitated calculation of the volume of each lysate representing 100µg protein (Appendix H).

As a quality-control measure, lysate proteins were also separated and visualised using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein (37.5-150µg) in sample application buffer, as well as 5µl of molecular weight (MW) ladder (PageBlue Ruler, Thermo Scientific), were loaded onto a 10% separating gel and run at 0.02 amperes for approximately one hour (or until the dye front approached the gel edge). Gels were washed to remove SDS, stained 15min using AquaStain (Vacutec), imaged (Canon CanoScan LiDE 200) at maximal resolution, and saved as a .TIFF file (fig. 2).

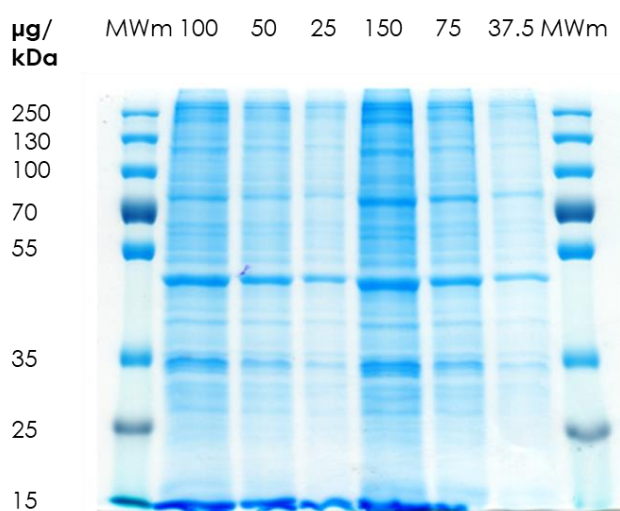


Figure 2 Representative SDS-PAGE of Neutrophil Lysate

3.4.2. Filter-assisted Sample Preparation (FASP)

Of each lysate, 100µg was subjected to reduction of protein disulphide bonds via addition of 10mM DTT (Omnipur) for 30min at room temperature. The reduced protein sample was then loaded onto a 500µl-capacity 30kDa MWCO spin-filter (Millipore) pre-washed with urea (14.4g urea in 0.1M Tris pH 8.5) for FASP [5]. Briefly, RIPA buffer was replaced with urea buffer (4 cycles: 100µl, 14 000g, 15min, room temperature), cysteine thiol groups were alkylated (100µl iodoacetamide (0.02775g in 3ml urea buffer), 20min in the dark, room temperature), iodoacetamide was washed off (3 cycles: 100ul urea buffer, 14 000g, 15min, room temperature), pH was increased (2 cycles: 100ul ammonium bicarbonate (0.02955g in 7.5ml dH₂O, pH 8), 14 000g, 10min, room temperature), and 5µl (5µg) of mass spectrometry-grade trypsin as well as 40µl (filter dead volume-equivalent) supplied CaCl₂ buffer (New England BioLabs) were added to a final 1:20 trypsin:protein ratio (to avoid sub-standard digestion observed in neutrophil lysates during optimisation). Samples were digested overnight for 18hrs at 37°C.

3.4.3. Peptide Elution, Acidification, & Desalting

Resultant tryptic peptides were eluted (14 000g, 10min, followed by 40µl ammonium bicarbonate, 14 000g, 10min) and acidified (3 cycles: 50µl 2% acetonitrile (ACN; containing 0.1% formic acid (FA); both Sigma Aldrich), 14 000g, 10min). Desalting StageTips were packed in-house, using 2 x 1mm C18 discs (Millipore) and solvent-friendly 20ul pipette tips (Eppendorf) [6]. StageTips were equilibrated (3 cycles: 50µl 80% ACN (0.1% FA), 1500g, 1min, room temperature) and washed (2 cycles: 50µl 2% ACN (0.1% FA), 1500g, 1min, room temperature), discarding StageTips with unmatched flow-rates (resulting from variability in C18 compression). Peptides (20µg) were loaded onto separate StageTips and washed (3 cycles: 50µl 2% ACN (0.1% FA), 1500g, 1min, room temperature) to desalt. Peptides were then eluted into glass inserts (3 cycles: 50µl 80% ACN (0.1% FA), 1500g, 1min, room temperature) and vacuum-dried using a Speedivac (~40min, room temperature) prior to immediate resuspension (for improved peptide solubility) in 2% ACN (0.1% FA) to a final peptide concentration of 200ng/µl. Desalted peptides, ready for HPLC-MS/MS analysis, were stored at -20°C.

3.4.4. HPLC-MS/MS: Operation and Acquisition Parameters

Resuspended peptide samples were again randomised to batches for analysis by HPLC-MS/MS. Each sample was analysed using a nanoLC instrument (Dionex HPLC, Thermo Fisher Scientific) coupled to a Q-Exactive quadrupole-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific).

3.4.4.1. HPLC

Samples were loaded into the Dionex autosampler tray, and software was programmed to load 1ul of each 0.2µg/µl peptide sample into the sample loop at a flow rate of 20µl/min in 2% ACN (0.1% FA) for application onto a 40cm C18 reverse-phase analytical column (packed in-house, Aeris 3.6µm C18 solid-core beads, Phenomenex). Initially 1µl of one sample from each class was loaded to determine approximate loading volumes of subsequent samples required to achieve average TIC intensities of 5E9 (usually ~6µl = 1.2µg peptide). Samples were eluted from the analytical column using a neutrophil-optimised 167min gradient (fig. 3). Due to biological variability exceeding technical variability as well as to instrument availability constraints, each biological sample was injected only once (notwithstanding re-runs necessitated by occasional poor spray stability).

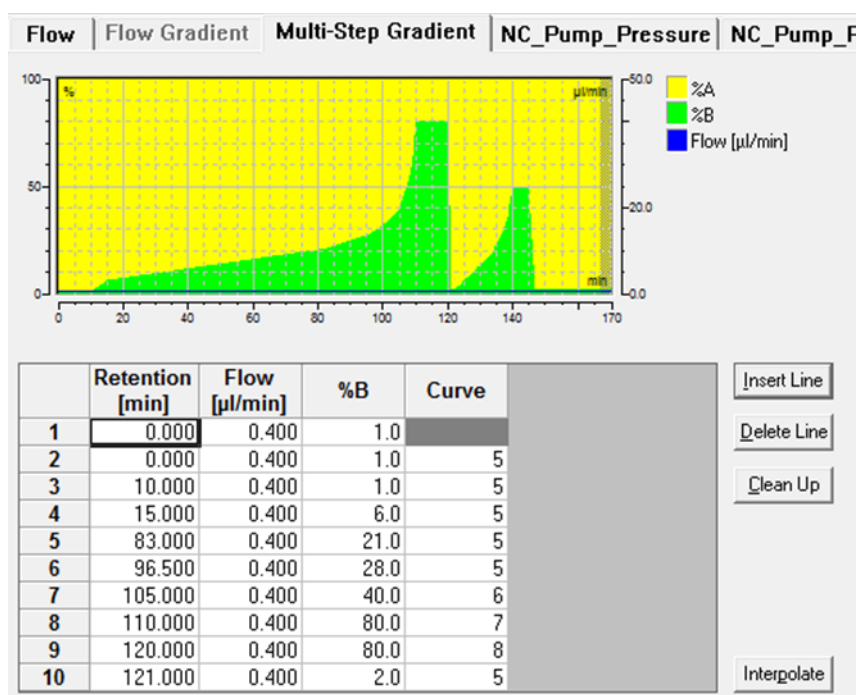


Figure 3 Neutrophil-optimised HPLC Gradient (Xcalibur method G90_OG9_6-21-28-40_167min)

3.4.4.2. Mass Spectrometry

Ions produced by the electrospray ionisation (ESI) source (Thermo Scientific) entered the mass analyser. ESI was operated at varying voltage (1.7-2.1kV, without sheath gas flow) in order to stabilise spray. Mass spectra were collected with almost identical settings to those described by Pirmoradian et al. (2013) [7]. Briefly, mass spectra were acquired in a data-dependent manner. Survey scan acquisition was performed in all-ion fragmentation, positive ion, and Top20 modes. Resolution of parent ion spectra acquisition was 70 000, with an automatic gain control (AGC) target of 3×10^6 ions and a maximum integration time of 250ms. Scan range spanned 300 – 1750 m/z. Up to the 10 most abundant precursor peptide ions were selected for higher-energy collisional dissociation (HCD) fragmentation at a normalised collision energy (NCE) of 25eV. A 4 m/z unit window was set for ion selection, with underfill ratio set at 1% and exclusion of ions with unassigned charge or charge outside the boundaries 2+ to 4+, inclusive. Resolution of fragment ion spectra acquisition was 17 500, with an AGC target of 2×10^5 ions and a maximum fragment ion accumulation time of 120ms, and lower m/z bound 100. Peptide matches were preferred and dynamic exclusion was set at 30sec.

Due to instrument availability constraints, samples were run in two large batches separated in time: February 2018 (Dataset A, fig. 4A) and June 2018 (Dataset B, fig. 4B). This should have enabled combination of the datasets for a single analysis, but HPLC drift between February and June produced inconsistent (i.e. not batch-correctible) changes to peptide retention times (fig. 5), precluding combined-batch data analysis (i.e. data resulting from each batch was analysed separately, allowing batches to serve as independent validation “cohorts”).

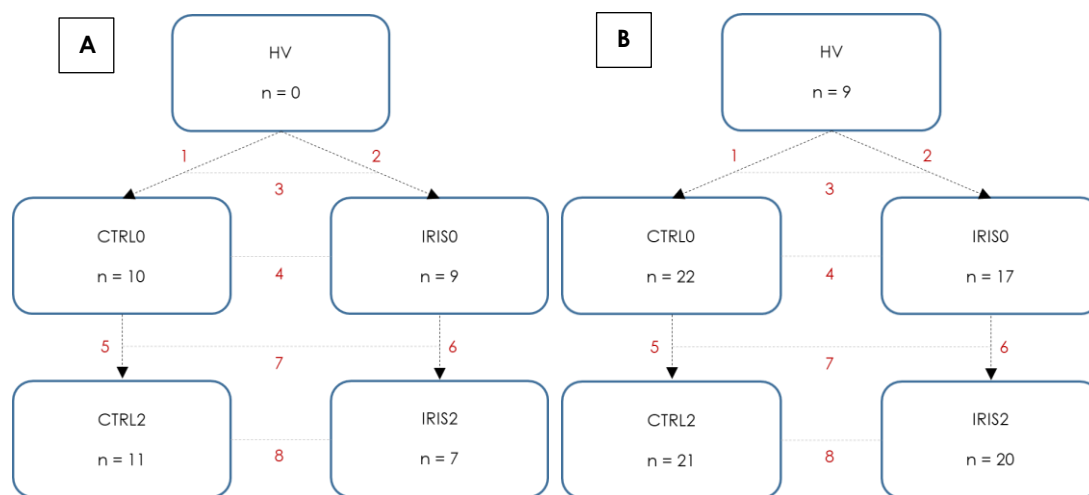


Figure 4 Dataset Sources and Sample Sizes. No HV group was included during generation of Dataset A.

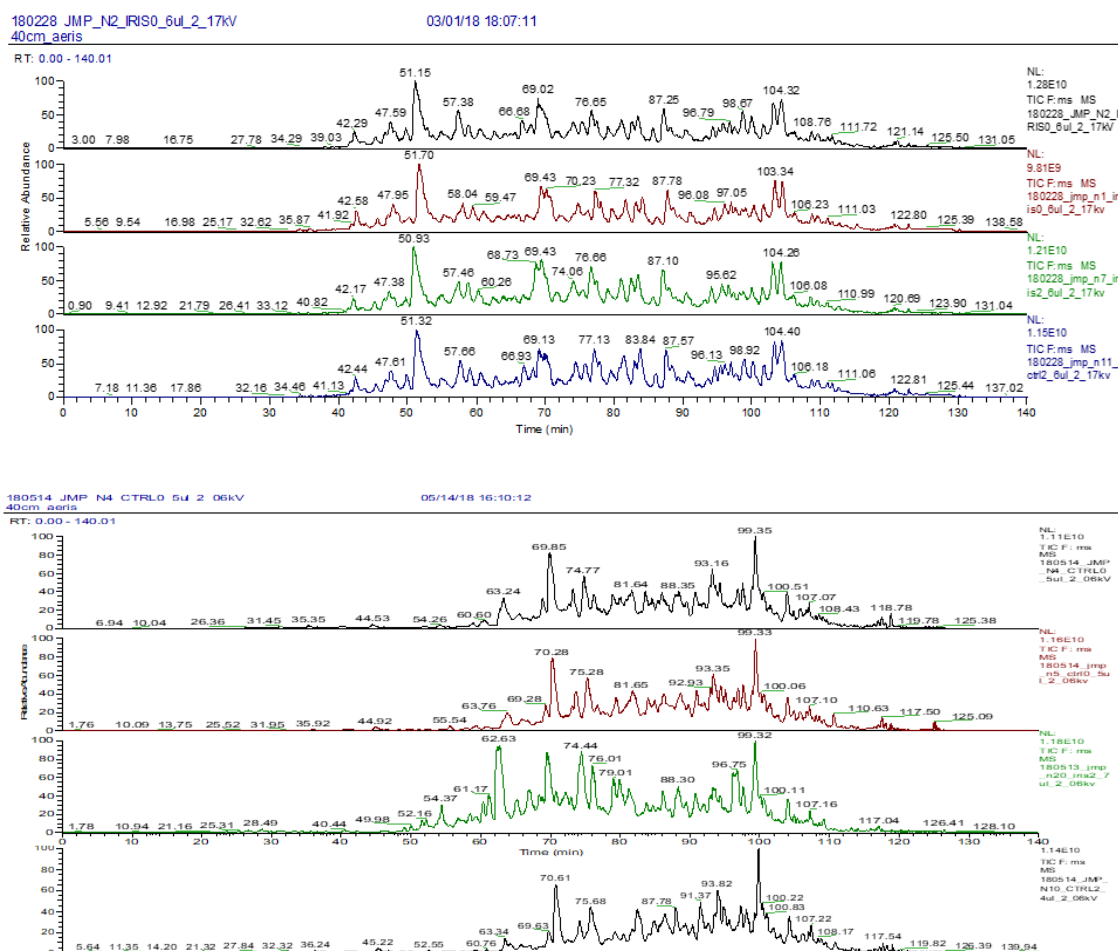


Figure 5 Total ion chromatograms (each panel containing one CTRL0, one IRIS0, one CTRL2, and one IRIS2 sample TIC) demonstrating peptide retention time drift between February 2018 (180228; Dataset A) and June 2018 (180514; Dataset B).

3.4.5. Protein Identification, Quantitation, & Data Quality Control

For each of the abovementioned batches, all Thermo .RAW MS/MS spectra were simultaneously submitted to MaxQuant v1.5.3.12 [8] for protein identification and label-free quantitation. MaxQuant's integrated search engine, Andromeda, assigns peptide identities (along with a probability score) to fragment ion spectra, then compares these to theoretical peptides generated by *in silico* trypsin digestion of the reference protein database (*vide infra*), as well as to a reversed decoy database concatenated to the target database, in order to infer identities of proteins represented by identified peptides, and compute the false-discovery rate (FDR). Furthermore, PTM analysis relies on expected peptide mass alterations to infer the potential presence of a given PTM. Quantitation is achieved via application of MaxQuant's Label-free Quantitation (LFQ) algorithm to raw intensities (including normalising individual protein intensity to total protein intensity, based on the assumption that only a small proportion of the total proteome will change significantly in response to any given perturbation).

To serve as reference databases, the most up to date (August 2017) human and *Mycobacterium tuberculosis* complete proteomes were downloaded from UniProtKB [9]. MaxQuant was operated with default parameter settings, including the following modifications: tryptic digestion, maximum two missed cleavages, multiplicity one (no isotopic labels), carbamidomethylation (C) as a fixed modification (due to alkylation with iodoacetamide during sample preparation), peptide and protein false discovery rates (FDRs) of 1%, a minimum of 1 unique peptides required, match-between-runs (to match retention times between biological replicates), and second peptide re-quantification (to maximise proteome coverage).

Selection of variable modifications to include in the analysis required more in-depth consideration. Ideally, *all* most commonly-expected variable modifications would be included in the final database search to maximise peptide identification, as well as maximising quantitation accuracy. However the larger the search space/reference database becomes, the longer the search will take, and the higher risk of false positive identifications concomitantly increases analysis stringency, which can artificially reduce the number of identifications meeting true positive criteria. Because of the search space/identification cut-off stringency trade-off, it is ideal to include a maximum of five variable modifications per Andromeda search. Typically, mass spectrometry-based PTM investigations first enrich for modified peptides, as they are present at relatively low abundance (only a subset of the total units of any single protein will be modified in a particular way at a particular time, and only a fraction of the peptides resulting from the parent protein will display such modifications). However, certain covalently modified peptides may still be detectable without enrichment (detection of highly-dynamic reversible modifications requires additional experimental steps). Although quantitation of such low-abundance species is likely to be somewhat inaccurate, certain trends may emerge (as we have observed for citrullination, for example, during analysis of other clinical cohort samples), and presence of the hypothesised modification (as well as the resulting functional inference) can be validated through targeted analyses.

Default variable modifications (common in conjunction with the experimental workflow described here) included in MaxQuant are: oxidation (M), deamidation (N), and protein N-terminal acetylation. However, an unbiased preliminary analysis of neutrophil proteome PTMs using Byonic software [10] (data not shown) identified that while oxidation (M) is indeed very common, deamidation (N) is more common than protein N-terminal acetylation. Furthermore, we hypothesised that enhanced neutrophil activation may elevate the number of phagolysosomal- and respiratory burst-dependent PTMs observed, and preliminary proteomic data analyses suggested significant alterations in ubiquitination and SUMOylation flux. Therefore, to provide an additional layer of evidence supporting our hypotheses, we performed two separate MaxQuant PTM analyses with parameters as described above (not exceeding five total variable modifications per analysis) in an attempt to locate such PTMs within the dataset. Both analyses included the two most common neutrophil protein PTMs as detected by Byonic (oxidation (M) and deamidation (N)). The first analysis also included phagolysosomal modifications (chlorotyrosine (Y), iodination (Y), and nitrosylation (C)), while the second analysis also included ubiquitin cleavage tags LRGG (K) and diglycine (K), as well as citrullination (R). These selected

modifications of interest were initially investigated in neutrophils from the subgroup of patients for whom samples from both timepoints were available (data not shown) to determine which should be included in the final analysis. Although SUMOylation is of interest given preliminary dataset analysis suggestions of differential dysregulation in control and IRIS patients during ARV exposure, this PTM was not analysed, because only a small proportion of SUMO substrates is SUMOylated, and deconjugating enzyme activity leads to rapid deSUMOylation. Expected mass changes induced by modifications were extracted from UniMod [11]. To limit false negatives, we consider total number of sites of each modification (overall and per sample), the number of modified proteins identified with high confidence (charge state 2-3+, mass error <1 ppm, PEP score <0.05, localisation score ~1, no other modifications on the same peptide), and in how many of the samples these modified proteins appear. Ideally, identified modified proteins should be inferred from multiple modified peptides, which are detected by MS/MS rather than by matching. Ultimately, if a newly-noted PTM has already been characterised by UniProt, this provides added confidence and understanding. An additional investigation of the effect of PNGase-mediated peptide deglycosylation on the neutrophil peptide identification rate of ~21% had no significant impact (data not shown) - likely due to the relatively larger population of non-glycosylated peptides - suggesting that variable glycosylation is not responsible for this relatively low peptide identification rate (which indeed may actually be due to lower rates of *resubmission* of the same peptides for identification).

MaxQuant output (proteinGroups.txt) was parsed for instrument performance and sample data quality using an R MaxQuant summary script generously provided by Dr. Karsten Krug (Proteome Centre, University of Tübingen, Germany), prior to detailed analysis. Biological multivariate data commonly “arise when multiple” variables (e.g. expression of multiple genes or proteins) are measured per sample [12]. Advanced high-throughput technologies necessarily generate large, high-dimensional biological datasets which pose an analytical challenge: making explicit patterns latent within the data in ways that are statistically appropriate, rigorous, and replicable [13] [14]. In order to obtain biologically meaningful and clinically-relevant output results, high-quality input data are indispensable. It is of critical importance that the experimenter be confident in the quality of their HPLC-MS/MS data, from patient selection through sample collection, processing, and analysis. Biological datasets are inherently noisy (large inter-individual variability), but this is minimised by including only the highest-quality samples from patients clearly meeting study and diagnostic criteria, as well as *ad hoc* discarding of poorly-prepared or –analysed samples. There is nothing to be gained in a high-throughput ‘omics experiment by pursuing quantity over quality (in this case experience strongly suggests that biological quality outweighs statistical requirements for larger sample numbers). We optimised every step of the experimental workflow, and – as detailed in Chapter 2 – discarded inappropriate samples/sample data to ensure only the highest possible quality of the final dataset.

3.4.5.1. Protein Identification Quality & Proteome Coverage Depth

In dataset B, MaxQuant identified 2414 protein groups, of which 2394 were legitimate identifications (not contaminants or reverse hits). Of 24 214 spectra submitted, 22.6% were identified, with 100% of identified peptides unique to a protein group. Instrument performance for all analyses fell within optimal parameters (e.g. ms1:ms2 scan ratio). Across all analyses, up to 95.26% of proteins exhibited no missed cleavages (indicating excellent tryptic digestion efficiency), the protein group-level FDR approximated a value of one, and mass error levels were insignificant. Sample and equipment performance quality control measure data (not shown) indicate good performance and consistency (e.g. TIC profile and retention time, number of peptides/protein groups identified per sample, number of MS versus MS/MS spectra generated, mass spectrometer sensitivity and mass resolution, etc.).

Regarding performance of technical replicate injections spaced in time (days), protein identity agreements are excellent (fig. 6), at over 90% for dataset A (February 2018) and over 83% for dataset B (June 2018). Imperfect protein identity agreements are unsurprising, as the mass spectrometer analyses only a non-comprehensive sample of peptides from the injected mixture during each run (such that an infinite number of runs would get infinitely close to complete coverage of analysable peptides present).

Also unsurprising is the marginally poorer performance seen in dataset B, as HPLC performance was less reliable during June than February 2018.

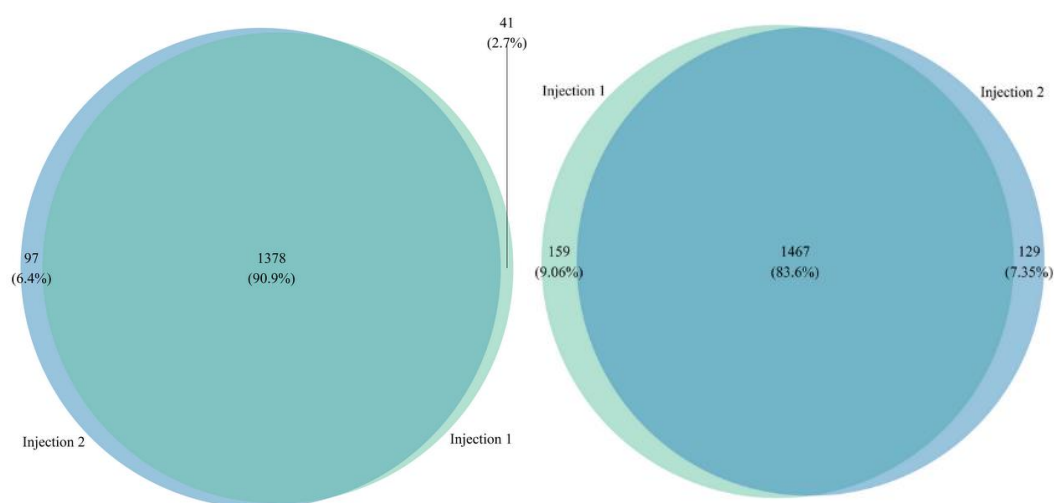


Figure 6 Technical Replicate Protein Identity Overlap for Datasets A (left) and B (right)

Regarding agreement of identity (based on all non-zero raw-intensity protein groups without application of unique-peptide cut-off thresholds), datasets A and B exhibit nearly 71% agreement (fig. 7), representing excellent concordance in neutrophil proteome coverage despite differential HPLC performance between February and June 2018.

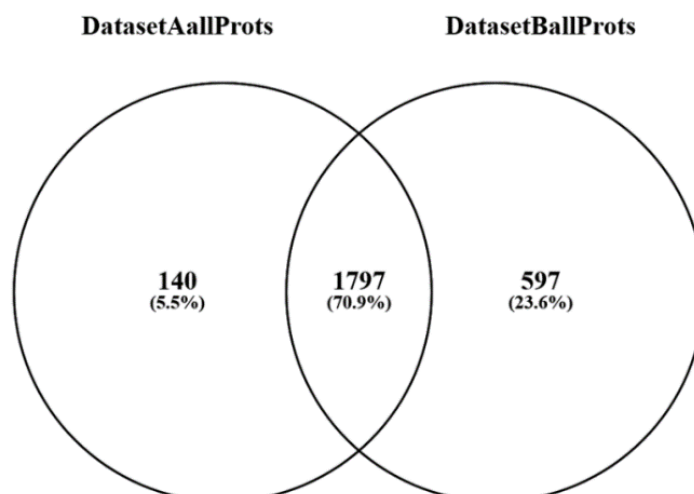


Figure 7 Comparison of Proteins Identified in Datasets A and B

Also unsurprisingly, many more protein groups were identified in Dataset B (which contained over double the number of biological replicates per comparison class, as well as a healthy volunteer class) than in Dataset A (2394 versus 1937 protein groups, combined total 2534 – achieved without sample fractionation). This approximates the neutrophil proteome coverage depth (2536 proteins) achieved by

Zhou et al. (2013) [15] employing extensive SCX fractionation (25 fractions per sample). Only one other granulocyte data-dependent proteomic study to date (via employing stable isotope peptide labelling and pooling, extensive sample fractionation – 28 SCX fractions - *and* a decision tree peptide fragmentation algorithm) achieves greater coverage depth of over 3000 protein groups [16] (Table 2).

Table 2 Studies with Highest Neutrophil Proteome Coverage Depth

Protein Groups	Methods	Reference
3656	28 SCX fractions isotopic labelling and pooling decision-tree fragmentation	[16]
2536	25 SCX fractions	[15]
2534	no pre-fractionation	current study
1332	SCX	[17]
850	2DE	[18]

While the exact number of proteins encoded within the human genome remains contentious, estimates range from 19 901 to 21 306 protein genes [19]. However, due to alternative mRNA splicing, single amino acid polymorphisms (SAPs), and post-translational processing (e.g. PTMs), up to 100 different proteoforms can theoretically result from a single gene (numbers between 10 000, several million, and several billion different protein species have been estimated), and expression patterns of many are dynamic, responding to environmental conditions [20]. In-depth analyses of human cell lines estimate the total detectable human proteome at >10 000 proteins [21] [22] [23] [24] [25], many of which are not expressed by every cell type (nor are all proteoforms), or are not expressed constitutively or at high abundance. Therefore, the current study (2534 proteins) covers a maximum of between only 11.89 and 12.73% of protein-coding genes (and <25.34% of the human proteome), but compares favourably with other neutrophil-interrogating LC-MS/MS-based proteomic experiments. Given the high complexity and dynamic range ("at least seven orders of magnitude") of the human proteome, comprehensive coverage is more difficult than for lower-order organisms [7].

3.4.5.2. Protein Quantitation Quality

Of the total of 2394 legitimate protein group identifications in dataset B, 100% were quantifiable (non-zero LFQ values) in at least one sample. To determine consistency of quantitation via the present experimental instrumentation, we previously compared the coefficient of variation for selected proteins across triplicate injections of the same sample (Table 3). As expected, lower and higher quantitative variability is observed for high- and low-abundance neutrophil proteins, respectively. However, overall, quantitative variance is low between replicate injections.

Table 3a Top 5 High-abundance Proteins: Intensity Variance Across Three Technical Replicates (Sample 220B)

Top 5 High-Abundance Proteins	Coefficient of Variation
neutrophil defensin 3/2	0.43
protein S100-A9	0.48
protein S100-A8	0.46
actin, cytoplasmic 2	0.24
protein S100-A12/calcitermin	0.44

Table 3b Bottom 5 Low-abundance Proteins: Intensity Variance Across Three Technical Replicates (Sample 220B)

Bottom 5 Low-Abundance Proteins	Coefficient of Variation
maltase-glucoamylase, intestinal	0.64
spectrin beta chain, brain 1	0.92
aminopeptidase N	0.98
utrophin	0.86
dedicator of cytokinesis protein 2	0.39

Regarding performance of technical replicate injections spaced in time (days), quantitative agreement appears excellent (fig. 8), with raw intensity correlations of 90% and LFQ correlations of 95% for dataset A (February 2018) and raw intensity correlations of 90% and LFQ correlations of 86% for dataset B (June 2018).

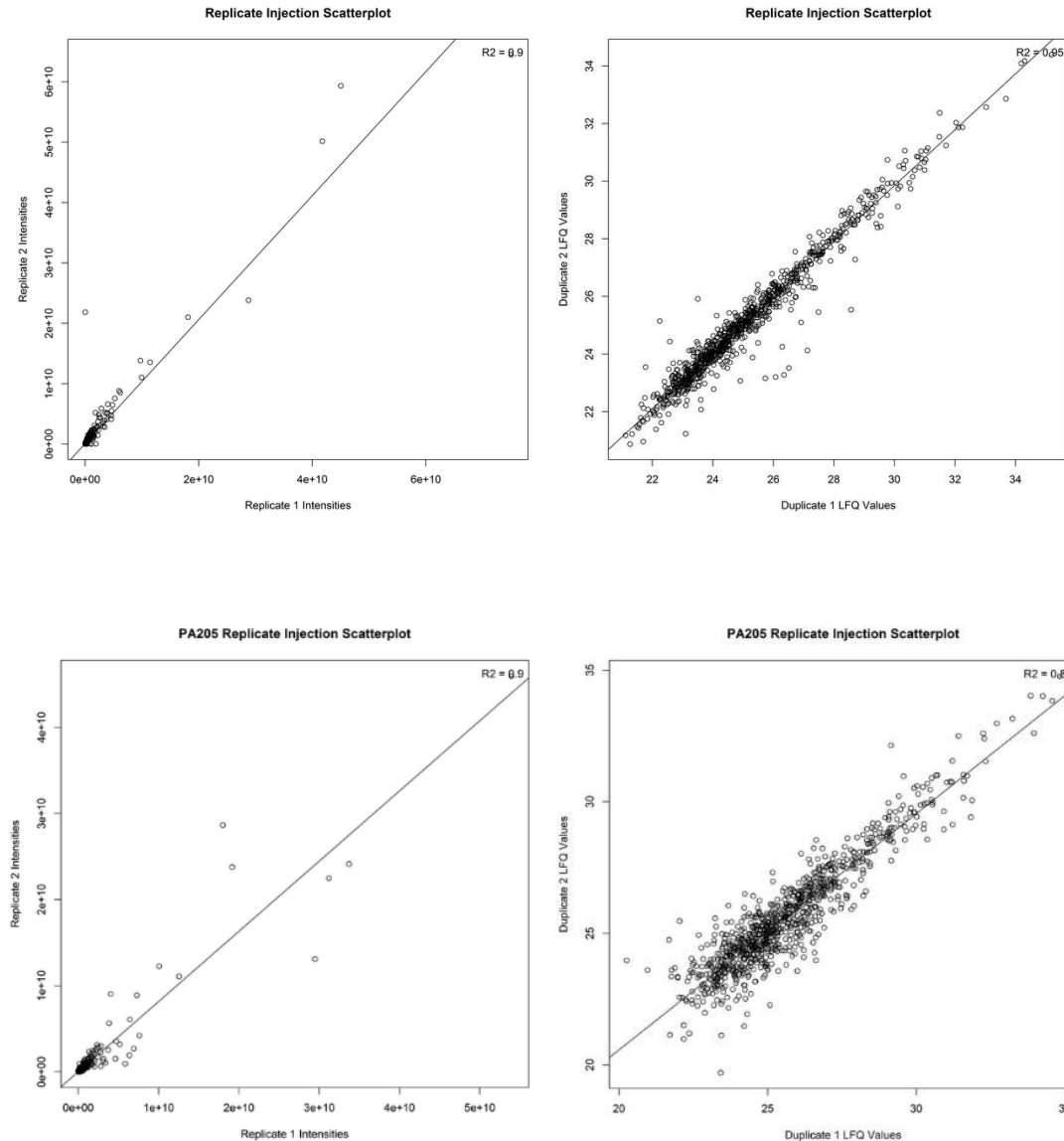


Figure 8 Technical Replicate Intensity Correlations for Datasets A (top) and B (bottom)

3.4.5.3. PTM Analysis

Phagolysosomal: Halogenation (Iodination, Chlorination) & Nitrosylation

Conventionally-activated neutrophils rapidly engage a respiratory burst to generate hydrogen peroxide and – via a cascade of interdependent reactions – an array of additional oxidants. For example, transition metal (e.g. Fe)-dependent Fenton reactions produce hydroxyl radicals, NO reacts with hydroxyl radicals to generate various RNS (although literature suggests inhibited NO production in TB-IRIS patients), and the enzyme MPO uses hydrogen peroxide to oxidise halogens (Cl_2 , I_2 , Br_2 , and possibly even F_2) to various hypohalous acids (e.g. HOCl and HOBr). These are potent halogenating agents which re-react with hydrogen peroxide to generate yet additional oxidants. Overall, oxidants modify biological macromolecules (e.g. proteins, lipids, nucleic acids) in ways that are detrimental to microbes (when reactions are confined to subcellular compartments such as phagolysosomes) as well as hosts (when reactive species exist outside these compartments). For example, HOCl chlorinates amino groups to form chloramines (RNHCl), themselves toxic chlorinating agents that react with local tyrosyl residues to produce chlorotyrosine (3-chlorotyrosine is a stable and specific marker for activated

MPO-HP-halide system-mediated inflammatory damage). Other hypohalous acids produce additional halogenation products (e.g. 3-bromotyrosine), which can convert to 3,5-dihalotyrosine. Hypohalous acids also react with thiols and thioethers to generate various products. Furthermore, the RNS peroxynitrite generates 3-nitrotyrosine, which becomes chlorinated by HOCl to produce 3-chloro-5-nitrotyrosine. Cyanate/isocyanate carbamylates proteins, and MPO can also cause protein cross-linking. It is thus expected that microbial and host proteins present within the phagolysosome of an activated neutrophil will exhibit oxidation, halogenation, and/or nitrosylation. While single amino acids can undergo multiple modifications (e.g. di- or tri-chlorination or -iodination), we focus here on single halogenation: chlorotyrosine, iodotyrosine, and nitrotyrosine. Previous optimisation analyses also examined bromination, fluorination, dichlorotyrosination, and trichlorotyrosination (results not shown), in order to determine which modifications to include in the final analysis. Interestingly, INH (and paracetamol) inhibits MPO. MPO action on thiocyanate (which links to the endogenous H₂S/cyanide detoxification system of neutrophils described in Chapter 4) also generates hypothiocyanous acid and subsequently the minor products cyanate/isocyanate.

Full proteomic functional results (Chapters 4 and 5) strongly suggest down-regulation of the respiratory burst in all clinical classes relative to healthy volunteers, suggesting that resultant PTMs such as the inflammatory marker 3-chlorotyrosine may unfortunately not be readily observed during TB/HIV-coinfection or TB-IRIS. In the analysis of neutrophil proteins from the subset of patients for whom samples from both time-points were available (N = 24), only two apparently-chlorotyrosinated proteins met stringency criteria and were SwissProt identifications (KRT27, FAM105A), but none were detectable in >2 patient samples. Similarly, only three apparently-nitrosylated proteins met stringency criteria and were SwissProt identifications (TRIM66, FAM105A, KIAA1456), but none were detectable in >3 patient samples. However, 14 apparently-iodinated proteins met stringency criteria and were SwissProt identifications (POTEJ, HSPA8, LYZ, HIST1H4A, ACTG, ACTA1, HBA1, MRM2, and an Mtb leucine-tRNA ligase), and one (ACTG, an actin) was quantifiable in sufficient patient samples (n = 11, all from the IRIS group) to examine the potential effect of ARV therapy on iodination over time (fig. 9). Indeed, six patients exhibit decreasing iodinated ACTG peptide levels during ARV therapy, though five exhibit increasing iodinated ACTG peptide levels. Differential changes are apparently not accounted for by placebo/prednisone status, though only three patients received prednisone, while seven received placebo/nothing. That only IRIS samples apparently exhibit ACTG iodination may suggest that phagolysosomal content iodination is more common in this group.

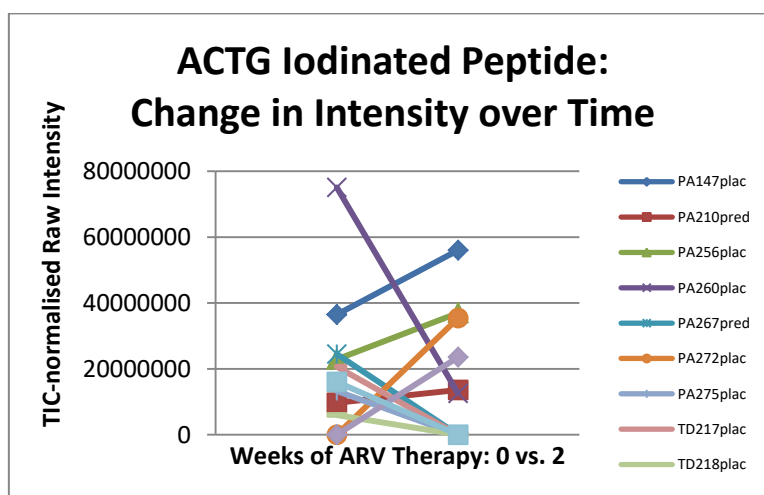


Figure 9 Change in Putative Iodinated ACTG Peptide Intensity Over Two Weeks' ARV Therapy

Ubiquitination

Ubiquitination by ubiquitin ligases targets proteins for proteasomal degradation, though deubiquitinating enzymes can remove ubiquitin, leaving behind an LRGG tag (which becomes a diglycine tag post-trypsinisation) [26]. Depending on the specific lysine residue which becomes ubiquitinated, functional outcomes include lysosome function, DNA repair, ERAD, etc. Full proteomic functional results (Chapters 4 and 5) strongly suggest altered ubiquitination flux in all clinical classes, relative to healthy volunteers. In the analysis of neutrophil proteins from the subset of patients for whom samples from both time-points were available ($N = 24$), only three apparently-diglycine-tagged proteins met stringency criteria and were SwissProt identifications (USP9Y, BMP3, RPS27A), and two (USP9Y, RPS27A) were detectable in a sufficient number of patient samples ($n_{\text{USP9Y}} = 10$, with only PA257 from the CTRL group; $n_{\text{RPS27A}} = 11$, all from the IRIS group) to examine the potential effect of ARV therapy on iodination over time (fig. 10). Differential changes are apparently not accounted for by placebo/prednisone status, though only two and three patients received prednisone, while seven received placebo/nothing. That mainly IRIS samples apparently exhibit USP9Y and RPS27A ubiquitination may suggest that ubiquitination (or indeed ubiquitin-mediated protein degradation flux) is more common in this group.

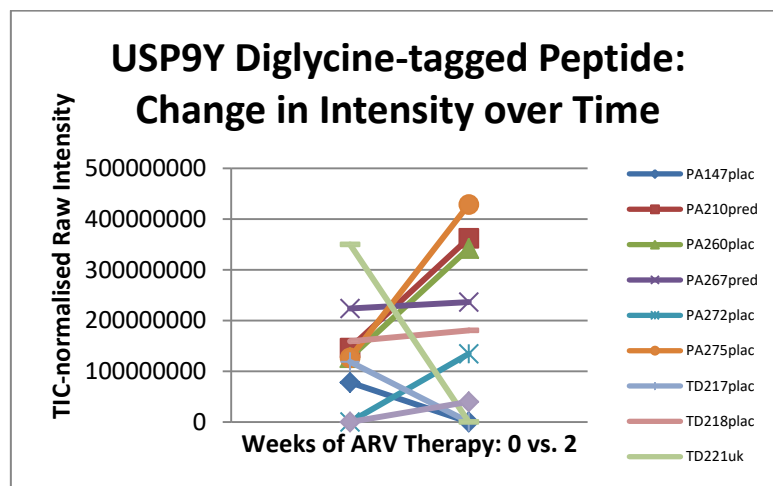


Figure 10a Change in Putative Diglycine-tagged USP9Y Peptide Intensity Over Two Weeks' ARV Therapy

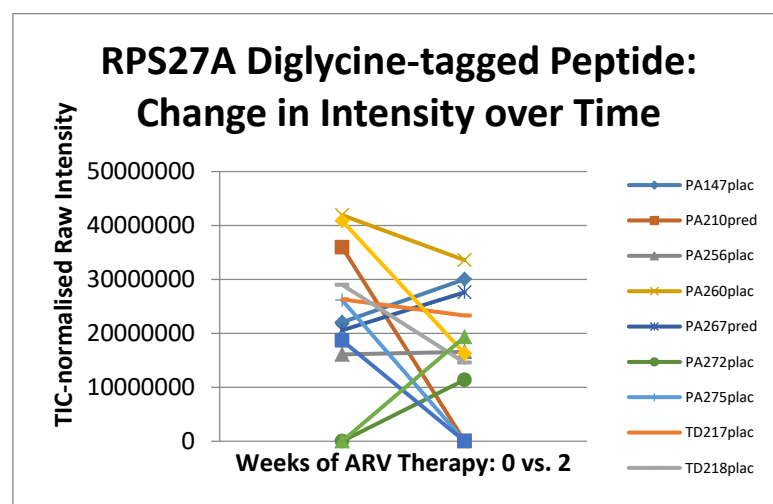


Figure 10b Change in Putative Diglycine-tagged RPS27A Peptide Intensity Over Two Weeks' ARV Therapy

Indeed seven patients exhibit increasing (and only three exhibit decreasing) diglycine-tagged USP9Y peptide levels during ARV therapy, suggesting increased turnover of USP9Y (which normally deubiquitinates targets, for example enhancing SMAD4-SMAD2/3-TGF β signalling). On the other hand seven samples exhibit decreasing (and only three exhibit increasing) diglycine-tagged RPS27A peptide levels, suggesting decreased turnover of RPS27A (normally processed to generate free ubiquitin and a constituent of the ribosomal 40S subunit involved in protein expression). Together, this suggests an increase in protein translation capacity, and both increased ubiquitin availability and deubiquitination (i.e. increased ubiquitination flux).

Citrullination

Citrullination (R) is a Ca²⁺-driven modification performed by peptide arginine deiminase (PAD) enzymes; it can form immunogenic epitopes and is implicated in NETosis [27]. In the analysis of neutrophil proteins from the subset of patients for whom samples from both time-points were available (N = 24), only six apparently-citrullinated proteins met stringency criteria and were SwissProt identifications (S100A8, MYCTU_THYX – a mycobacterial flavin-dependent thymidylate synthase involved in purine metabolism, MYCTU_PIMA – a mycobacterial phosphatidyl-myoinositol mannosyltransferase, HNRNPU, SPTBN1, SLFN5, SF3A3, and DUSP19), but only MYCTU_THYX was detectable in a sufficient number (n = 6, all from the IRIS group) of patient samples to examine the potential effect of ARV therapy on iodination over time (fig. 11). Differential changes are apparently not accounted for by placebo/prednisone status, though only two patients received prednisone, while three received placebo/nothing. That mainly IRIS samples apparently exhibit Mtb THYX citrullination may suggest that levels of Mtb-derived proteins are higher and/or that citrullination is more common in this group. Full proteomic functional results (Chapters 4 and 5) strongly suggest altered *host* purine and pyrimidine metabolism.

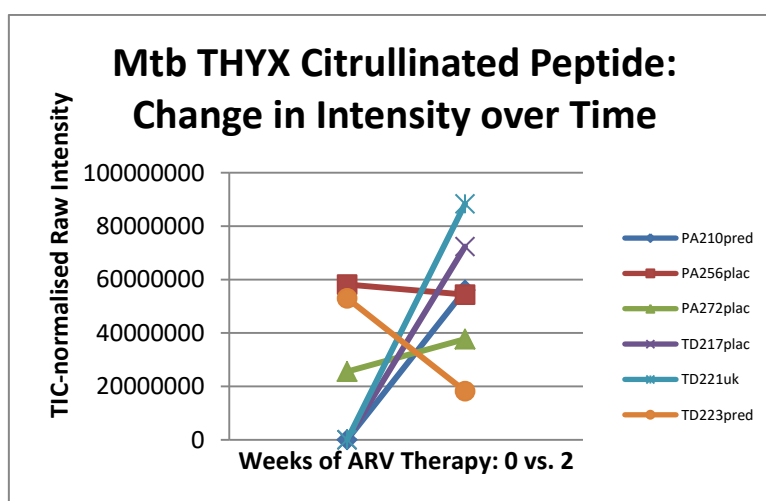


Figure 11 Change in Putative Citrullinated Mtb THYX Peptide Intensity Over Two Weeks' ARV Therapy

Indeed four patients exhibit increasing (and only two exhibit decreasing) citrullinated Mtb_THYX peptide levels during ARV therapy, suggesting potential generation of novel antigenic epitopes which could elicit antibodies. Interestingly, active TB patients often exhibit anti-citrullinated protein antibodies [28]. Results of full dataset A (N = 37) and B (N = 74) PTM analysis are enclosed in Appendix I.

3.4.5.4. Data Wrangling, Transformation, & Meeting Statistical Assumptions

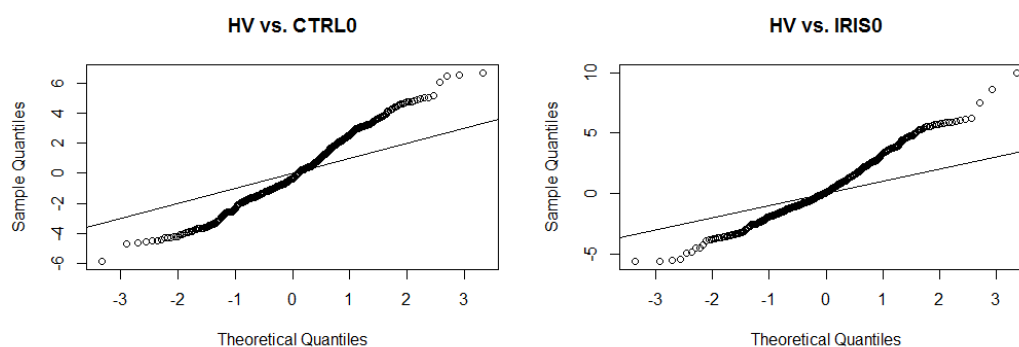
Subsequently to ensuring its high quality, data must undergo appropriate pre-processing. This includes conversion into formats required by different analyses and application of the minimum number of appropriate transformations, which facilitate comparison by bringing values within range, centering distributions, and stabilising variance [29] [30] [31] [32] [12]. Selected statistical and functional analyses must be appropriate not only to the inherent nature of the dataset, but also to the research question(s) at hand [29]. We apply analytical techniques appropriate to a hypothesis-generating case-control proteomic profiling study, and ensure assumptions of these techniques are met. Although our data are technically of a multivariate nature, we focus on observing only how binary disease state/time-point (independent variable) influences protein expression (dependent variable). Because our independent variable (disease state) is categorical, and our dependent variable (protein expression level) is continuous, the correct statistical test of significance in this context is the independent t-test. We thus perform differential expression analysis by means of a moderated form of Student's independent samples t-test. An argument could be made for use of the multivariate analysis of variance (MANOVA) to compare all five comparison classes (healthy volunteer, CTRL0, IRIS0, CTRL2, IRIS2) simultaneously (in order to minimise type I error), but in order to maximise data utility for hypothesis generation, we explicitly favour avoiding type II errors (false negatives) over avoiding type I errors (false positives), opting for sequential two-group comparisons using the independent samples t-test, but pending downstream hypothesis validation by orthogonal means.

The independent samples t-test assumes sample independence, normal distribution of data within each comparison class, and comparable variance of data between comparison classes (homoskedasticity). **Independence** is an absolute requirement, and is a feature of study design: it requires that the comparison classes from which measurements are taken are independent of each other [33]. This is true of our study design, in which CTRL and IRIS groups (and even time-points *within* disease groups, for the vast majority of participants) represent separate participants. T-testing also assumes data parametricity; usually **normality**. When sample data are derived from a population whose behaviour can be adequately modelled by known probability distributions (e.g. uniform, Poisson, geometric, normal/Gaussian, chi-squared, etc.), the data are said to be parametric, because each distribution functions within a fixed and predictable set of parameters. A good initial check for normality is visual inspection of data distribution via a histogram. Another graphical technique to determine whether data come from a stated distribution is the quantile-quantile (QQ) plot, a scatter plot which compares fitted with empirical distributions [34]. When all points on the plot fall approximately on the 45° reference line, data come from the stated distribution [34]. Of various statistical tests for normality (e.g. Anderson-Darling, Martinez-Iglewicz, Kolmogorov-Smirnov, D'Agostino Omnibus), we employ the Shapiro-Wilk W test, which is powerful for smaller sample sizes [34]. It calculates the ratio of two estimates of variance, and the closer the W-statistic is to 1, the more normally-distributed the dataset (and the straighter the QQ plot) [34]. Furthermore, if the p-value is high, we accept the null hypothesis that data come from the stated distribution. In the present dataset, comparison class distributions are roughly normal (*vide infra*). Finally, t-testing assumes **homoskedasticity**. Simplistically, this ensures comparison of "apples with apples", but real-world biological datasets often contain comparison classes with unequal variance. Variance may be estimated by the coefficient of variation (CV). Distribution of CVs in a control group are commonly less broad than those in a disease group (though the inverse can also be true), and CVs from humans are greater than from laboratory animals [35]. Furthermore, CV for high-abundance proteins is approximately constant (because the standard deviation of the response is approximately proportional to the mean response), while CV for low-abundance proteins is higher [36] [37]. To test for equal variance, a linear regression model is created and residuals (deviations from the line) are plotted. When variance is equal, the plot will appear 'blob-like' in its distribution, although normality of the residual distribution can be more formally examined by histogram. It is difficult to test for equal variance when CV is very small [38]. In the present dataset, comparison class variances for the top five most-abundant and least-abundant proteins indicate approximately equal variance (Table 3, *vide supra*).

In practice, the independent samples t-test is in fact quite robust to violations of both normality (provided distributions are not extremely skewed or do not have very heavy tails due to outliers) and the least important assumption: homoscedasticity (especially when sample sizes are equal and degrees of freedom are adjusted accordingly) [39] [40]. Nonetheless, non-parametric alternatives to the t-test (but which typically have less power to detect true differences) include for example the Mann-Whitney U-test [39] [41] [40], and alternative tests (e.g. Welch's t-test) are also available for heteroskedastic cases.

Regarding data transformation, *normalisation* represents an early transformation typically applied to expression data to adjust individual intensities (which may otherwise have been affected by unequal starting peptide quantities, differential detection efficiency, and systematic bias), remove noise (stabilise variance to improve power to detect smaller fold-changes), and balance them such that meaningful comparisons are possible [30] [32]. However, since we experimentally control starting peptide quantities at the level of cell number, estimated protein quantity, estimated peptide injection quantity, and total ion chromatogram (TIC) intensity, and since MaxQuant's LFQ algorithm already normalises individual intensity values to total protein intensity, we do not apply normalisation (although during optimisation we tested MaxQuant's iBAQ algorithm – which fixes the median at zero in log space to normalise raw intensities – and quantile normalisation, which does not fix the mean at zero in log space [32] [30] [42]). Repeat-normalisation at the LFQ intensity level (especially using an aggressive algorithm such as quantile normalisation) may obscure true significant differences (multiple testing correction (MTC) already results in many p-values becoming non-significant), and a hypothesis-generating study such as this aims to cast as wide a net as possible while still maintaining stringency.

We do, however, apply **log₂ transformation**. Due to the assumptions inherent in hypothesis testing methods used for differential expression analysis, it is typically performed “on the log scale” [31]. Many real-world datasets are non-normally distributed, and significant violation of the assumption of normality increases type I or II error rates, whether employing parametric or non-parametric tests [43] [29]. Log transformations improve normality of positively-skewed distributions [29] (LFQ distributions are typically left-skewed due to missing values, but we compare only proteins for which non-zero LFQ values are shared between classes). Log transformations compress the right side more than the left side of a distribution [29]. Although ratios are intuitive expression level measures, they treat up- and down-regulation differently (e.g. two-fold up-regulation results in an expression ratio of 2, while two-fold down-regulation results in an expression ratio of -0.5) [30]. Log₂ transformation also corrects this skewing, since $\log_2 2 = 1$, and $\log_2 0.5 = -1$ [30]. The choice of log base is important [29]. Commonly used bases include 10, e, and 2 [29]. Datasets with extreme range can benefit from log transformation in base 10, as higher bases more drastically compress extreme values [29]. Log-type data transformations can also alter variance (reducing it for high levels and increasing it for low levels), further increasing efficacy of downstream statistical methods [36] [37]. We compare only proteins for which non-zero LFQ values are shared between classes, and – after log₂ transformation – these are indeed approximately normally distributed (based on the top five most-abundant and least-abundant proteins) by QQ plot (fig. 12) and the Shapiro-Wilk test (data not shown).



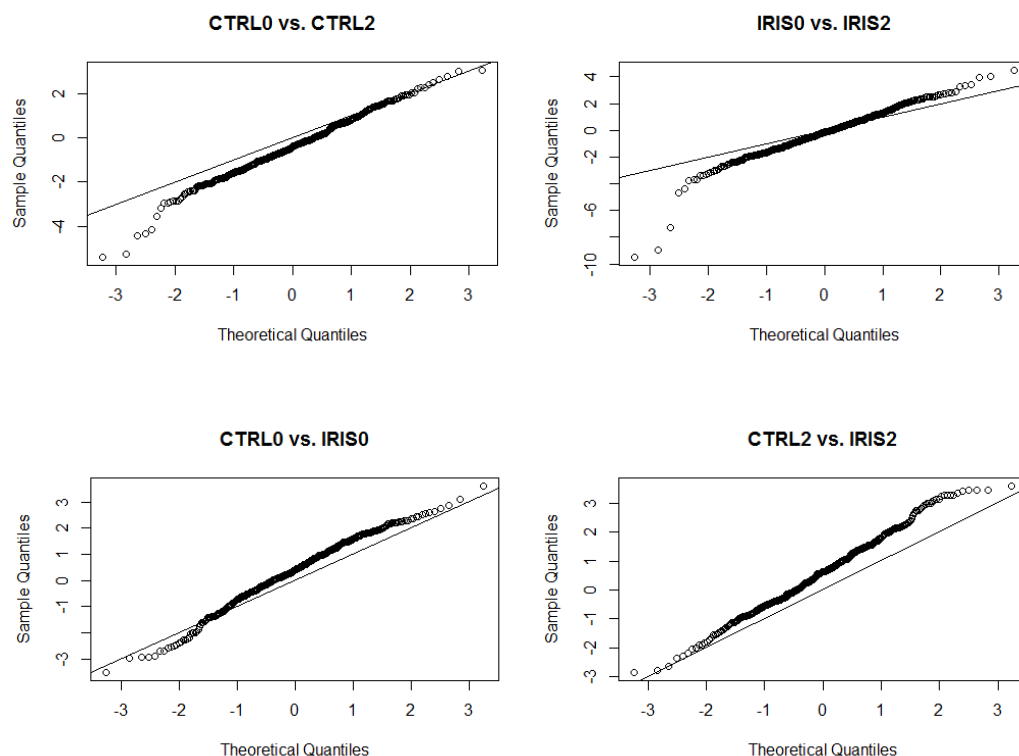


Figure 12 Study Comparison QQ Plots Indicate Approximate Normality of log₂LFQ Distributions

3.4.6. Data Analysis: Comprehensively Understanding Between-class Neutrophil Functional Differences

Data analysis (including differential proteomic profiling) was performed using a custom-built R [44] pipeline (fig. 13). Data pre-processing (log₂ transformation, statistical assumption check, comparison of technical and biological replicate performance) has already been discussed above. Briefly, subsequent data analysis incorporated the following steps: principal components analysis (PCA), detectable/undetectable proteins analysis with output visualisation by means of Venn diagrams [45], linear modelling incorporating independent two-samples t-testing ($p < 0.05$) using Limma [46], application of MTC to Limma's TopTable function using the Benjamini-Hochberg FDR method [47] ($p < 0.05$), generation of a Volcano plot to visualize significantly differentially expressed proteins, functional enrichment analysis (FunRich [48], STRING [49]), pathway enrichment analysis (STRING) and pathway activation state prediction (SPIA [50]), deep manual analysis of neutrophil-relevant and data-specific functional modules (focusing on inference-friendly proteins, pathways, and modules), RandomForest-based identification of biomarker candidates [51], and ROC-based evaluation of biomarker candidate performance [52]. Protein-protein expression correlation, heatmapping, clustering analysis, non-negative matrix factorisation (NMF), and geneset enrichment analysis (GSEA) added little to findings and are therefore not included here.

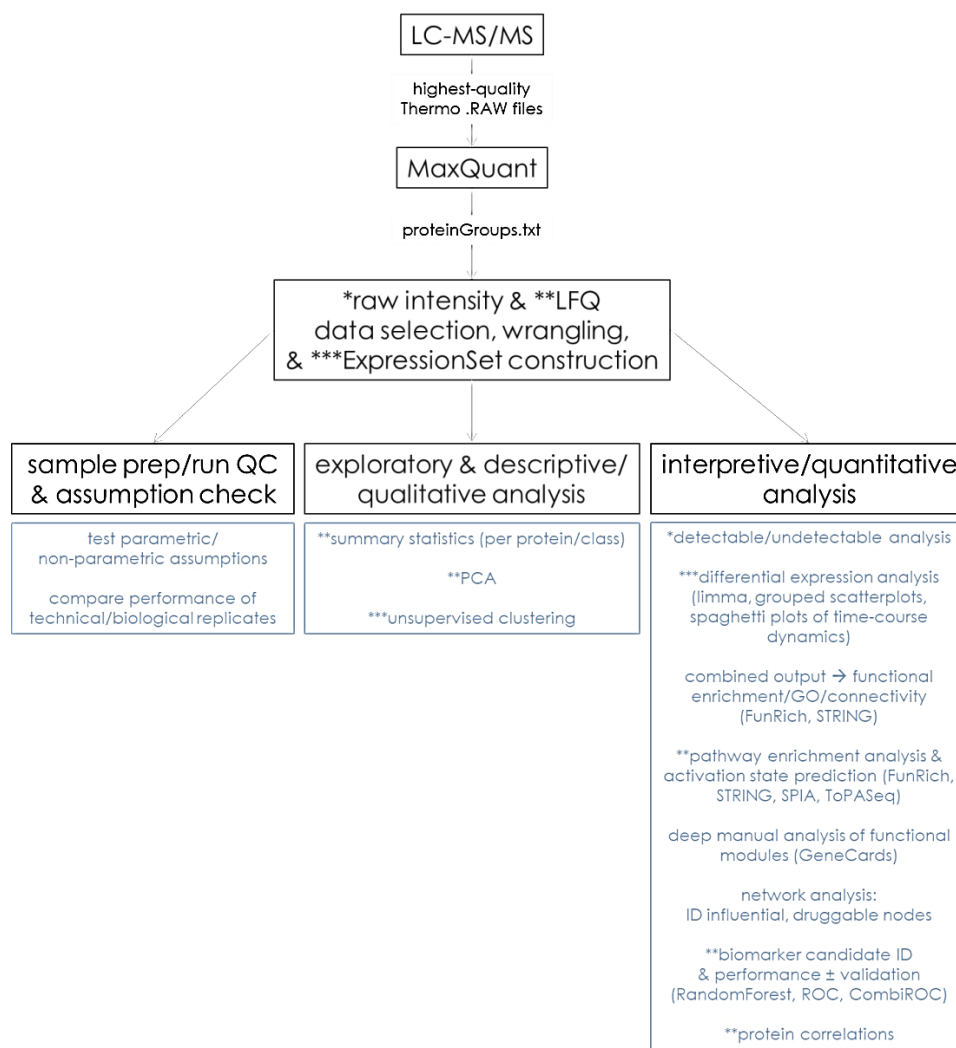


Figure 13 Custom Data Analysis Pipeline. Quality of .raw files was manually assessed during LC-MS/MS run-time based on equipment performance (e.g. spray stability); total ion chromatogram (TIC) profile, intensity, and retention time (RT); and file size. Within proteinGroups.txt, protein group annotations were parsed for gene names, used here as 'protein identifiers' during downstream analysis to maximise cross-compatibility between references databases. The first protein in the protein group (i.e. for which most evidence exists) was taken as the true identification. Furthermore, protein groups were filtered for contaminant and reverse identifications, and only protein groups identified on the basis of ≥ 2 unique peptides were carried forward for analysis. Data wrangling consisted of placing final participant proteomic identification and intensity data into appropriate data formats with correctly-named rows and columns for downstream analysis, as well as log₂ transformation to bring values within range and further normalise data distribution. ExpressionSets refer to data constructs required by downstream R packages NMF and limma (*vide infra*). All information regarding protein function and expression compartments derives from GeneCards [53] unless otherwise specified. Appropriate statistical measures of confidence have been considered at each step of study design and results analysis, and applied where appropriate for a hypothesis-generating study. **LFQ** label-free quantitation, **QC** quality control, **PCA** principal component analysis, **GO** gene ontology, **ID** identify/identification, **ROC** receiver-operator characteristics. R packages are described in the text above.

3.4.6.1. Exploratory Analysis: PCA & Clustering (Dataset B)

Biological multivariate data must be represented in a utilitarian manner which “makes latent structure in the data explicit”, often by minimising dimensionality [54]. Various unsupervised learning algorithms based on matrix factorization or clustering (e.g. principal component analysis (PCA), independent component analysis (ICA), singular value decomposition (SVD), vector quantization, etc.) are commonly used to identify “natural group structures [extant within] high-dimensional” data [55] [56]. Differing constraints inherent within each algorithm, produce factors with different representational properties [55].

A good overview of data structure may be attained via construction of a PCA bi-plot (e.g. as an initial check that available data actually do reflect comparison class differences, prior to committing to further in-depth data analysis). PCA is a statistical technique (unsupervised learning algorithm similar to

clustering) which – through orthogonal transformation into/projection onto linearly-uncorrelated principal components (linear combinations of the original variables) - reduces the dimensionality of multivariate data while retaining as much variance as possible (i.e. summarises major data features) [12]. This (usually, but not always) facilitates detection of important latent trends and patterns – too complex to interpret by manual inspection - within the data, “without prior knowledge about” sample groups [12]. Principal component selection “is equivalent to carrying out multiple linear regression on the projected data against each variable of the original data” (albeit with concomitant minimization of “perpendicular distance between a data point and the principal component, whereas linear regression minimises distance between the response variable and its predicted value”), aiming for the optimal summary of data features with the fewest principal components [12].

The first few principal components typically account for most variability present in the data, with each subsequent component thereafter accounting for additional (but less) variability. Here, R package FactoMineR [57] was used to implement PCA only on the subset of proteins quantifiable (i.e. non-zero LFQ values) between comparison classes. Results of PCA are reported in Chapters 4 and 5. When visualising a PCA bi-plot, each loading (in this case protein-representing) vector has a positive and a negative side, and the closer a sample falls to the positive side, the higher the abundance of that protein in that sample (and vice versa). An ideal outcome includes maximal original-data variance capture by the fewest possible principal components (with the first few components accounting for most of the variance), as well as tight and separate clusters explained (indeed, hopefully driven) by some pre-existing independent variable of interest. Occasionally the first few principal components capture another source of data variance (e.g. gender, age, or an independent variable that is unaccounted for/not of interest), and sometimes variance is distributed across many components (i.e. it is hard to decrease dimensionality of the original data, which does not exhibit a small number of significant features). In such cases, a three-dimensional PCA tri-plot (i.e. using three rather than just two principal components) may be useful. Additional limitations of PCA: input data structure must be linear, highly-correlated patterns may not be resolvable, and because PCA aims to maximize variance it is not actually intended for clustering [12].

Many non-PCA algorithms are specifically intended for unsupervised clustering (i.e. identification of clusters and, occasionally, of the differences that drive clustering). One of these is non-negative matrix factorisation (NMF), which – like PCA – is a “multivariate data decomposition technique” based on matrix factorisation, and which finds a parts-based linear representation of the original data [55] [54]. One of its more unique constraints – in contrast with PCA and ICA - is non-negativity (requiring entries of matrices derived from the original data to be non-negative, such that the original data are summarised by representations which are additive, not allowing for subtractive components) [54]. This constraint is motivated by many real-world quantities being non-negative (which can sometimes complicate interpretation of components analysis results) and because, intuitively, parts are combined (not subtracted) to produce a whole [54]. One of NMF's most useful properties is that it usually produces a “sparse” representation of the data (data vectors are sufficiently approximated using only a few basis vectors), producing easily-interpretable results [55] [54]. Thus, “NMF decomposes a large input dataset into a small set of highly interpretable and additive parts” [14]. NMF has been successfully applied to many problems (e.g. in signal processing and image recognition, including identifying intuitive major facial features e.g. eyes, nose, mouth, etc.) [54] [14]. Indeed, NMF is appealing because of its “empirical success in learning meaningful features from a diverse collection of real-life datasets” [54]. Its properties make it particularly effective in biological pattern discovery, such as in gene expression (e.g. microarray) data, identification of disease (e.g. cancer) subtypes, and in various ‘omics techniques [58] [14].

And yet, as for PCA, certain real-world datasets are resistant to NMF-mediated generation of a useful data representation, and it fails to decompose the data into intuitive features [54]. Researchers have pointed out various shortcomings of NMF (e.g. a tendency towards finding only global rather than local features, and an inability to explicitly control parameters such as sparseness) and suggested extensions which can correct these in order to facilitate improved NMF performance for difficult datasets [54]. In such cases, explicitly controlling parameters such as representational sparseness improve NMF performance in identifying intuitive data features [54]. A researcher from our institution created an R package called NMF [59] which enables explicit control over such parameters, as well as empirical *a priori* determination of the ideal parameters given a specific dataset. This package also facilitates identification of the features (in this case proteins) responsible for clustering. Although this algorithm was successfully applied during pilot studies within this project, during analysis of the full dataset it did not add any additional utility beyond PCA, and thus NMF results are not reported.

3.4.6.2. Detectable/Undetectable Analysis (Dataset B) & Attempted Identification of Mtb Proteins (Datasets A & B)

To determine which proteins are differentially detectable/undetectable between classes, we made use of raw intensities rather than protein LFQ values (some of which are zero despite being detectable). Proteins were defined as 'detectable' if $\geq 80\%$ of samples within a class exhibited non-zero raw intensity values (increased to 100% for dataset A, due to smaller sample numbers). Venn diagrams were constructed using Venny [45] to compare overlaps of identifiable proteins between relevant comparison classes. The implicit assumption here is that detectable proteins are *present* in neutrophils within a particular class, and that undetectable proteins are *absent*. However, we are mindful that many technical reasons other than true absence may result in non-detection of proteins, such as non-accessibility to trypsin (e.g. due to incorporation into membranes, incomplete denaturation, etc.), poor ionisation of peptides, peptide modifications not included in search engine parameters, low peptide abundance below the limit of detection of the system, or selection bias/spectral interference by co-eluting high-abundance peptides. However, given the similarity of the samples, their preparation methods, and their TIC profiles, many detectable/undetectable events will indeed indicate true relative presence/absence in neutrophils of origin. Results of detectable/undetectable analysis are incorporated into Tables reported in Chapters 4 and 5.

Because protein reference databases are incomplete/imperfect, careful selection is paramount. In order to avoid misattribution, reference databases employed by the search engine to which spectra are submitted for identification should include all proteins likely present in the samples of interest. For this reason, and because – to our knowledge – it has never before been shown whether Mtb-derived proteins are detectable within neutrophil samples from infected humans (and pathogen protein/antigen levels are potentially relevant to TB-IRIS), we include both human and Mtb proteomes when submitting spectra to MaxQuant for peptide identification and subsequent protein inference.

In datasets A and B, respectively, four and six potential unmodified Mtb proteins are identified (Table 4). However all are inferred based on only a single unique peptide, and most (three of four, five of six) are from Uniprot's TrEMBL database (whose entries, once reviewed, continually migrate to the more reliable UniProt SwissProt database). Although pilot datasets from this project previously identified potentially-interesting Mtb proteins (*vide infra*) from human neutrophil samples based on multiple unique peptides, identifications in the full dataset (Table 4) are made based on fewer evidences. We can be confident, however, that these proteins are unlikely to be carry-over on the HPLC column from other studies in our laboratory employing mycobacteria, as evidenced by either running samples on a fresh column or submitting spectra resulting from blank column runs (prior to experimental runs) to MaxQuant for protein group identification.

Table 4 Potential Mtb-derived Proteins Identified in Human Neutrophil Datasets

Dataset	Database	Protein	Gene
A	sp	uncharacterized HTH-type transcriptional regulator	Rv1985c
	tr	probable transmembrane alkane 1-monooxygenase	alkB
	tr	probable conserved lipoprotein	lppR
	tr	possible sensor-transducer protein	blaR
B	sp	uncharacterized HTH-type transcriptional regulator	Rv1985c
	tr	probable transmembrane alkane 1-monooxygenase	alkB
	tr	conserved protein	Rv1754c
	tr	possible osmoprotectant ATP-binding transporter	proV
	tr	probable conserved lipoprotein	lppR
	tr	possible sensor-transducer protein	blaR

These results are disappointing in light of earlier findings based on two high-quality pilot datasets analysed when the instrument was operating at peak efficiency. A pilot comparison of three versus three CTRL2 and IRIS2 samples using a standard HPLC gradient not optimised for neutrophils and incorporating both human and Mtb reference proteomes (as well as allowing phagolysosome-specific variable modifications) during MaxQuant analysis identified 40 apparent Mtb proteins, with one (SwissProt entry Rv3377c gene product, a type B diterpene cyclase which converts GGPP to tuberculosinyl pyrophosphate) LFQ-quantifiable in both classes and significantly more abundant in IRIS

than CTRL samples ($p = 0.0035$). This protein was identified by MS/MS (rather than matching) in 4 out of 6 samples, on the basis of multiple peptides (including 1 unique). No homologous human protein exists, and visual inspection reveals relatively high spectral quality (evidence #418).

This was an especially-tantalising finding since Rv3377c is involved in a mycobacterial state change in response to host immunity: its partner Rv3378c conjugates tuberculosinyl pyrophosphate to adenosine to produce 1-tuberculosinyladenosine (a.k.a. substance A), which alters macrophage lysosomal architecture, prevents phagosome maturation and phagolysosome acidification, may inhibit autophagy, and may also favour formation of foamy macrophages. Both these genes are specific to virulent mycobacterial species [60], and substance A is present at high levels in clinical Mtb strains (while being absent from most other mycobacteria and bacteria). Were this finding (the Rv3377c gene product being present at higher abundance at the time of TB-IRIS manifestation) confirmed, it would suggest that residual (uncleared) *viable* Mtb are responding to enhanced myeloid activation with increased virulence factor expression, and thus that it may be antigens produced by live, more virulent Mtb which contribute to the TB-IRIS phenomenon (as suggested by RD1-encoded α -crystallin findings in Chapter 1).

Furthermore, a more recent 2016 comparison of four versus four CTRL2 and IRIS2 samples using a neutrophil-optimised HPLC gradient and incorporating both human and Mtb reference proteomes (as well as allowing phagolysosome-specific variable modifications) during MaxQuant analysis identified 14 apparent Mtb proteins, two of which (both SwissProt entries) were identified in all samples, and based on >5 unique peptide: EFTU (elongation factor Tu, also involved in the responses to hypoxia, Fe, and redox stress) and CH602 (60kDa chaperonin 2, GRoEL, 65kDa Ag, HSP65; involved in the responses to heat, hypoxia, and redox stress by preventing protein aggregation). The latter is also a major mycobacterial immunogenic antigen, which (via TLR4 and MyD88-dependent and -independent pathways) activates NFkB. LFQ-based abundance of both proteins trended higher in IRIS samples, which – if confirmed – would again suggest a response by *viable* Mtb (via RD1 expression) to increased hypoxia/redox stress due to enhanced myeloid activation at the time of TB-IRIS manifestation. An alternate explanation for findings of both pilot datasets would be enhanced Mtb virulence inhibiting antigen clearance to a greater degree in IRIS-developers than CTRLs.

3.4.6.3. Differential Expression Analysis: Linear Modelling with Moderated *t*-tests (Dataset B)

Typically, differential protein expression analysis involves modelling, a relevant statistical test of significance, and MTC [30] [32]. The independent *t*-test provides a measure of significance of differential expression data. Its output, the *p*-value, represents the probability that a protein's mean expression level is different between two comparison classes only by chance. For example, if 2000 proteins are tested, a *p*-value of 0.01 signifies that 1% (20 proteins) may appear significantly differently expressed by chance. The independent *t*-test requires that datasets encompass at least three replicates (in this case biological) of each dependent variable. As discussed above, it also assumes certain properties of the dataset, all of which are met here.

We applied linear modelling and Student's independent *t*-test, as implemented by the R package Limma. The linear model provides a measure of the difference in protein expression between two comparison classes (the mean of each protein's expression level is compared between classes), and of how likely it is that these values could have arisen by chance [61]. The output 'multiple R^2 ' is a measure of the variance accounted for by the model; the closer it is to 1, the higher the percentage of observations explained by the model (i.e. the likelier it is that protein expression level is a function of disease state) [61]. However, protein expression levels are usually determined by multiple factors, not all of which are known *a priori* and not all of which are of interest, but which (ideally) should nevertheless be taken into account by the model. This may be achieved using "a hierarchical variance model estimated in empirical Bayes fashion" [36] [37], and we may in future attempt to employ surrogate variable analysis (SVA) to identify variables representing unknown sources (both technical and biological) of heterogeneity – based on dataset structure – to incorporate into the linear model [62]

[63]. This has been shown to enhance both reproducibility and biological accuracy of expression-based studies [62] [63]. Outputs given by Limma's TopTable function are log of the fold-change (logFC), p-value, the moderated t-statistic, and the B-statistic. The latter represents log odds (the natural logarithm of the odds ratio, a measure of how strongly associated two conditions are) of differential expression and is the default method for ranking genes [64]. Here, we are interested in fold-change and p-value.

Subsequent to the t-test, MTC minimises type I error that may otherwise arise from the large number of independent measurements performed simultaneously (i.e. thousands of per-protein comparisons) [32]. An analogy may be drawn to picking a coin (match) out of a container holding 10 identical coins, 9 of which are copper (correct matches) and one of which is silver (false match). A single sampling represents a 1 in 10 chance of picking the silver coin, while 10 simultaneous samplings (but not sequential samplings with replacement) increases this chance substantially. Thus, MTC bases significance on probability magnitude rather than relative enrichment magnitude, which can be due to chance [65]. Of the various MTC methods (incl. Bonferroni, Sidak, Holm, Hochberg, Hommel, Benjamini-Yekutieli etc.) we utilised Benjamini-Hochberg (a.k.a. FDR), a modified step-down type method, which ranks the protein list by p-value from smallest to largest, then multiplies each p-value below the largest one by (total number of proteins in list divided by that protein's rank) to produce an adjusted p-value corresponding to the FDR [65]. This method does not assume independent variables, and is less conservative (i.e. will produce fewer false negatives) than for example Bonferroni, which multiplies each p-value by the total number of proteins in the list [65]. Assuming very strong dependencies and a high category number, bootstrapping or Monte-Carlo simulation may be more appropriate [65].

In gene or protein expression studies, it is common for the majority of p-values to become non-significant after adjustment, which severely limits the potential of systems biology studies to engage in deep biological functional profiling. There are various arguments both for and against MTC, including ongoing debates regarding its appropriateness for mass spectrometry-based discovery proteomics with label-free quantitation (which is more of a handedness rather than a sampling problem). Indeed, an over-reliance on statistical rather than biological significance (such as when many independent lines of evidence converge on a common conclusion) causes studies to miss true biological differences [66] [67], thereby failing to maximise the utility of rare and extremely valuable data. Especially, since this is a hypothesis-generating study, we define all proteins with $p < 0.05$ as SDE and carry these forward for functional analysis. This is done on the assumption that the majority of identified differential expression is likely biologically real, while accepting the requirement for downstream orthogonal validation of any resulting hypotheses. Furthermore, functional analysis may be better at indicating outliers, whereas many observed protein changes often converge on the same biological conclusions, supporting that involved proteins are indeed differentially expressed between comparison classes.

A good first step in visualising differential expression analysis results is a volcano plot. Results of differential expression analysis are reported in and incorporated into Tables reported in Chapters 4 and 5. The next challenge is extracting biological meaning from obtained lists of differentially expressed proteins in order to better understand underlying bio-molecular phenomena [65]. In order to maximise data utility, we combine detectable and up-regulated proteins in order to generate a list of proteins thought to represent those relatively more abundant within that class, and undetectable and down-regulated proteins in order to generate a list of proteins thought to represent those relatively less abundant within that class. These lists are carried forward for interrogation of biological meaning.

3.4.6.4. Inferring Differential Biological Function

Many knowledgebases and tools are available to annotate dataset proteins with subcellular location, function, pathway/functional module membership, expression control, and disease association, among other attributes. A truly satisfactory automated method for deep and comprehensive functional analysis remains lacking, however. We have employed progressively more sophisticated methods for functional meaning inference: gene ontology (GO) annotation, enrichment/over-representation analysis (ORA), pathway/functional module membership and activation state, and deep manual

integration of functional modules based on their inter-relationships captured in literature. Despite the small healthy volunteer class size ($n = 10$), results from a more balanced comparison (via randomised selection of $n = 10$ clinical samples) remain highly-similar to the full analysis (results not shown).

3.4.6.4.1. GO Annotation

The standard starting point for drawing biological conclusions from label-free high-throughput proteomics data is based on annotation of each protein with GO terms [65] [68]. The GO project provides a standardised vocabulary describing three levels of attributes, from hierarchically most specific to most general [69]:

- (a) cellular component(s) (subcellular location)
- (b) molecular function(s) (e.g. ATPase, chaperone, or peptidase activities)
- (c) biological process(es) (e.g. energy metabolism or apoptosis).

Automated annotation of SDE proteins with GO terms generates a superficial functional profile [65] [69], but GO has various limitations. Imposing an artificial system of only three tiers on biology is obviously incomplete (especially given that GO tools weight all biological processes equally [65]). Examining GO annotations in isolation can: provide too broad a functional overview to be of any practical use (especially at the biological process level, whose labels can be so general that they are meaningless, e.g. "immune system" or "metabolism"), be misleading (while certain proteins are annotated as being involved in 'Haematological Malignancies', this is by no means their primary function – more likely that this was the earliest-discovered or most well-researched area functionally related to the protein), and fail to provide specific information about the protein's role in individual functional modules/pathways/pathway networks. Certain proteins (e.g. upstream adaptors) and pathways are also promiscuously involved in many cellular functions, limiting their usefulness. Furthermore, "some functions are implicit in activity but not explicitly annotated and thus will be missed by directed queries" [65], and GO categories do not take into account the fact that proteins and pathways are often functionally dependent. Additional drawbacks include incomplete database annotations (only a subset of known genes are functionally annotated and there is a time lag in accruing current information), imprecise/incorrect information (e.g. for humans, of 19490 total biological process annotations available, 11434 are inferred exclusively from electronic annotations without expert human involvement), and an inability to discover previously-unknown protein functions/functional module memberships [65].

Examples of GO annotation software include the Web Gene Ontology Annotation (WEGO) and DAVID [69] [68]. Newer software tools (e.g. FunRich, STRING) extend annotation beyond only GO terms to include, for example, cell types the protein is expressed by (but not necessarily anatomical or subcellular compartments the protein is expressed in), transcription factor programmes the protein is associated with, clinical phenotypes the protein is associated with (but not necessarily different physiological contexts in which the protein is expressed), and publications the protein is associated with. Of course, STRING also facilitates examination of protein interaction partners, which is relevant to network analysis (*vide infra*). Another classification tool, Protein ANalysis Through Evolutionary Relationships (PANTHER) is additionally able to annotate gene products with evolutionary family and subfamily, as well as pathway membership (in this case, the pathway "explicitly specifies the relationships between interacting molecules") [70].

However, understanding the locations and functions of proteins is only the start. In order to understand functional differences between comparison classes, another level of analysis is required.

3.4.6.4.2. Enrichment Analysis/Over-representation Analysis (ORA)

This compares a within-class list of SDE protein identities to a reference background (e.g. all known proteins of that organism/tissue type, or all proteins detectable by the platform) in order to evaluate which input annotations are statistically significantly over-represented (i.e. present at higher levels than

would be expected by chance) [69] [68]. Differential significantly over-represented annotations within comparison classes may highlight areas of the proteome/functional modules for closer examination [69] [68]. Indeed, enrichment analysis/ORA is usually combined with GO (or other annotations) as GO-ORA. As mentioned, various GO enrichment analysis tools are available (e.g. GOrilla) [69] [68]. FunRich and STRING also perform GO-ORA. The appropriate GO enrichment analysis tool must be chosen based on the given type of analysis [65]. A comprehensive list of GO tools is available from <http://www.geneontology.org>. Enrichment analysis/ORA, too, has obvious limitations. For example, it ignores the quantitative changes of SDE proteins, focusing only on their identities and annotations. Furthermore, while the above types of analysis focus on large disturbances in individual gene products, co-ordinated changes in functionally-related gene product sets can produce significant biological effects not detectable from examination of individual gene products.

The most relevant results of FunRich and STRING GO-ORA are reported in Chapters 4 and 5. Additionally, an example of Cellular Component annotation and enrichment by FunRich is provided here (Table 5), suggesting for example inhibited expression of nucleolar proteins but enhanced expression of mitochondrial proteins in response to ARV therapy and during IRIS pathogenesis.

Table 5 Top Three Subcellular Locations of Identified Neutrophil Proteins (Dataset A)

Unique to Class	Cytoplasm (%)	Lysosome (%)	Nucleolus (%)	Mitochondrion (%)	Centrosome (%)
CTRL0 (v CTRL2)	69.7	33.7	19.1		
CTRL2 (v CTRL0)	61.3	31.5		21.6	
IRIS0 (v IRIS2)	59.3	25.3	17.6		
IRIS2 (v IRIS0)	67.6	26.7		24.8	
CTRL0 (v IRIS0)	76	28	22.7		
IRIS0 (v CTRL0)	57.5	26.7		15	
CTRL2 (v IRIS2)	64.5	31.2		16.1	16.1
IRIS2 (v CTRL2)	63.8	30.8		19.2	

Unsurprisingly – since the most abundant, most digestible (i.e. not sequestered e.g. within membranes), individual-eluting, and most ionisable proteins are most likely to be detected via LC-MS/MS – most proteins detected in dataset A appear to have originated from neutrophil cytoplasm and lysosomes (although of course a single protein is often found in multiple subcellular compartments).

3.4.6.4.3. Approaching True Functional Modules: Pathway & Gene-set Analysis

Over-represented GO terms in a given dataset can be matched to specific biological pathways, and various non-GO tools rely on additional (sometimes proprietary) knowledgebases of cellular pathways (constructed from the scientific literature) or functional gene sets (whose expression is known to be co-ordinately regulated, e.g. gene cassettes controlled by a specific transcription factor) to match the input SDE protein list to pathways/gene sets, and in this way determine which pathways/functional modules most likely exhibit dysregulation under that class's condition [71] [72] [69] [68]. Nevertheless, even if many gene products in a pathway are differentially expressed, the pathway may not be significantly biologically perturbed (and vice versa), e.g. unless the perturbed gene product is a *significant decision-making node in the pathway*. Conversely, pathway pickup relies on the number of gene products in a pathway represented by the input list, so even if a key gene product in the pathway is dysregulated (but not many other pathway members are), the pathway will not be flagged as dysregulated. One user-friendly gene-set knowledgebase is the Molecular Signatures Database (MSigDB), for analysis using freely-available GSEA software [71] [72]. Examples of pathway knowledgebases (which also provide suites of analytical tools) include Reactome [73], the Kyoto Encyclopedia of Genes and Genomes (KEGG) [74], and IPA (proprietary) [75]. Standalone open-source analytical tools for use with existing freely-available pathway databases include the R packages SPIA and TopaSeq [76].

It is important to distinguish between functional class scoring (*pathway/functional module membership and over-representation within a dataset*) and pathway *topology/activation state* analysis. Pathway

membership and over-representation ignores dependence of member gene products, which is to some extent addressed through functional module scoring (e.g. GSEA). However, both consider only the number (and sometimes change in magnitude) of gene products, ignoring the directionality and nature of changes. To date, the most comprehensive automated tools for functional profiling based on systems biology data culminate in pathway activation state analysis. For example, open-source SPIA and proprietary IPA accept as input lists of genes/proteins with associated fold-changes/p-values, identify which pathways are most highly-represented by these, and *attempt to predict whether these pathways are activated or inhibited* (ultimately providing numeric pathway activation state scores and associated significance values). Thus, SPIA and IPA take into account gene product change magnitude and directionality, as well as functions and pathway topology. SPIA relies on the Reactome and/or KEGG databases, while IPA uses a proprietary database (whose terminology does not always correlate with that of GO) [77]. IPA classifies "the genes implicated in each function within sub-functions and provide a direct link of each molecule to the bibliographic reference where that relationship is described" and identifies altered established (canonical) pathways to provide clear pictographic representations that facilitate "easy identification of molecules implicated in each function" [77]. "Moreover, IPA generates networks where the differentially regulated genes can be related according to previously known associations between genes or proteins, but independently of established canonical pathways" [77]. "Ingenuity systems' pre-sorted, manually-curated information and search tools enable reliable identification and extraction of the pertinent entities and interactions" [78]. Selected IPA pathways or pathway subsets (with super-imposed gene products of interest), as well as radar plots, are a good way to visualise these types of data. Both SPIA and IPA allow viewing of the pathway of interest with input SDE gene-products within the pathway highlighted (including directionality of change).

Here, we include an example of SPIA pathway membership, activation state, and pathway map (with overlaid SDE) output for dataset A, as well as visualisation of class-differential pathway activation state by radar plot (Tables 6 and 7, fig.s 14-16). Findings for Dataset B are similar (not shown).

Table 6 Top 5 Pathways Dysregulated in IRIS0 vs CTRL0 (SPIA, Dataset A)

Pathway	Members	p _{NDE}	p _{PERT} (TA)	p _G	Predicted Status
amyotrophic lateral sclerosis	14	0.002	0.13 (0.76)	0.002	activated
legionellosis	25	0.002	0.19 (1.11)	0.002	activated
leukocyte transendothelial migration	43	0.001	0.57 (1.05)	0.003	activated
NOD-like receptor signalling	15	0.003	0.95 (-0.12)	0.02	inhibited
tuberculosis	51	0.06	0.3 (1.46)	0.09	activated

p_{NDE} small when SDE genes are true pathway members) p_{PERT} probability that perturbation is not due to chance p_G global significance score

Table 7 Top 5 Pathways Dysregulated in IRIS2 vs CTRL2 (SPIA, Dataset A)

Pathway	Members	p _{NDE}	p _{PERT} (TA)	p _G	Predicted Status
legionellosis	25	0.014	0.02 (2.38)	0.002	activated
amyotrophic lateral sclerosis	14	0.002	0.17 (0.69)	0.003	activated
tuberculosis	51	0.005	0.24 (1.98)	0.01	activated
NOD-like receptor signalling	15	0.003	0.54 (0.30)	0.01	activated
leukocyte transendothelial migration	43	0.059	0.56 (0.96)	0.15	activated

p_{NDE} small when SDE genes are true pathway members) p_{PERT} probability that perturbation is not due to chance p_G global significance score

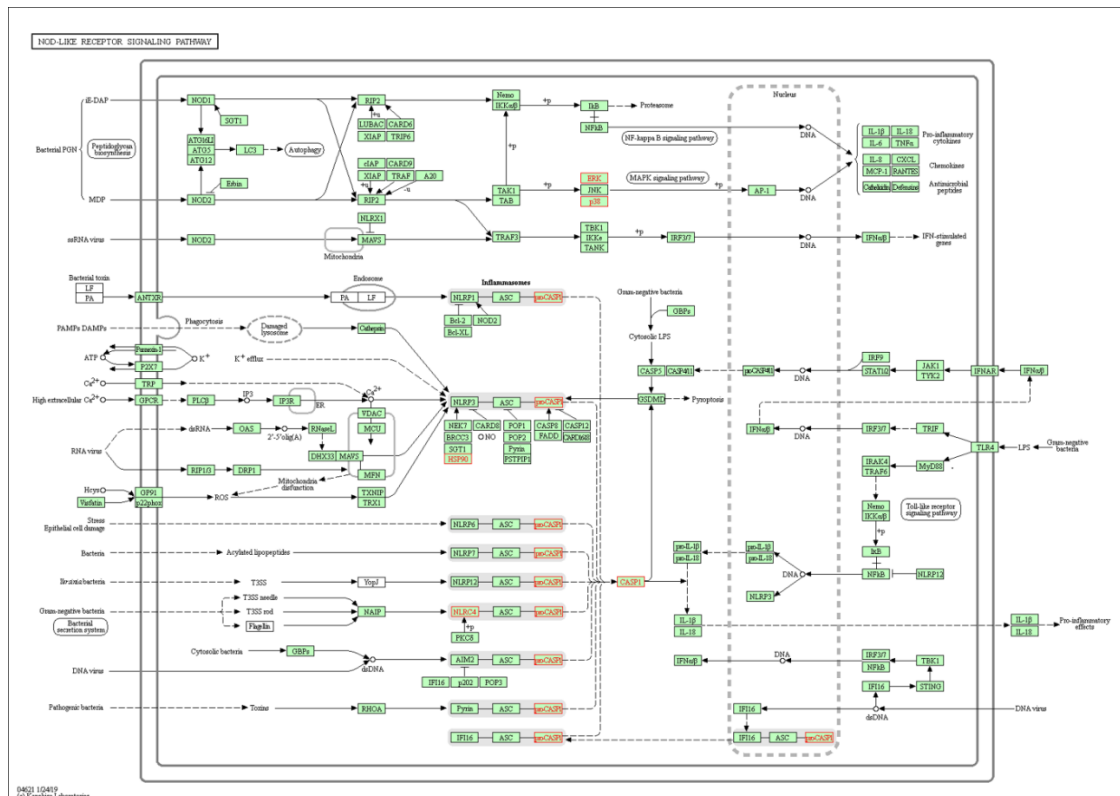


Figure 14 NOD-like receptor signalling pathway with overlaid SDE: IRIS0 vs CTRL0 (Dataset A)

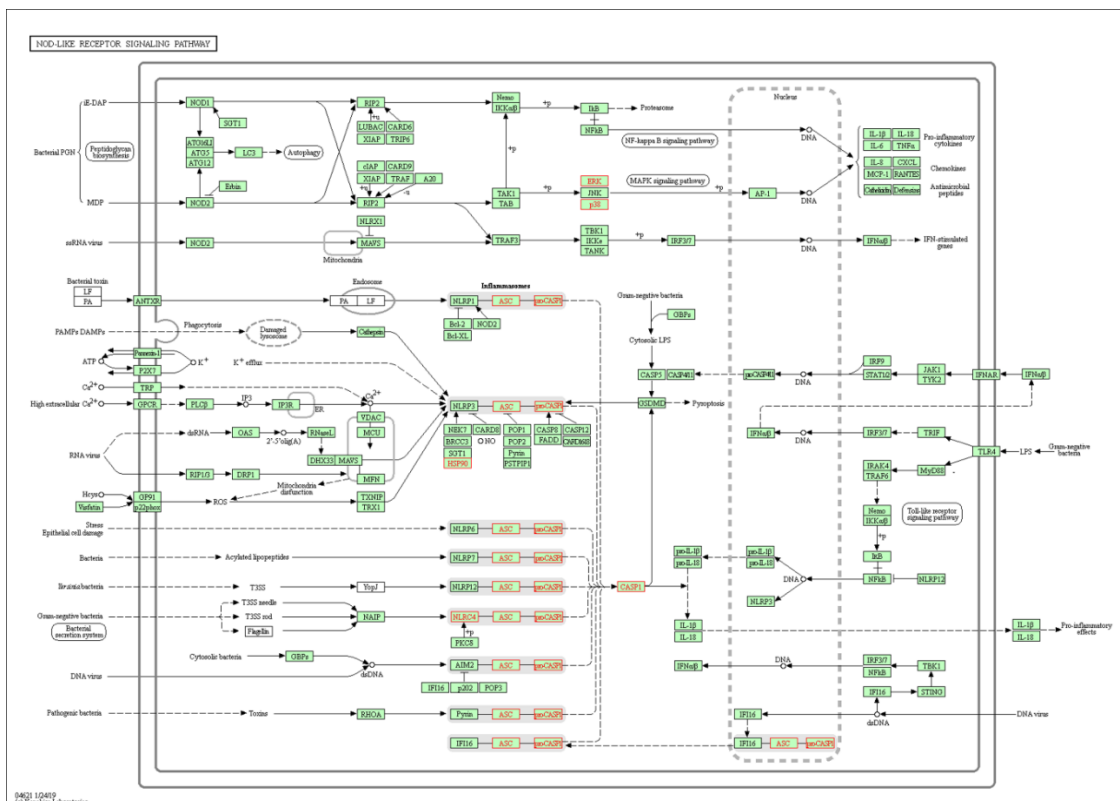


Figure 15 NOD-like receptor signalling pathway with overlaid SDE: IRIS2 vs CTRL2 (Dataset A)

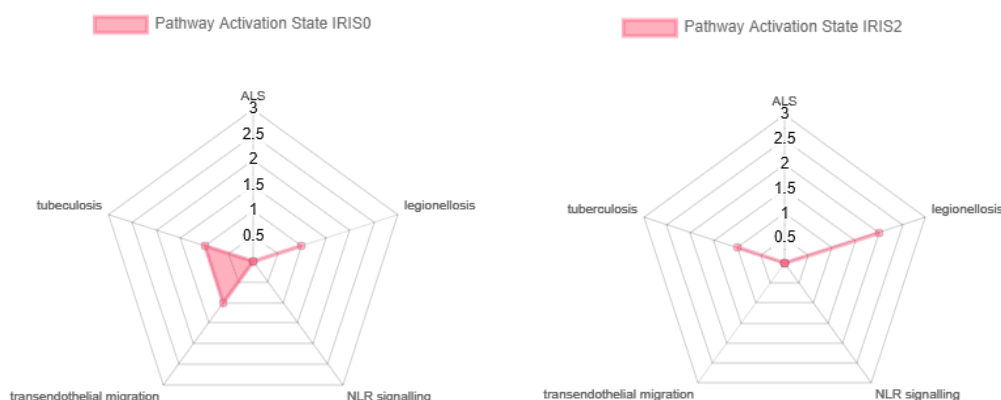


Figure 16 Predicted IRIS0 vs IRIS2 Pathway Activation States. Pathway name "Legionellosis" obscures its molecular nature (TLR and inflammasome signalling).

3.4.6.5. Supplementing Automated Analysis with a Novel Deep Manual Data Integration Approach

The true power of systems biology lies in its ability to effectively *reconstruct* the causal behaviour over time of a subcellular system, cell, tissue, organism, or any combination of these. This requires not only an understanding of every functional module represented, but also of the causal interactions between these functional modules, for example to disentangle which of the many differences between cases and controls are *drivers* of pathology that can inform testable novel hypothesis, and which are *incidental*, or occur as a *result* of pathology. Such high-level integration provides deep insight into the molecular mechanisms underpinning phenotype, and facilitates causal inferences regarding even higher-level events (e.g. inter-cellular interaction). However, especially at the hypothesis-generating level, it also requires extraction of *maximal utility* from a dataset (using as many protein identifications and quantitations to identify and characterise the functional state of as many subcellular pathways as possible), which no automated tool (or combination of tools) is currently able to achieve, despite the functions of most proteins and pathways being available in literature (though not necessarily in easily-queriable databases, in easily-accessible formats, and especially not in an unbiased manner).

The automated tools discussed and employed above are unquestionably useful, but tend to identify the most obvious superficial or broad-level patterns within datasets, perhaps highlighting one or a few functional modules with which to engage with more deeply. These may represent the modules most easily perturbed (i.e. general stress responses) rather than initial changes *causally underlying* pathology. Gene-set/pathway-level topology/activation state analysis (automated sorting of protein data into functional modules and prediction of their functional states) does begin to approach comprehensive understanding of the functional landscape in the system under study (for example, considering multiple evidences pointing in the same direction), but - like any broadly-applicable automated analytical process - has various limitations. Because biological pathways are not truly self-contained (i.e. have no objective boundary, given that different pathways intersect/interact with each other), pathway database construction is - to a certain extent - subjective. This is also true in the sense of pathway member weighting (often not done, or done in a non-transparent manner). By extension, associated statistics are also somewhat subjective (and decisions regarding significance cut-offs are arbitrary), and outputs from such automated tools sometimes predicts pathway activation state incorrectly. Furthermore, such analysis ignores key contextual protein information (which is, in any event, often not available to the researcher without further experimentation) of which we are particularly mindful (*vide infra*).

Publicly available knowledgebases employed by the above automated tools also have their limitations. Just as reference proteome databases are imperfect, so are annotation and pathway knowledgebases, exhibiting content that is: incomplete/fragmented, available only at a delay after

study publication, sometimes inaccurate (including e.g. some information deriving from electronic rather than manually-curated entries), missing condition- or tissue-specific information (e.g. pathway topology), and often biased (e.g. skewed towards well-funded/researched diseases, cell types, and pathways). As an example, consider research from The US National Library of Medicine (NLM), which flags ~all PubMed papers pertaining to gene/protein structure, function, and location [79]. In 2002, this amounted to 565 000 articles describing 27 000 genes (including RNA-coding and pseudogenes), but the top 10 most prolifically-studied genes (each represented by *thousands* of papers) were – unsurprisingly – TP53, TNF, EGFR, VEGFA, APOE, IL-6, TGFβ1, MTHFR, ESR1, and AKT1 [79]. The neutrophil proteome dataset reported in the current study exhibits direct dysregulation of TGFβ1, MTHFR, and AKT1, with multiple indications (possibly due to database skewing) of indirect connections to TP53, TNF, EGFR, VEGFA, APOE1, and IL-6. Interestingly, based on dynamic medical, economic, and political pressures, the popularity of different genes varies over time, with CD4 being popularised by its role in HIV infection, and APOE owing its popularity to roles in cholesterol metabolism and Alzheimer's Disease [79]. Even more tellingly, just 100 of the human genome's protein-coding genes account for over 75% of the NLM-tagged papers, leaving the majority of encoded genes under-studied or not studied at all [79].

Indeed, given the limitations of automated tools and knowledgebases, the most logical conclusions are drawn via (biological) expert interpretation of enrichment and pathway analysis results. This holds especially true with regards to identifying tissue-specific functional modules not appearing in general pathway databases (common for understudied cell types and functional modules), the causal relationships *between* functional modules (which do not act in isolation), and hypothetical extrapolation to overall cell/tissue/organ/organism phenotype (including causal interactions between these high-level entities). There is as yet no substitute for expert examination of the data, but true functional profiling is time-consuming, currently requiring deep manual engagement with both data and literature, and does not provide objective statistical measures of confidence. Most importantly, deep manual analysis makes possible an important type of protein weighting not employed by any automated method: *a focus on key inference-friendly (pathway state watershed) proteins* (e.g. rate-limiting enzymes, protein kinases and phosphatases, and transcription factors) which more easily facilitate functional inference. The manual approach has not yet been automated, but automation (including provision of statistical confidence measures) may be achievable (e.g. with deep learning techniques).

For these reasons as well as those stated below, we have supplemented the above automated analytical methods with a more comprehensive manual data analysis for deep functional profiling – as well as (in imitation of proprietary IPA) integration of these findings with existing literature – in order to generate multiple logical novel hypotheses. Briefly, expression-altered proteins were functionally annotated using GeneCards [80] and manually grouped into nine rational, neutrophil-relevant, and causally-understandable functional modules (largely immunometabolic) represented within the data (Table 8). This was followed by a focus on key inference-friendly proteins to – where possible – predict activation status of these functional modules, whose activities were then integrated to provide a complete mechanistic model accounting for neutrophil phenotype and function in every non-healthy class, facilitating generation of hypotheses specifically regarding TB-IRIS predisposition and pathogenesis. One early surprising finding resulting from this deep manual analysis approach is the significance of nitrogen-sulfur-B-vitamin metabolism in neutrophils. In addition, these are functional modules not commonly or comprehensively captured by traditional pathway knowledgebases, and which are therefore missed by automated analysis.

Table 8 Data-represented Neutrophil-relevant Functional Modules

Immune Modules	Metabolic Modules
major signal transduction pathways (including cAMP-PKA, cGMP-PKG, PI3K-PKB/AKT-mTOR, PLC-IP3/Ca2+/calmodulin-DAG/PKC, MAPK, and AMPK)	
gene expression (including chromatin architecture, RNA processing, and protein translation)	
PRR and cytokine signalling (including inflammasomes)	carbon-energy metabolism (including glycogen metabolism, glycolysis, the PPP, and nucleotide metabolism, the TCA cycle, neutrophil-specific oxidative phosphorylation,

	fatty acid and isoprenoid/cholesterol metabolism, and mitochondrial function/stress)
the eicosanoid cascade	protein homeostasis (including ER protein folding, chaperones, the UPS, the UPR, and autophagy)
neutrophil antimicrobial functions (including inhibitory receptors, recruitment, activation, and myeloid microbicidal activities)	integrated nitrogen-sulfur-B-vitamin and redox/xenobiotic/glyoxal metabolism (including amino acids, SAMe-cysteine-H2S pathways, glutathione functions, and iron metabolism)
cytoskeletal dynamics (including motility and vesicle trafficking)	

Recall that immune and metabolic activity are inextricably, bi-directionally linked as "immunometabolism". **AMPK** AMP-activated protein kinase **mTOR** mammalian target of rapamycin **PRR** pattern recognition receptors **PPP** pentose phosphate pathway **TCA** tricarboxylic acid cycle **ER** endoplasmic reticulum **UPS** ubiquitin-proteasome system **UPR** unfolded protein response.

Although manual analysis cannot provide statistical measures of confidence, convergence of multiple observations within the data on single conclusions is compelling (and may indeed – along with key inference-friendly protein-based weighting/scoring - contribute to future statistical confidence measures should this approach become automated). Furthermore, outcomes of manual analysis agree with and expand upon those of automated analyses and existing literature, enhancing confidence in findings. Results are reported in Chapters 4 and 5.

3.4.7. Clinical Translatability

3.4.7.1. Identification of Novel Druggable Target Candidates Using Network Analysis

In order to further interrogate data, major perturbed pathways may be extracted from literature and compiled into a cellular network model (e.g. using CellDesigner [81]) with dataset findings overlaid [82] [83] [84]. Even before the full complexity of the network can be modelled, analysis of such networks can help identify druggable nodes. Although we have not yet formally performed such an analysis, hypothetical approaches are discussed in greater detail in Chapter 7 to inform future analyses.

3.4.7.2. Identification of Biomarker Candidates Using RandomForest & Evaluation Using ROC Analysis

RandomForest (RF) [85] is a machine learning algorithm that employs bootstrap sampling (random sampling with replacement) [86] to maximise utility of all available data within small sample sizes, in order to decide which item (patient) belongs to which class, and to generate a list of class-discriminatory variable (protein biomarker) candidates.

The algorithm uses study data to construct a 'forest' consisting of many binary decision trees. To grow each tree, a random sample (two thirds of patients) of the data is selected. At each node (branch-point) of the tree, a small random subset of all dataset gene products is selected, and the gene product which performs best in classifying patients is used as the 'splitter variable'. The size of this small random subset is fixed throughout the process as splitting of tree branches continues using additional classification-capable gene products randomly selected from the remaining dataset gene products, until all terminal tree leaves represent only one of the comparison classes. The tree is then 'internally validated': used to predict classifications for the remaining one third of patients (each patient goes down one branch or another depending on their expression values for each node's splitter gene product until it reaches a terminal leaf); the tree is said to cast a 'vote' on these patients' classes. A new random two thirds-one third patient division repeats the process to build each additional tree. The desired number of trees in the forest can be set *a priori* (e.g. at 200), and "the number of input variables tried for each node is the square root of the number of total variables" [87]. Ultimately, each individual patient contributes to growing ~two thirds of the trees, and is classified by the other ~one third. Essentially, each tree is trained on a random two thirds of patients, and used to classify the other third.

The fraction of votes for one class or another is an estimate of the probability that that patient belongs to that class, and by comparing such estimates to *a priori* known patient class labels, forest accuracy can be determined (an accurate forest can be used to classify independent datasets, e.g. consisting of patients with unknown class). How often a gene product is used as the splitter variable, the higher its 'variable importance'; this can be used to determine which gene products are good candidates as

discriminatory biomarkers. One commonly-used measure of variable importance (relative contribution to predictive accuracy) is the Gini score: for each variable, it represents “the increase in prediction error if the values of that variable are permuted across” the one third of remaining observations [87]. Per-tree Gini is computed before averaging over the entire forest, and dividing by the forest’s standard deviation (Gini scores range between one and 100; the larger, the higher the variable’s importance).

The RF method is “simple, fast, stable, and insensitive to noise”, exhibits little to no overfitting, and automatically compensates for biased sample sizes [86]. It has performed well for example in microarray and single nucleotide polymorphism (SNP) studies [86]. When compared to other commonly-used classification tools – e.g. projection to latent structures (PLS), linear discriminant analysis (LDA), and support vector machine (SVM) learning – in classifying healthy participants versus colorectal cancer patients in a clinical metabolomics study, RF performs best (in terms of overfitting, receiver-operator characteristic (ROC) curve analysis, variable reduction, and Pearson correlation) during biomarker selection [86]. Decision trees do possess certain limitations, for example when data are highly skewed or class sizes are highly unbalanced (>5:1); in the latter case it favours accurate prediction only for the larger/dominant class (although this can be mitigated by random sampling from the dominant class to match the size of the smaller class, by adjusting class weighting to account for the imbalance, and by using a stratified sampling constraint to force ~equivalent sampling from both classes).

Luckily, such problems are not characteristics of the current dataset. Here we have used the R packages `randomForest` [87] and `ROCR` [88] to identify class-discriminatory biomarker candidates and assess their within-dataset performance. Only proteins in the overlap between SDE proteins and RF-predicted discriminators were taken forward for performance assessment. Well-performing biomarkers were then used to construct heatmaps (including hierarchical clustering) to roughly visualise their ability to classify samples (we are mindful that this approach does not properly take into account the combinatorial power of biomarkers – *vide infra*).

ROC analysis was devised during World War II, driven by the parallel emergence of signal detection theory and use of radar [89]. It originated after the Pearl Harbour attack during the effort to understand “how U.S. radar had failed to detect Japanese aircraft” [90]. Radar was being used to detect enemy aircraft, but would also detect noise – e.g. clouds, birds – that mimicked the presence of aircraft [89]. With gain set to zero, radar is not sensitive enough to detect true signals, while increasing gain increases identification of both true signals and noise (there is an overlap between the distribution of airplane signals and non-airplane signals) [89]. This produces a trade-off situation: at a certain point in gain increase, noise begins to outweigh signal [89]. No matter where the gain cut-off point is set, some aircraft will still be missed, and some non-aircraft will still be detected [89].

However, as long as an independent and accurate assessment of each signal (i.e. whether it is indeed an aircraft or not) is available, the sensitivity and specificity at each possible cut-off point can be calculated to determine which produces the optimal trade-off [89]. Plotting these values (x-axis: 1 – specificity, y-axis: sensitivity) produces the ROC curve, in which the lower left corner is analogous to gain set to zero (no signal pick-up) [89]; incidentally, plotting the false positive rate (x-axis) against the true positive rate (y-axis) produces an identical curve [90]. This curve visualises how well a certain signal detector (or diagnostic test) discriminates between signals (including signals indicating pathology). Indeed, it is commonly used in radiology and other clinical diagnostic contexts. The diagonal line represents results that would arise by chance (i.e. correct classification ~50% of the time in a binary classification system): if the plotted ROC curve falls above this line, discrimination is better than that expected by chance, and vice versa.

The hypothetical ‘perfect’ test (i.e. perfect discrimination at any cut-off point) would yield a curve that runs directly up the y-axis to the top, and then horizontally across (which also yields maximal area under the curve (AUC)) [89]. In this case, AUC (actually equivalent to the output of the Wilcoxon or Mann–Whitney U test) represents accuracy of the test: “the probability that the test will yield a higher value for a randomly chosen individual with the disorder than for a randomly chosen individual [without] the disorder” [89]. As a general guideline, an AUC of 0.5-0.7 represents low accuracy, 0.7-0.9 represents moderate accuracy, and >0.9 represents high accuracy [89]. An AUC = 1 implies a perfect ROC curve

(all predicted 'positive' samples are associated with higher signal values than all predicted 'negative' samples), but *not* perfect discrimination between classes. Furthermore, comparing two different ROC curves which cross at any point, however, requires use of partial AUC (pAUC) [89]. Indeed, ROC curves can be applied whenever there is a question regarding whether "a set of numerical values can predict a set of binary outcomes" [88], and are particularly useful for evaluating outputs from machine learning approaches such as logistic regression, RF, SVM, etc.

Sensitivity and specificity of any test are always a trade-off: as one increases, the other decreases [89]. A good diagnostic test has both high sensitivity and high specificity [89]. Clearly, class prediction depends on the chosen cut-off point. Although the cut-off point is often chosen to minimise the overall number of errors (i.e. false negatives (FN) and false positives (FP)), the costs of making an FN or FP mistake within the particular context in question must be considered [89]. For example, for very common and serious disorders which can be relatively easily treated (e.g. TB) and should be treated as early as possible, it may be worth having higher sensitivity and lower specificity. In order to select the best fit-for-purpose cut-off point, three curves should be plotted: ROC, recall-precision, and calibrating (resulting ROC curves for predicted candidate biomarkers are reported Chapters 4 and 5). As an especially clinically-useful statistical alternative to sensitivity and specificity, likelihood ratios can also be used to quantify diagnostic accuracy [91].

It is possible to boost diagnostic test performance by combining highly-performing biomarkers into a single panel with improved sensitivity and specificity, but this requires combinatorics (including considerable computational power) to assess their combined discriminatory performance (e.g. using combiROC [92]). An examination of which proteins co-vary – plotting all-versus-all expression associations – also contributes to identification of biomarkers which are unlikely to exhibit additive combinatorial value. We have used R package corplot [93] for this purpose (data not shown).

It is worth briefly considering the clinical characteristics of an ideal clinical biomarker/diagnostic test beyond appropriate sensitivity and specificity, in order to focus on validating and developing only the most promising identified candidate biomarkers, and only in contexts where the pathology lends itself to biomarker-based predictions. Ideal biomarker characteristics generalizable to the TB-IRIS context include: peripheral (non-invasive) accessibility (e.g. blood or urine), reliable detectability early in the course of disease (but persistence throughout the disease course) – ideally as a result of a known pathophysiological mechanism, and correlation with severity of disease [94]. Biomarkers should also: be confined to a narrow physiological range, be specific to the condition of interest (i.e. should not also be affected by other common disorders), and should perform consistently across clinical populations (e.g. regardless of gender, race, co-morbidities, and co-treatments). Regarding diagnostic tests, the condition must firstly represent a significant healthcare problem within the population of interest, and it must be in some way difficult to predict, diagnose, or monitor clinically (e.g. having a latent/asymptomatic stage, or being a diagnosis of exclusion) [95]. Secondly, facilities should be available for ongoing case-finding, diagnosis, and treatment (preferably within a well-established national policy for that condition) [95]. Thirdly, the test must be acceptable to patients (i.e. non-invasive, in agreement with cultural norms – e.g. a urine/blood-based point-of-care lateral flow assay), and must successfully identify patients who will benefit from treatment [95] [96]. Fourthly, a positive result must inform treatment, and effective treatment (also acceptable to the patient) must be available [96]. Finally, the diagnostic test and treatment must be economically balanced relative to overall national healthcare budget considerations [95] (e.g. avoidance of unnecessary over-diagnosis). Ultimately, the utility of a given diagnostic test depends on whether it can guide treatment decisions in a way that improves patient health outcomes [96] but does not require unrealistic healthcare resources. A set of generic criteria termed 'ASSURED' (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users) can guide selection of "appropriate diagnostic tests for resource-constrained settings", but should be adapted to the specific needs of each context [97].

Once candidate biomarkers have been selected (or a diagnostic test has been developed), they must be validated on an independent (and preferably larger) cohort to assess generalisability of utility. Prior

to undertaking the expense and effort required to develop and validate a diagnostic test, it is also possible to validate biomarkers on pre-existing independent datasets within the context of interest. In our case, we may be able to use the following pre-existing TB-IRIS datasets (although they do not all derive specifically from neutrophils, it is likely that at least some neutrophil-derived proteins or their corresponding transcripts will be present in circulation/urine, or that the overall participant condition will induce similar responses in other phagocytic leukocytes or even lymphocytic PBMC): dataset A from this study, a proteomic analysis of isolated PBMC [98] a whole-blood transcriptomic profiling study [99], and monocyte transcriptomic/whole gene expression profiling studies [100] [101]. A similar approach can be taken – with relevant extant datasets - to help validate biomarkers identified for discrimination of partially ATT-treated TB/HIV-coinfection from healthy volunteers, which could perhaps uncover helpful biomarkers for diagnosing active TB on a background of HIV (paucibacillarity usually makes conclusive diagnosis more difficult). Biomarkers are not only useful for risk stratification/diagnostic/prognostic/treatment monitoring purposes, but can also provide useful surrogate end-points for clinical trials.

Finally, the performance and utility of candidate biomarkers/diagnostic tests must be evaluated via clinical trials, with sensitivity and specificity reported along with [102] their 95% confidence intervals. In the case of TB-IRIS, such evaluation would currently occur in the absence of a 'gold standard' diagnostic test, and must, accordingly, adhere to relevant guidelines [102].

3.4.8. Human Proteins Never Before Demonstrated to be Expressed by Neutrophils or Never Before Empirically Observed

We first compiled a list of the most recent high-quality transcriptomic and proteomic human neutrophil studies (n = 20) up to 2017 [103] [104] [16] [105] [106] [107] [108] [109] [110] [111] [112] [113] [114] [115] [116] [117] [118] [119] [120] [121] with data freely available from public repositories, filtered these datasets for the highest-quality identifications, and downloaded the corresponding human proteins from UniProt to compile the most comprehensive neutrophil-specific protein database (derived from 13 561 unique transcripts and 3907 unique proteins) to date (named "NeutroFile"). When compared to NeutroFile, a 2017 preliminary analysis of only local healthy volunteer neutrophil proteomic data (N = 10) identifies multiple proteins apparently never before observed to be expressed by neutrophils (fig.s 17 and 18), including a few additional 'missing' proteins (*vide infra*): OXER1 and ZNF560.

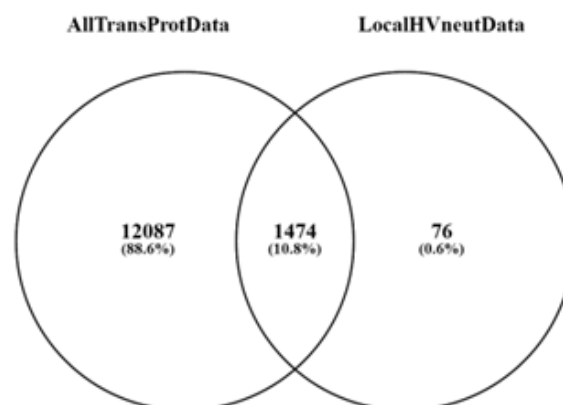


Figure 17 Local Neutrophil Proteomic vs. Existing Neutrophil Transcriptomic Data
The local healthy volunteer neutrophil proteome exhibits 76 unique proteins, including for example HRAS precursor (involved in the activation of Ras protein signal transduction) and ARNT2 (aryl hydrocarbon receptor nuclear translocator 2, about whose function in neutrophils little is known).

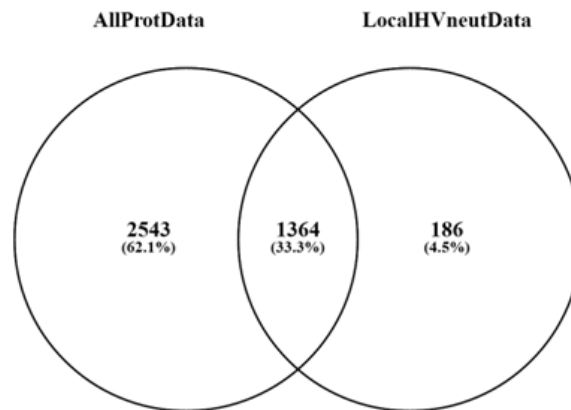


Figure 18 Local Neutrophil Proteomic vs. Existing Neutrophil Proteomic Data

The local healthy volunteer neutrophil proteome exhibits 186 unique proteins, including for example NOX3 (NADPH oxidase 3, which produces superoxide upon formation of a complex with CYBA/p22phox) and GLUD2 (mitochondrial glutamate dehydrogenase 2, involved in glutamate recycling).

Approximately 82.4% of human genes currently have products with a PE1 assignment: experimental evidence at the protein levels [122]. The remaining 14.7-18% of predicted but unobserved proteins (HPP/neXtProt PE2-4, inclusive) have been dubbed 'missing' [122]. These comprise mainly transmembrane G-protein-coupled receptors (GPCRs), the largest family of human proteins (with >800 members) as well as being common pharmaceutical targets [122]. Five major GPCR sub-families exist: rhodopsin, secretin, adhesion, glutamate, and Frizzled/taste; most missing proteins hail from the rhodopsin group (especially the olfactory receptors, probably due to a lack of relevant tissue samples) [122]. Other missing protein groups include: zinc finger, coiled-coil domain, homeobox, keratin-associated, ankyrin-repeat- and death-domain-containing, leucine-rich repeat-containing, transcription factors, cadherins, sperm-related, testis-expressed, endogenous retroviral, solute carrier family, nuclear, ubiquitin-like, beta-defensins, and PRAME family, among others [122]. Many of these may be of clinical/pharmacological utility [123].

The MissingProteinPedia (<http://www.missingproteins.org>), a low-stringency communal-submission database, gathers all PE2-4 proteins into a central, queriable/sortable knowledgebase to accelerate coverage of the missing proteins through encouraging researchers to submit new as well as not-yet-public legacy data [122]. Additionally, ProteomicsDB includes an 'adopt-a-protein' feature to encourage follow-up on missing proteins [124]. To achieve coverage of the final 10% of the proteome (and indeed for optimal-depth coverage in any proteomics experiment), researchers must take into account the reasons why some proteins may not be easily detectable/observable (e.g. present only in highly-specialised/rare tissues, expressed only at very low quantities/certain times, expressed only in response to rare stimuli, insoluble/not amenable to tryptic digestion, peptides which do not efficiently ionise or are "outside observable mass range detection settings", non-identification of high-quality spectra (including bioinformatic factors discussed in sections 7 and 9), proteins possibly "retired by evolution", and additional factors we may not yet have considered) [125] [126] [122]. Observing such proteins may require novel research approaches (e.g. better subcellular/class enrichment, more extensive pre-fractionation, etc.) [122].

While Kim et al. (2014) detected ~84% of human protein-coding gene products via label-free quantitation of normal tissues [125] [127] and Wilhelm et al. (2014) detected ~92% of human protein-coding gene products via new MS experiments combined with existing public datasets [125] [124], these two groups have been criticised for non-compliance with communal stringency criteria – e.g. FDR consensus, minimum proteotypic peptide length – imposed by HPP (and because they reported olfactory receptors even though nasal tissues were not analysed [128]). However, it has equally been argued that such strict metrics preclude identification of some bioactive peptides/proteins [122] (e.g. several neuropeptides and other inter-cellular signalling peptides are shorter than nine amino acids long, and proteins not amenable to tryptic cleavage will never yield at least two proteotypic peptides at least nine amino acids in length) [122]. Furthermore, "the availability of two independent datasets facilitates some degree of cross-validation" [125]. Given their extant stringency criteria, it is projected

that HPP will achieve PE1 status of $\geq 95\%$ of remaining proteins around 2020, while extrapolations from the more stringent PeptideAtlas criteria suggests a timeline closer to 2030–40 [122]. The present neutrophil proteomic dataset putatively observes three currently missing proteins (Table 9), including a very likely-real identification: SLC35A4 (a solute carrier which likely transports UDP-galactose).

Table 9 Putatively Observed Missing Human Neutrophil Proteins

Protein	Dataset	Database	# Unique Peptides	Unique Seq. Coverage (%)	Seq. Length (aa)	# Samples ID'd by MS/MS	# Samples with Non-zero Raw Intensity Value	**Spectral quality	Do peptides match any other proteins?	Likely Real?	*Sufficient evidence to report?
SLC35A4	A	SwissProt	2	14.6	103	3/37	34/37	good	N	Y	Y
KATNAL2	A	trEMBL	1	9.4	128	18/37	6/37	moderate	N	maybe	N
VWA3B	B	trEMBL	1	9.5	105	10/90	7/90	moderate	N	maybe	N

*The human proteome project (HPP) requires MS-based protein identification via a minimum of two uniquely-mapping, non-nested peptides of at least nine amino acids each; peptide **spectral quality components include high mass-accuracy, high signal-to-noise ratio, absence of typical signs of misidentification, and consideration of potential alternate explanations for the peptide-spectral match [129].

Appendix H BCA Results

Preparation Batch	Clinical Sample Code	Class	Laboratory Sample Code	BCA Result (µg/µl)
1	PA273	IRISO	1	1.143
	PA211	IRISO	2	1.123
	TD251	IRISO	3	1.194
	PA151	CTRL0	4	1.264
	PA246	CTRL0	5	1.120
	PA201	CTRL0	6	1.078
	PA141	IRIS2	7	1.177
	TD223	IRIS2	8	1.248
	TD217	IRIS2	9 (80µl)	0.896
	PA140	CTRL2	10	1.098
	PA177	CTRL2	11	0.974
	PA252	CTRL2	*12	1.207
2	TD217	IRISO	13	1.026
	TD221	IRISO	14	0.998
	TD244	IRISO	15	0.917
	PA250	CTRL0	16	1.156
	TD245	CTRL0	*17	1.635
	PA261	CTRL0	*18 (150µl)	1.913
	TD260	IRIS2	19	1.128
	PA210	IRIS2	20	1.237
	PA154	IRIS2	21	1.161
	TD245	CTRL2	*22	1.245
	PA201	CTRL2	23	1.111
	TD224	CTRL2	24	1.046
3	TD243	IRISO	25	1.060
	PA244	IRISO	26	1.190
	TD218	IRISO	27	1.089
	PA257	CTRL0	*28	1.310
	TD208	CTRL0	29	1.285
	PA193	CTRL0	30	1.143
	TD218	IRIS2	31	0.990
	TD220	IRIS2	32	1.040
	PA147	IRIS2	33	1.127
	PA206	CTRL2	34	1.180
	TD142	CTRL2	35	1.536
	TD261	CTRL2	*36	1.321
4	TD225	IRISO	37	0.935
	PA241	IRISO	38	1.006
	PA245	IRISO	39	0.960
	PA205	CTRL0	40	1.178
	PA146	CTRL0	*41	1.460
	PA251	CTRL0	42	1.202
	TD203	IRIS2	43	1.036
	PA244	IRIS2	44	1.137
	TD195	IRIS2	45	1.044
	TD219	CTRL2	46	0.964
	PA261	CTRL2	47	1.029
	PA250	CTRL2	*48	1.426
5	PA208	IRISO	49	1.130
	PA285	IRISO	50	0.990
	TD250	IRISO	51	1.000
	PA251	CTRL0	*52	1.370
	TD246	CTRL0	53	1.186
	TD224	CTRL0	54	0.870
	TD252	IRIS2	55	0.930
	PA196	IRIS2	56	1.140
	PA204	IRIS2	57	1.310
	TD246	CTRL2	58	1.000
	PA206	CTRL2	59	1.120
	PA135	CTRL2	*60	1.300
6	PA256	IRISO	61	1.260
	TD223	IRISO	62	1.060
	PA260	IRISO	63	0.920
	PA269	CTRL0	64	0.820
	PA202	CTRL0	65	1.240
	PA155	CTRL0	66	1.190
	PA263	IRIS2	67	0.940
	PA256	IRIS2	68	1.210
	TD221	IRIS2	69	0.970
	TD222	CTRL2	70	0.980
	TD235	CTRL2	71	1.230
	PA144	CTRL2	*72	1.320
7	PA248	IRISO	73	0.810
	PA272	IRISO	74	1.120
	TD257	IRISO	*75	1.400
	TD235	CTRL0	76	1.020
	PA157	CTRL0	77	0.980
	PA264	CTRL0	78	1.040
	PA255	IRIS2	79	1.020
	PA267	IRIS2	80	1.030

	PA270	IRIS2	81	0.950
	PA269	CTRL2	*82 (1.5ml)	1.400
	PA178	CTRL2	83	1.140
	TD242	CTRL2	*84	1.240
9	PA266	IRISO	85	0.854
	PA143	IRISO	86	0.934
	PA259	IRISO	87	1.061
	TD142	CTRL0	88	0.747
	PA294	CTRL0	89	1.025
	PA292	CTRL0	90	0.900
	PA272	IRIS2	91	0.870
	PA253	IRIS2	92	0.734
	PA247	IRIS2	93	0.862
	PA289	CTRL2	94	0.825
	PA146	CTRL2	95	0.731
	PA257	CTRL2	96	0.915
	PA263	CTRL2	97	0.787
	PA274	IRISO	98	1.112
	PA175	IRIS2	103	0.989
	PA182	IRIS2	104	0.989
	PA278	IRISO	*99	1.380
	PA188	IRISO	100	1.380
9	PA210	IRISO	101	1.100
	PA258	IRISO	102	0.810
	PA266	IRIS2	105	1.060
	PA275	IRIS2	106	1.070
	PA260	IRIS2	107	0.870
	TD257	IRIS2	108	1.060
	PA275	IRISO	109	0.900
	PA280	CTRL2	*110	1.300
	PA286	CTRL2	111	0.830
	PA207	IRISO	112	1.050
	PA267	IRISO	113	1.060
	PA148	IRISO	114	1.160
	PA187	IRISO	115	1.510
	PA282	IRISO	116	1.220
	PA204	IRISO	117	1.117
	PA185	IRISO	118 (1M cells in 300µl)	0.240
	PA195	IRIS2	119	1.965
	PA192	IRIS2	120	1.130
10	PA191	IRIS2	121	1.290
	PA147	IRISO	122	0.669
	PA147	IRIS2	123	1.000
	TD251	IRISO	*127	1.960
	TD222	CTRL0	128	0.720
	PA289	CTRL2	131	1.023
	Average BCA result (protein µg/µl)			1.107 ± 0.220
	Average protein yield (µg) per 5x10 ⁶ neutrophils in 100µl/vial			111

Outlying BCA values are due to limiting/overabundant neutrophil numbers in lower/higher volumes, indicated in parentheses, or frank *rifampicin staining of the sample. Apparently less-ordered randomisation categories in the final few batches is due to samples being prepared prior to PredART study unblinding.

Appendix I PTM Analysis Results

Please note that - as discussed above - this experiment was not optimized for PTM peptide enrichment, and thus quantitation of low-abundance covalently-modified species is likely to be somewhat inaccurate. However, PTM data resulting from this study are intended only as preliminary explorations of novel context-based hypotheses (rather than final data on which to base inferences). Therefore, no actual mass spectra are presented, pending validation through targeted analyses.

Phagolysosomal PTMs

Full dataset A (N = 37) analysis identifies only three apparently-chlorotyrosinated proteins (non-identical to those identified above, i.e. in participants for whom samples from both time-points were available) meeting stringency criteria, but only a single trEMBL entry (ANKZF1, with a role in maintaining mitochondrial integrity under conditions of oxidative stress) was quantifiable in near-sufficient patient samples (4/10 CTRL0, 5/9 IRIS0, 7/11 CTRL2, 2/7 IRIS2) to facilitate analysis of quantitative change during ARV therapy (fig. 1). Although observations suggest that ARV therapy may have differential effects on chlorotyrosination in CTRL and IRIS groups, trends are non-significant.

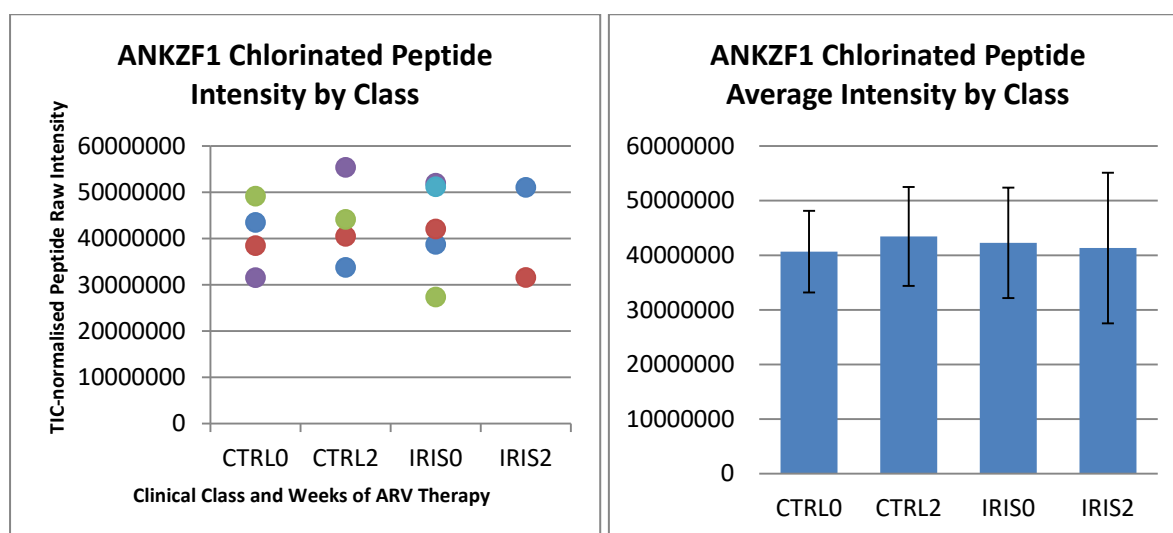


Figure 1 Putative Changes in ANKZF1 Chlorinated Peptide Intensity During ARV Therapy

Similarly, only seven apparently-nitrosylated proteins (non-identical to those identified above) met stringency criteria, but only two trEMBL entries (ubiquitin-like protein modifier UBD and centrosomal protein CEP128 fragment) were quantifiable in sufficient patient samples (UBD: 9/10 CTRL0, 8/9 IRIS0, 11/11 CTRL2, 7/7 IRIS2; CEP128: 7/10 CTRL0, 7/9 IRIS0, 10/11 CTRL2, 7/7 IRIS2) to facilitate analysis of quantitative change during ARV therapy (fig. 2). Although observations suggest that ARV therapy may result in decreased nitrosylation in both CTRL and IRIS groups, trends are non-significant. It is notable that UBD regulates multiple immune components implicated in TB-IRIS: TNF α -induced NF κ B activation, the response to IFN γ , and caspase-dependent apoptosis, among others.

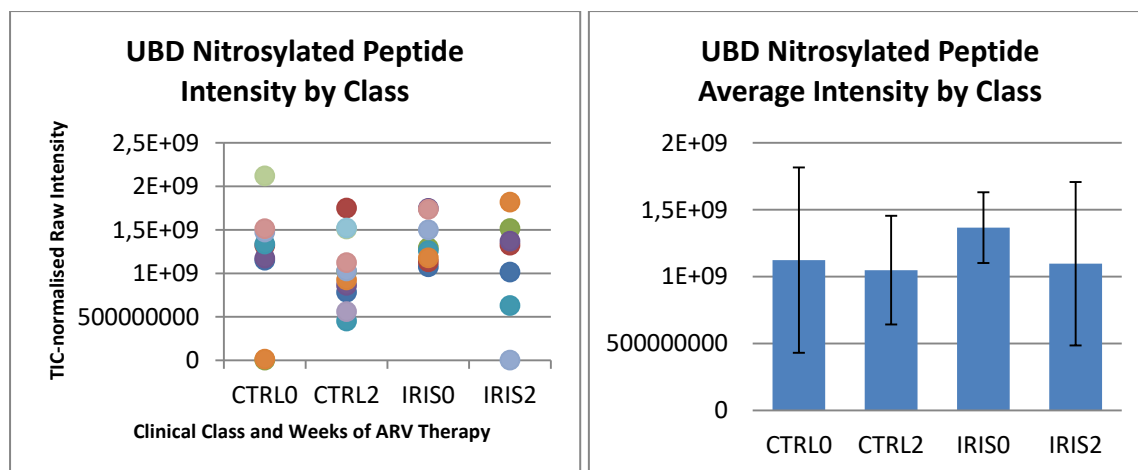
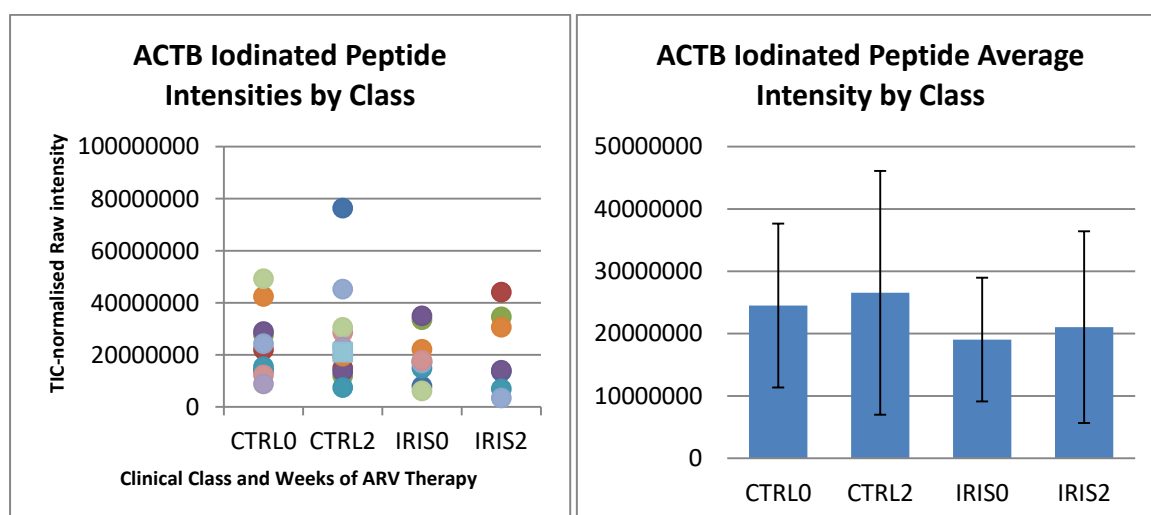
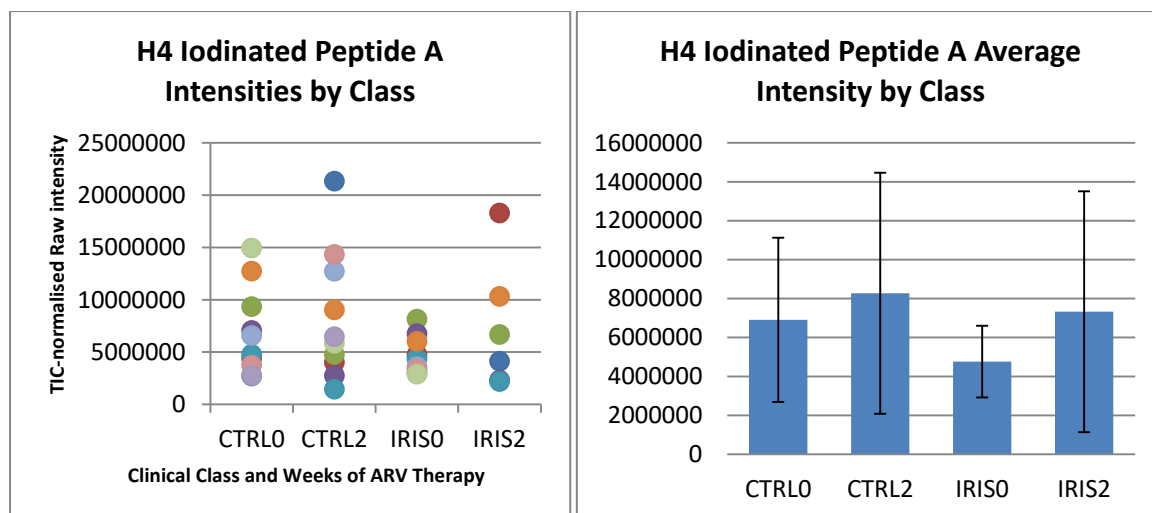


Figure 2 Putative Changes in UBD Nitrosylated Peptide Intensity During ARV Therapy

However, 15 apparently-iodinated proteins (some identical to those identified above: LYZ, HIST1H4A, HBA1, and many being known phagolysosomal enzymes) met stringency criteria, and 13 of these (POTEF, MPO, S100A9, INHBB, RNASE2 and 3, EPX (eosinophil peroxidase), HIST1H4A and H1 (histone H4 A and B), ACTB, and HBA1) were SwissProt identifications (one of the trEMBL entries was LTF (lactotransferrin)). Many (RNASE3, ACTB, H4A and B, HBA) were quantifiable in sufficient patient samples (RNASE3: 8/10 CTRL0, 5/9 IRIS0, 5/11 CTRL2, 3/7 IRIS2; ACTB: 10/10 CTRL0, 9/9 IRIS0, 11/11 CTRL2, 7/7 IRIS2; H4A: 10/10 CTRL0, 9/9 IRIS0, 10/11 CTRL2, 6/7 IRIS2; H4B: 8/10 CTRL0, 6/9 IRIS0, 10/11 CTRL2, 7/7 IRIS2; and HBA: 10/10 CTRL0, 6/9 IRIS0, 11/11 CTRL2, 6/7 IRIS2) to facilitate analysis of quantitative change during ARV therapy (fig.s 3 and 4). Although observations suggest that ARV therapy may result in increased iodination in both CTRL and IRIS groups, trends are non-significant.





Figures 3 and 4 Putative Changes in ACTB and H4A Iodinated Peptide Intensity During ARV Therapy

Full dataset B (N = 74) analysis identifies only four apparently-chlorinated proteins (FAM105A, RNF220, NSD2, and FMNL2), only six apparently-nitrosylated proteins (**UBD**, TRIM66, FAM105A, **CEP128**, CTSD, and KIAA1456), and 19 apparently-iodinated proteins (HBD, LTF, GAPDH, S100A8, **MPO**, **S100A9**, ENO1, HIST1H1E, HSPA8, **EPX**, HIST1H1B, SERPINB1, PGD, **HIST1H4A**, ACTG, ACTA, **HBA1**, MRM2, CCDC151) meeting stringency criteria (though most peptides are identified by matching rather than by MS/MS). Proteins indicated in bold were also identified as being modified in independent dataset A.

Thus, both datasets A and B exhibit only few putative chlorotyrosinated and nitrosylation protein identifications meeting stringency criteria (though UBD and CEP128 are identified as being nitrosylated in both). Full proteomic functional analysis (Chapters 4 and 5) suggest relative under-utilisation of the phagolysosomal respiratory burst in all clinical classes (and TB-IRIS literature suggests decreased NO production), which is consistent with a lack of chlorotyrosinated and nitrosylated proteins. Alternative possible non-biological reasons include such peptides being present at lower abundance, site occupancy being low (requiring pre-enrichment), or type II error (erroneous exclusion of genuine modified protein identifications). There is also the possibility that different phagolysosomal-specific modifications occur at differing rates, because multiple putative iodinated proteins (many of which are known phagolysosomal enzymes) are exhibited by both datasets (with MPO, S100A9, EPX, HIST1H1B and E, HBA1, and LTF identified as being iodinated in both). Perhaps iodination is somehow less dependent on a functional respiratory burst than the other phagolysosomal modifications.

Ubiquitination

Full dataset A (N = 37) analysis identifies 16 apparently-diglycine-tagged proteins (some identical to those identified above; i.e. for participants for whom samples from both time-points were available) meeting stringency criteria, of which nine (STXBP3, USP9Y, TLL1, BMP3, MRC1, RGS6, SPTBN1, FSIP2, SQOR) were SwissProt entries. Three apparently modified proteins (USP9Y, BMP3, MRC1) were quantifiable in sufficient samples (USP9Y: 10/10 CTRL0, 9/9 IRIS0, 11/11 CTRL2, 7/7 IRIS2; BMP3: 7/10 CTRL0, 8/9 IRIS0, 11/11 CTRL2, 6/7 IRIS2; MRC1: 10/10 CTRL0, 9/9 IRIS0, 11/11 CTRL2, 7/7 IRIS2) to facilitate analysis of quantitative change during ARV therapy, but only USP9Y modified peptides were identified mainly by MS/MS rather than matching (fig. 5; the other two follow similar patterns of differential change between CTRL and IRIS during ARV exposure, however). Although observations suggest that ARV therapy may result in differential ubiquitination changes between CTRL and IRIS groups, trends are non-significant. It is notable that USP9Y is a probably ubiquitin carboxyl-terminal hydrolase, and that MRC1 is macrophage mannose receptor (MR) 1 (a C-type lectin).

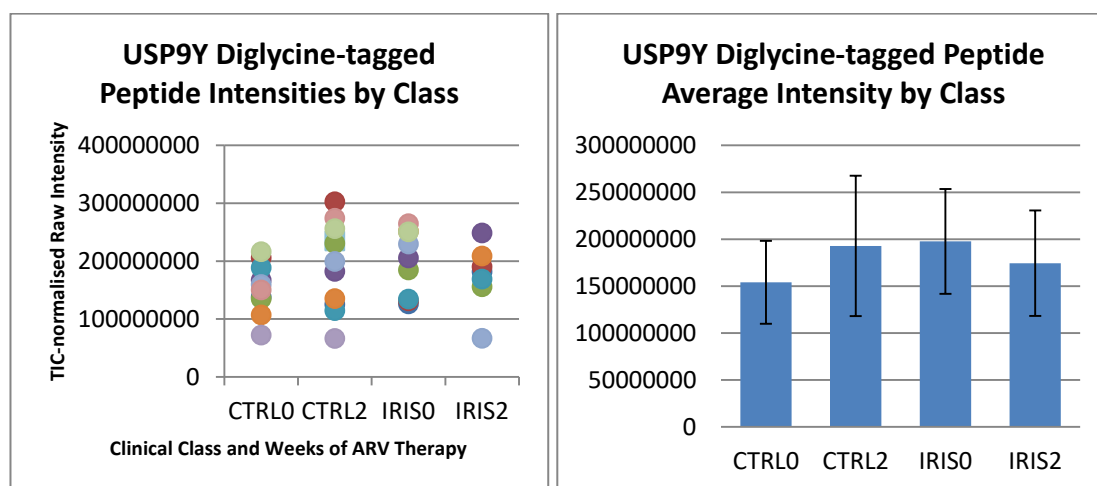


Figure 5 Putative Changes in USP9Y Ubiquitinated Peptide Intensity During ARV Therapy

Full (i.e. not just post-translational modification) proteomic data analysis indeed suggests increased UPS flux. Indeed, in both the (non-independent) dual-timepoint dataset (i.e. participants for which samples are available from both time-points) and dataset A, some putative ubiquitinated protein identifications (one of which itself is a ubiquitin-like modifier) meet stringency criteria, with USP9Y being comparable across multiple patients in both datasets. Dual-timepoint data potentially suggest that the abundance of a USP9Y ubiquitinated peptide increases in both classes during ARV exposure, while dataset A data potentially suggest an increase in the CTRL but a decrease in the IRIS class during ARV exposure. Certain putative ubiquitinated protein identifications meeting stringency criteria are common between the dual-timepoint dataset and dataset A: USP9Y and BMP3.

Citrullination

Full dataset A (N = 37) analysis identifies 16 apparently-citrullinated proteins (some identical to those identified above: USP9Y, MYCTU_THYX, SPTBN1) meeting stringency criteria, of which nine (USP9Y, HDC, MYH9, MYCTU_THYX, MYCTU_Rv2250c, SPTBN1, CBX2, OR4K2, and MTIF3) were SwissProt entries. Two apparently-modified Mtb proteins (MYCTU_THYX, MYCTU_Rv2250c) were quantifiable in sufficient samples (THYX: 9/10 CTRL0, 6/9 IRIS0, 10/11 CTRL2, 5/7 IRIS2; Rv2250c: 7/10 CTRL0, 9/9 IRIS0, 10/11 CTRL2, 6/7 IRIS2) to facilitate analysis of quantitative change during ARV therapy, but only MYCTU_THYX peptides were identified mainly by MS/MS rather than matching (fig. 6). Although observations suggest that ARV therapy may result in increased citrullination in both CTRL and IRIS groups, trends are non-significant. It is notable that MYCTU_THYX is involved in dTT biosynthesis (pyrimidine nucleotide metabolism): it catalyses methylation of dUMP to dTMP, using MTFH as a methyl donor, and NADPH/FADH2 as reducing agents. Indeed, Chapter 4 and 5 full proteomic findings suggest altered host purine/pyrimidine nucleotide and folate/methylation metabolism.

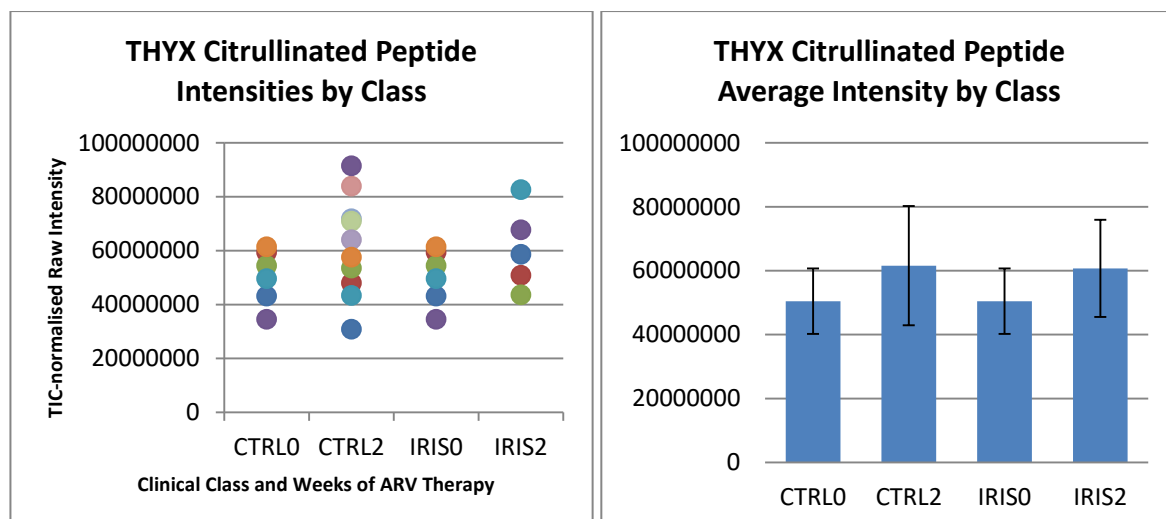


Figure 6a Putative Change in Citrullinated Mtb THYX Peptide Intensity During ARV Therapy

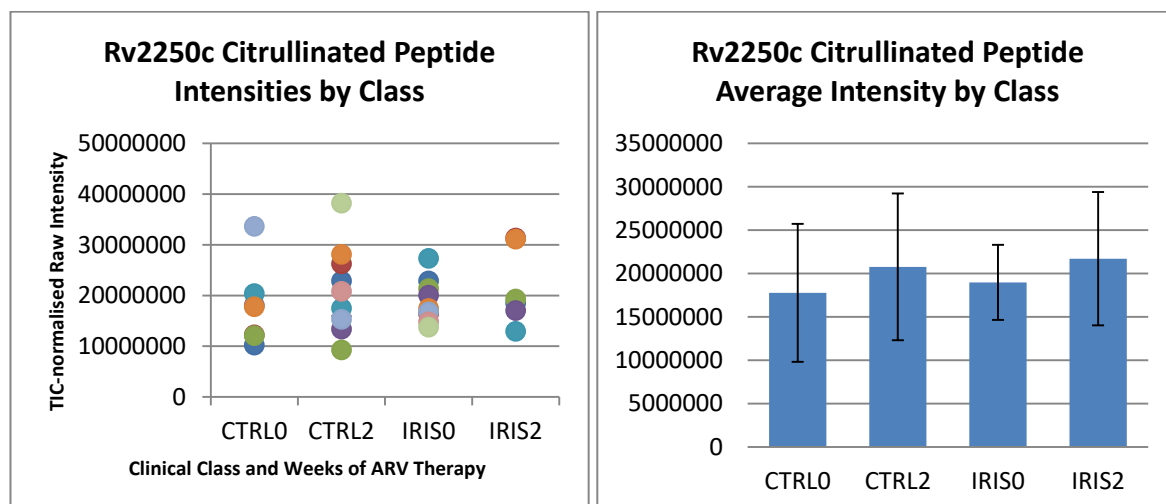


Figure 6b Putative Change in Citrullinated Mtb Rv2250c Peptide Intensity During ARV Therapy

Literature-derived hypotheses concerning inflammation-induced NETosis suggest that protein citrullination may be elevated in the neutrophils of TB-IRIS developers. Indeed, in both the dual-timepoint dataset and dataset A, multiple putative citrullinated protein identifications (including of putative mycobacterial proteins) meet stringency criteria, with one (Mtb THYX, involved in pyrimidine metabolism) being comparable across multiple patients in both datasets. Dual-timepoint data potentially suggest that the abundance of a THYX citrullinated peptide increases in both classes during ARV exposure, and dataset A findings agree (as well as suggesting the same for the abundance of an Mtb Rv2250c citrullinated peptide). Certain putative citrullinated protein identifications meeting stringency criteria are common between the (non-independent) dual-timepoint dataset and dataset A: THYX and SPTBN1.

Examples of Modified Peptide Characteristics (Dataset B)

Proteins (Tables A1-A3) were identified in most samples by matching (rather than directly by MS/MS). All localisations ~1.

Table A1 Putative Proteins Exhibiting Chlorotyrosine Modifications

Protein	Mass Error	Charge	PEP	Number of Chlorotyrosines	Position(s)
FAM105A inactive ubiquitin thioesterase	0.33	2	0.001	1	NLSVEAEVDLLSY(1)CAREWK
RNF220 E3 ubiquitin-protein ligase (fragment)	-0.48	3	0.025	1	FEFY(1)EWCGQKRIR
NSD2 histone-lysine N-methyltransferase	0.57	3	0.023	1	LGNY(1)RWWPAAEVCHPK
FMNL2 formin-like protein 2	-0.99	3	0.004	2	ILEILALGNY(1)MNSSKRGAVY(1)GFK

Table A2 Putative Proteins Exhibiting Nitrosylation

Protein	Mass Error	Charge	PEP	Number of Nitrosylated Cysteine Residues	Position(s)
UBD ubiquitin D	-0.20	2	0.008	1	TGIPTQIVTC(1)NGK
TRIM66 tripartite motif-containing protein 66	0.26	3	0.001	1	LLETSC(1)INTDPGSPWSIR
FAM105A inactive ubiquitin thioesterase	0.33	2	0.001	1	NLSVEAEVDLLSY(1)AREWK
CEP128 centrosomal protein of 128kDa (fragment)	-0.48	2	0.03	1	LQWLC(1)EELK
CTSD cathepsin D	-0.53	2	0.04	1	LLDIAC(1)WIHHK
KIAA1456 probable tRNA methyltransferase 9-like protein	-0.61	2	0.01	1	GCEAMVC(1)DNLNLPFR

Table A3 Putative Proteins Exhibiting Iodination

Protein	Mass Error	Charge	PEP	Number of Iodinated Tyrosine Residues	Position(s)
HBD haemoglobin subunit delta	0.75	3	6.95e-05	1 (histidine)	VLGAFSDGLAH(1)LDNLK
LTF lactotransferrin	0.48 -0.11	2 2	0.005 3.41e-05	1	YLGPPQY(1)VAGITNLK FDEY(1)FSQSCAPGSDPR
GAPDH glyceraldehyde-3-phosphate	-0.42	2	1.55e-08	1	WGDAGAEY(1)VVESTGVFTT MEK
S100A8 protein S100-A8	0.59	3	9.11e-44	1	LLETCPQY(1)IR
MPO myeloperoxidase	-0.57	2	0.003	1 (also has an oxidation)	DY(1)LPLVLGPTAMR
S100A9 protein S100-A9	-0.83	2	0.004	1 (histidine; also has a deamidation)	VIEH(1)IMEDLTNADK
ENO1 alpha-enolase	-0.34	2	0.006	1	YISPDQLADLY(1)K
HIST1H1E histone H1.4	0.1	2	0.002	1 (also has a deamidation)	ALAAAGY(1)DVEK
HSPA8 heat shock cognate 71kDa protein	-0.52	2	1.24e-05	1	SINPDEAVAY(1)GAAVQAAI LSGDK
EPX eosinophil peroxidase	-0.12	2	0.004	1	IVY(1)EGGIDPILR
HIST1H1B histone H1.5	0.38	2	0.004	1	ALAAAGY(1)DVEK
SERPINB1 leukocyte elastase inhibitor	0.56	3	4.06e-07	1	TY(1)GADLASVDFQHASEDA R
PGD 6-phosphogluconate dehydrogenase	-0.60	2	0.0003	1	LVPLDGTGDIIDGGNSEY(1)R
HIST1H4A histone H4	0.06	2	0.003	1	TVTAMDVVY(1)ALK
ACTG1 actin, cytoplasmic 2	0.77 -0.32	2 2	5.3e-10 0.002	1 (also has an oxidation and an iodination) 1 (also has an oxidation and an iodination)	SY(1)ELPDGQVITIGNER DSY(1)VGDEAQS
ACTA1 actin, alpha skeletal muscle	0.32	2	1.1e-11	1	Y(1)PIEHGIITNWDDMEK
HBA1 hemoglobin subunit alpha	-0.04	3	8.2e-15	1 (also has an oxidation)	VGAHAGEY(1)GAEALER
MRM2 rRNA methyltransferase 2, mitochondrial	0.29	2	0.001	1 (histidine; also has a deamidation)	LLEVNERH(1)QILR
CCDC151 coiled-coil domain-containing protein 151	-0.62	2	0.004	1 (histidine)	RLEELQLQH(1)SLR

S100A8, ACTG1, and histone H4 were identified largely by MS/MS

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Chapter 4: Results & Discussion

Effects of Partially ATT-treated Co-infection on Neutrophil Functional Profiles, Including Biomarker Candidate Performance

"It is not easy to convey, unless one has experienced it, the dramatic feeling of sudden enlightenment that floods the mind when the right idea finally clicks into place. One immediately sees how many previously puzzling facts are neatly explained by the new hypothesis." - Francis Crick

Abstract

Background Neutrophils are the major circulating leukocyte population and the earliest innate system responders to be recruited. Various functional specialisations facilitate their antimicrobial and immune functions, including unusual metabolic traits. Partial ATT treatment of TB/HIV-coinfection may partially ameliorate the effects of *Mycobacterium tuberculosis* (Mtb) - perhaps with a concomitant relative increase in the effects of HIV - on immune function, not to mention the direct immunometabolic impacts of ATT. **Methods** The proteomic profile of neutrophils from healthy (TB-HIV-) volunteers (HV) was compared to that of partially ATT-treated TB/HIV-coinfected participants. Using outputs from this comparison, proteomic differences between neutrophils from participants who did or did not go on to develop TB-IRIS (IRIS0, CTRL0) were identified. Based on identified level-altered proteins and their parent pathways, eight major functional modules were more deeply interrogated to generate class-specific neutrophil functional profiles: (1) signalling pathways, (2) PRR and cytokine signalling, (3) the eicosanoid cascade, (4) neutrophil antimicrobial functions, (5) carbon-energy metabolism, (6) protein homeostasis, (7) integrated nitrogen-sulfur-B-vitamin and redox/xenobiotic/glyoxal metabolism, and (8) miscellaneous (including gene expression and cytoskeletal dynamics). Findings were integrated to generate hypotheses regarding TB-IRIS predisposition. **Results** Neutrophil proteomic profiles successfully distinguish between healthy HV and IRIS0 or CTRL0. Both of the latter classes exhibit significant differences from HV, with level-altered proteins representing mainly energy-nutrient metabolism and immune response functional pathways. Many such differences from HV were shared between IRIS0 and CTRL0 (i.e. driven by partially ATT-treated co-infection), but some were class-unique (i.e. driven by factors predisposing participants to or protecting them from later TB-IRIS development). Briefly, neutrophil functional profiling suggests a dominant type I interferon and TGF β (rather than a Th1 cytokine-type) response, resulting in relative neutrophil inactivity (including down-regulation of T-cell activation), dysregulated cachectic-type metabolism (likely including increased mitochondrial stress and ROS generation), elevated endoplasmic reticulum-mediated lipid (including isoprenoid) biosynthesis, and a xenobiotic response. Specifically, IRIS0 neutrophils exhibit: an apparent inability of caspase 1-mediated IL-1 β and IL-18 maturation (unexpected); enhanced Th2 cytokine-type signalling; enhanced responsivity to type I IFN, TGF β , and insulin; relatively higher capacity to engage the early steps of leukotriene biosynthesis; poorer capacity for restraint of lectin-based complement activation; and various relative metabolic differences (e.g. earlier interruption of the respiratory chain, higher ammonia levels, relatively more normal B-vitamin synthesis capacity (unexpected), and decreased steroidogenesis - including possibly lower availability of microenvironmental epinephrine and glucocorticosteroids. Further significantly differentially-expressed (SDE) proteins identified during a head-to-head comparison of the CTRL0 and IRIS0 proteomes support and enhance findings of the full analysis (e.g. under the IRIS0 condition: the up-regulated response to Th2 cytokines, down-regulated activation of antimicrobial functions, and endoplasmic reticulum-mediated protein-folding quality-control). The head-to-head comparison *additionally* suggests that IRIS0 neutrophils are more prone to necrosis (and pathogen/antigen release) in response to smaller increases in cytosolic Ca²⁺, and that an absence of BTK may lead to decreased nucleic acid-mediated TLR-9 (possibly up-regulated by ammonia) activation (and thus type I IFN and IL-10 production, which may thus predispose to a less-controlled Th1 response occurring at the time of TB-IRIS) and/or impaired baseline TLR/cytokine (e.g. TLR/IL-1R-MyD88-NF κ B) signalling. Various candidate biomarkers distinguish CTRL- and IRIS-class partially ATT-treated neutrophils from HV neutrophils and from each other. **Conclusions** Findings suggest that individuals who go on to develop IRIS enter into ATT at a more advanced stage of TB/HIV-coinfection, at which: (a) the pathogen (and thus antigen) load is higher, (b) the immunological profile is skewed from a Th1/Th17 towards a more type I IFN/Th2/Treg profile somehow more permissive to subsequent unrestrained TLR- and complement-mediated Th1-type inflammation, and (c) various metabolic deficits perhaps contribute to impaired antigen clearance or result in pro-inflammatory intracellular changes or cell death (which may additionally release pro-inflammatory substances extracellularly). A subset of candidates exhibits optimal biomarker characteristics. Both mechanistic hypotheses and biomarker findings require validation in an independent cohort, preferably via orthogonal methods.

1. Proteomic Overview

Neutrophil proteomic differences distinguish between partially ATT-treated co-infected participants (both CTRL and IRIS groups) and healthy volunteers (fig. 1).

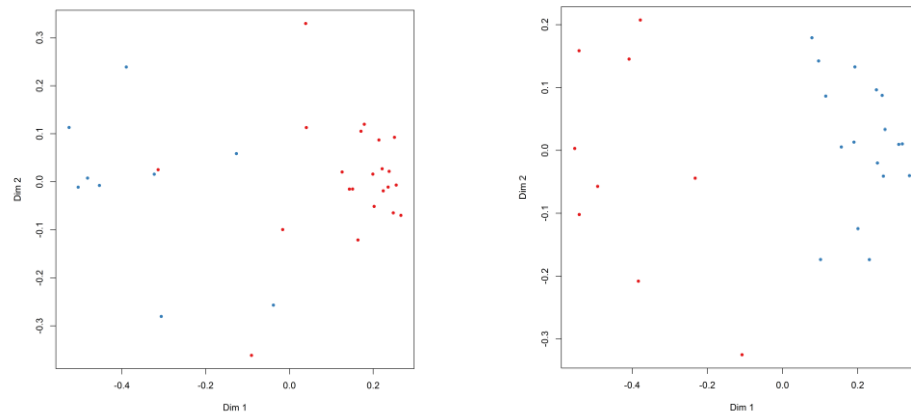


Figure 1 Multidimensional Scaling (MDS) Plots Demonstrating Between-class Discrimination for both CTRL0 (red, left panel) and IRIS0 (blue, right panel) versus HV.

Indeed, the analysis determining detectable/undetectable proteins in each class (fig. 2) and the differential expression analysis both demonstrate that partially-treated co-infection alters the levels of multiple neutrophil proteins.

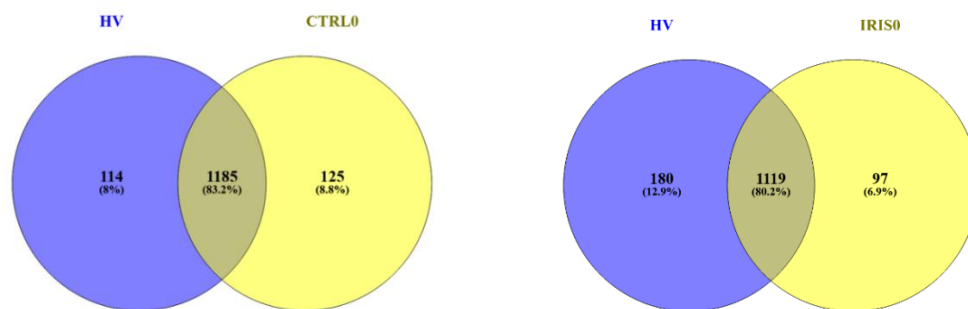


Figure 2 Venn Diagrams Demonstrating Numbers of Detectable/Undetectable Proteins per Class.
In the CTRL0 (relative to the HV) class, 125 and 114 proteins are uniquely detectable and undetectable, respectively.
In the IRIS0 (relative to the HV) class, 97 and 180 proteins are uniquely detectable and undetectable, respectively.

Differential expression analysis comparing CTRL0 and HV (Appendix J, Table 1) indicates that 182 proteins are significantly differentially expressed (SDE, $p < 0.05$), and that 145 of these remain SDE after multiple-testing correction (MTC, adjusted $p < 0.05$) (fig.s 3 and 4). Differential expression analysis comparing IRIS0 and HV (Appendix J, Table 2) indicates that 183 proteins are SDE ($p < 0.05$), and that 95 of these remain SDE after MTC (adjusted $p < 0.05$) (fig.s 3 and 4).

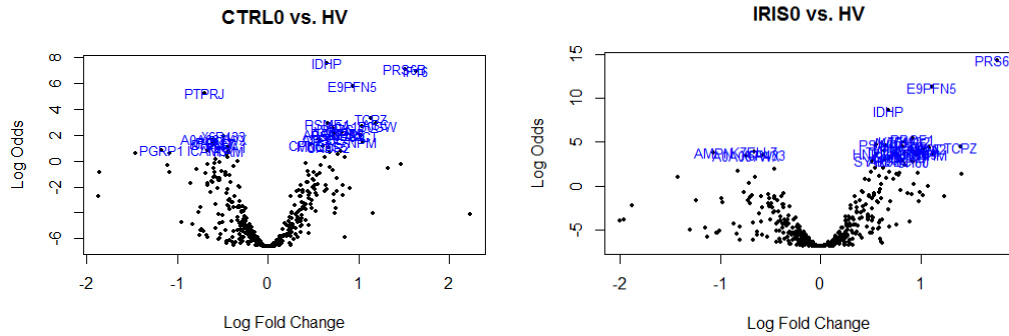


Figure 3 Volcano Plots Demonstrating Distribution of Between-class Proteomic Differences. Positive logfold-change indicates a relatively higher protein abundance in CTRL0 or IRIS0 (versus HV) and vice versa.

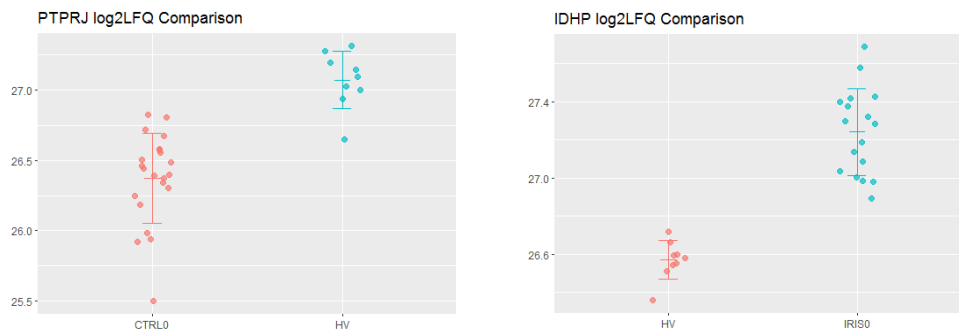


Figure 4 Representative Grouped Scatter Plots Comparing Inter-class Protein log2LFQ Values. Protein PTPRJ (gene PTPRJ), relatively less abundant in the CTRL0 class, is a receptor-type protein tyrosine phosphatase which regulates leukocyte function. Protein IDHP (gene IDH2), relatively more abundant in the IRIS0 class, is mitochondrial isocitrate dehydrogenase, which catalyzes conversion of isocitrate to α -ketoglutarate as part of the tricarboxylic acid (TCA) cycle.

Of all level-altered proteins (i.e. detectable/undetectable *and* SDE) in the CTRL0 class, many (Table 1a) are 'inference friendly' (as defined in Chapter 3): 19 rate-limiting enzymes, 10 protein kinases, 8 protein phosphatases, and 3 major transcription factors. Of all level-altered proteins in the IRIS0 class, many (Table 1b) are also 'inference friendly': 13 rate-limiting enzymes, 11 protein kinases, 6 protein phosphatases, and 5 major transcription factors.

Table 1a HV vs. CTRL0: Inference-friendly Level-altered Proteins

	Rate-limiting Enzymes	Protein Kinases	Protein Phosphatases	Transcription Factors
up	STT3A PHGDH ACADM COASY GFPT1 MAT2B IMPDH2 NAMPT ARG1	LYN	PRKAR2A PTPN11 PTPN6 DUSP23 PPP1R7	STAT1 STAT2
down	PFKM PPCS GLUL PNPO CPT1A PDXK LTA4H ALOX5 AMPD3 G6PD	CAMKK2 CSNK2A2 PRKAG1 PHKG2 AKT1 MAPKAPK2 LRRK2 PRKCD STK4	PTPRJ PTPRC PPP2CA	RELA

up uniquely detectable + significantly up-regulated. **down** undetectable + significantly down-regulated.

Table 1b HV vs. IRIS0: Inference-friendly Level-altered Proteins

	Rate-limiting Enzymes	Protein Kinases	Protein Phosphatases	Transcription Factors
up	STT3A PHGDH GFPT1 GPAT3 IMPDH2 ARG1	CMPK2 LYN NEK9	PPA1 PRKAR2A PTPN6 PTPN11	STAT1 STAT2 STAT3 STAT6
down	ADK CPT1A GLUL G6PD LTA4H PFKM PPCS	BTK CAMKK2 CSNK2A2 MAPKAPK2 PRKAG1 PRKCD PIK3CG TAOK3	PTPRC PTPRJ	RELA

up uniquely detectable + significantly up-regulated. **down** undetectable + significantly down-regulated.

Level-altered proteins represent various functional pathways (Table 2). For the CTRL group, members and functioning of each pathway marked by an asterisk (signal transduction – including AMPK signalling, interferon signalling, antimicrobial responses – including NOD-like receptor signalling and eicosanoid metabolism, carbon/energy metabolism – including potential mitochondrial stress, and protein homeostasis – including altered protein quality control mechanisms) are considered in greater detail below as part of the eight neutrophil-relevant functional modules identified by deep manual analysis. Similarly, for the IRIS group, members and functioning of each pathway marked by an asterisk (protein turnover, NOD-like receptor signalling, cytokine signalling, and carbon/energy metabolism) are considered in greater detail below as part of the functional modules identified by deep manual analysis. Endo- and exocytosis are unfortunately too complex to fit within the scope of this thesis, but are briefly considered.

Table 2a Top 5 Pathways Representing Proteins Up- and Down-regulated During Co-infection + ATT (CTRL0)

	KEGG	FDR	Reactome	FDR
represented by ↑ proteins	*proteasome	1.12e-6	immune system	1.19e-27
	*metabolic pathways	1.21e-6	cytokine signalling in immune system	2.21e-18
	*NOD-like receptor signalling pathway	7.81e-6	innate immune system	2.05e-17
	carbon metabolism	9.5e-6	neutrophil degranulation	1.32e-16
	biosynthesis of amino acids	1.2e-5	*interferon signalling	3.95e-12
represented by ↓ proteins	*AMPK signalling pathway	0.00015	neutrophil degranulation	4.96e-28
	leukocyte transendothelial migration	0.00018	innate immune system	9.01e-28
	endocytosis	0.00018	immune system	3.04e-22
	starch and sucrose metabolism	0.00018	adaptive immune system	5.99e-6
	insulin resistance	0.00084	metabolism	1.46e-5

KEGG Kyoto Encyclopedia of Genes and Genomes **FDR** false discovery rate

Table 2b Top 5 Pathways Representing Proteins Up- and Down-regulated During Co-infection + ATT (IRIS0)

	KEGG	FDR	Reactome	FDR
represented by ↑ proteins	*proteasome	7.7e-07	immune system	7.97e-31
	*NOD-like receptor signalling pathway	7.15e-06	innate immune system	1.22e-19
	protein processing in endoplasmic reticulum	2.51e-05	*cytokine signalling in immune system	2.61e-19
	Parkinson's disease	4.06e-05	neutrophil degranulation	4.05e-18
	Epstein-Barr virus infection	9.57e-05	*metabolism of proteins	1.34e-15
represented by ↓ proteins	*endocytosis	1.98e-08	innate immune system	2.57e-23
	tuberculosis	0.0019	*neutrophil degranulation	4.76e-22
	Fc gamma R-mediated phagocytosis	0.0019	immune system	1.06e-17
	phagosome	0.0019	metabolism	1.33e-08
	*metabolic pathways	0.0020	clathrin-mediated endocytosis	4.36e-05

KEGG Kyoto Encyclopedia of Genes and Genomes **FDR** false discovery rate

2. How Do CTRL0 (Relative to HV) and IRIS0 (Relative to HV) Neutrophils Differ?

Just over half the up-regulated protein signature (including ISGs, kinase Lyn, etc.) and just under half the down-regulated protein signature (including LTA4H, LTBR4, CAMKK2, BAX, G6PD, etc.) is shared by both groups, driven by partially ATT-treated co-infection rather than by inherent differences specific to the CTRL0 or IRIS0 neutrophil phenotypes (fig.s 5 and 6).

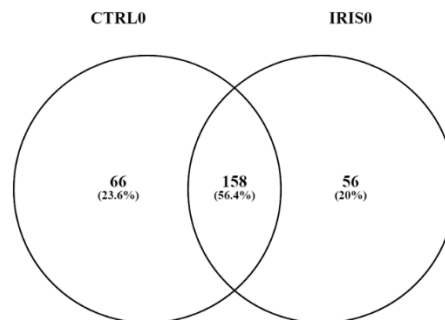


Figure 5 Proteins Up-regulated Relative to HV
Up-regulation unique to CTRL0 includes HSD17B10 and CD300LF. Up-regulation unique to IRIS0 includes USP15, OAS1, STAT3, and STAT6. Of the proteins up-regulated in IRIS0, none are overtly oppositely-regulated (i.e. down-regulated) in CTRL0 (not shown).

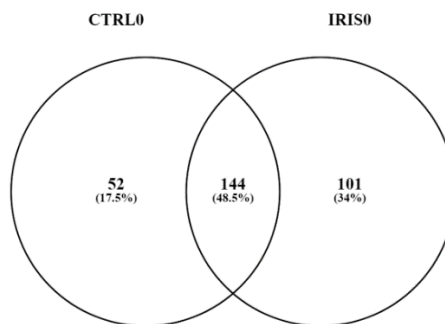


Figure 6 Proteins Down-regulated Relative to HV
Up-regulation unique to CTRL0 includes STK4, GYS1, ALOX5, LRRK2, ICAM3, PGLYRP1, S100A12. Up-regulation unique to IRIS0 includes CD55, PTPN1, CASP1, SIGLEC5, MAP2K3, LILRB2, BTK, and TAOK. Of the proteins down-regulated in IRIS0, none are overtly oppositely-regulated (i.e. up-regulated) in CTRL0 (not shown).

Level-altered proteins specific to the IRIS group (i.e. not shared with the CTRL group) again represent various functional pathways (Table 3), now apparently differing somewhat from those in Table 2b. While protein turnover and endo/exocytosis still feature prominently, proteins now also represent oxidative phosphorylation (oxphos) and mRNA-processing pathways.

Table 3 Top 5 Pathways Representing Proteins Uniquely Up- and Down-regulated In IRIS0 (Relative to HV) Compared to CTRL0 (Relative to HV)

	KEGG	FDR	Reactome	FDR
represented by uniquely up-regulated proteins	Huntington's disease	0.0209	neutrophil degranulation	2.46e-07
	pathogenic Escherichia coli infection	0.0237	immune system	2.46e-07
	Parkinson's disease	0.0237	platelet degranulation	4.59e-07
	phagosome	0.0237	innate immune system	1.01e-06
	oxidative phosphorylation	0.0237	metabolism of proteins	4.65e-05
represented by uniquely down-regulated proteins	endocytosis	9.43e-06	neutrophil degranulation	1.23e-06
	ribosome	0.0049	innate immune system	1.66e-06
	N/A	N/A	clathrin-mediated endocytosis	9.97e-05
	N/A	N/A	formation of a pool of free 40S subunits	0.00011
	N/A	N/A	nonsense mediated decay...	0.00015

KEGG Kyoto Encyclopedia of Genes and Genomes FDR false discovery rate

Neurological disorder-relevant KEGG pathways featured in Table 3 are an excellent example of the knowledgebase shortcomings discussed in Chapter 3. Although these pathways are named for popularly-researched disorders, naming is misleading. It obscures the molecular nature of these pathways, which encompass various 'discrete' cellular functions (often overlapping/repeated between pathways). For the Huntington's disease pathway, this includes: Ca^{2+} signalling, vesicle transport, protein folding, mitochondrial dysfunction, oxidative stress, caspase-mediated cell death, and transcriptional regulation. For the Parkinson's disease pathway, this includes: adenosine-regulated PKA signalling, altered tyrosine metabolism, mitochondrial dysfunction (with resultant oxidative stress, disruption of the ubiquitin-proteasome system (UPS), and caspase-mediated cell death). Note the underlined commonalities. Thus, the sub-components of these pathways begin to more closely resemble those pathways observed in Table 2b, including: proteasome, protein processing in the endoplasmic reticulum (ER), metabolism of proteins, endocytosis, and metabolic pathways.

3. Altered Neutrophil Immunometabolic Functional Pathways

As noted in Chapter 3, all tabulated information regarding protein function compartments is provided by GeneCards (unless otherwise specified). Fold-changes and p-values for all proteins are provided in Appendix J.

3.1. Signal Transduction

Changes exhibited by both participant neutrophil classes (Table 4) suggest increased homeostatic/anabolic (e.g. GPCR-PKA-glucose/lipid metabolism) and decreased stress response/inflammatory (e.g. GPCR/enzyme-linked receptor-induced PLC-mediated generation of arachidonate, IP3, and DAG) signalling. The latter effect would decrease: inflammatory eicosanoid production, Ca^{2+} /calmodulin signalling, PKC-mediated inhibition of (anabolic/motility-regulating) AKT1, and activation of (stress response) MAPK14 or NCF1 (involved in the NOX-mediated respiratory burst). Another PKC substrate, Lyn, is apparently activated (perhaps via enzyme-linked receptors such as TLR-2/4, $\text{Fc}\epsilon\text{R}$, or those for growth factors/cytokines), but also has inflammation-restraining roles, for example dampening neutrophil integrin signalling.

Table 4 Common Dysregulated Proteins Relevant to GPCR- and Enzyme-linked Receptor-mediated Signal Transduction

Protein	Function	Status	Hypothesised Outcome
PRKAR2A	PKA regulatory subunit	unique	↑ AC-mediated GPCR signalling ↑ PKA mediated glucose/lipid metabolism regulation
PLCB2, G2	produce DAG + IP3, MIP3 from PI/ML-4,5-BP	down	↓ PLC-mediated GPCR and enzyme-linked receptor signalling ↓ DAG, IP3, MIP3
DGKZ	decreases DAG levels and thus PKC activity (also - via producing phosphatidic acid - activates PIP5K1A, which converts PIP4 to PI-4,5-BP)	unique	↓ DAG ↓ PKC activity
KRT	regulates PKC activity (via binding ITB1 and RACK1)	unique	↓ PKC activity
PRKDC	PKCδ Ser/Thr kinase	down	↓ PKC activity
LYN	SFK; activated by PKC/enzyme-linked receptors (incl. various immune receptors) dampens downstream (esp. integrin) signalling; phosphorylates ITIMs to recruit PTPN6/SHP-1, PTPN11/SHP-2, and INPP5D/SHP-1 phosphatases, though PTPN11/SHP-2 positively regulates MAPK signalling	up	↑ LYN activity ↓ integrin/↑ MAPK signalling
PTPRC, J	phosphatases which dephosphorylate receptor cascade targets (e.g. LYN)	down	↓ LYN dephosphorylation ↑ LYN activity

The CTLR0 class exhibits additional unique changes (Table 5) suggestive of decreased PLC, DAG, and PKC activity (perhaps indicating more pronounced restraint of eicosanoid signalling and respiratory burst capacity). However, the IRIS0 class also exhibits additional unique changes (Table 6) suggestive of decreased PLC activity and PKC downstream signalling (thereby compromising motility and endocytosis). While absent BTK suggests IRIS0 neutrophils may be less responsive to TLR-9 ligands, there are simultaneous indications that TLR-4 signalling may be more prominent (and decreased sensitivity to progesterone may de-repress inflammatory capacity). Thus, IRIS-class findings are consistent with hypotheses from first principles (Chapter 1): retarded antigen clearance and predisposition to unrestrained inflammation.

Table 5 Control Group-specific Dysregulated Proteins Relevant to GPCR- and Enzyme-linked Receptor-mediated Signal Transduction

Protein	Function	Status	Hypothesised Outcome
DGKZ	decreases DAG levels and thus PKC activity (also - via producing phosphatidic acid - activates PIP5K1A, which converts PIP4 to PI-4,5-BP)	unique	↓ DAG ↓ PKC activity
KRT	regulates PKC activity (via binding ITB1 and RACK1)	unique	↓ PKC activity

Table 6 IRIS Group-specific Dysregulated Proteins Relevant to GPCR- and Enzyme-linked Receptor-mediated Signal Transduction

Protein	Function	Status	Hypothesised Outcome
IMPA1	provides inositol for ultimate phosphoinositide synthesis	absent	↓ phosphoinositide availability ↓ PLC-mediated conversion of PIP2 to IP3 and DAG
BTK	non-receptor Tyr kinase required for normal immunoreceptor signalling via activation of PLCG2 and thus PKC also facilitates recruitment of proteins involved in signalling from cytokine receptors and TLR induces TRAP degradation, links TLR-8 and -9 activation to NFκB activation, and is required for NA-activated TLR-9-mediated production of type I IFN, but not for the same effect by TLR-7) [1] in B-cells, lack of BTK increases IL-12 and decreases IL-10 production on TLR-9 stimulation [2]	absent	↓ PLC and PKC activity ↓ responsiveness to TLR-9 stimulation (possibly increasing IL-12 and decreasing type I IFN and IL-10 production)
MARCKS	most abundant PKC substrate binding to Ca ²⁺ /calmodulin or phosphorylation by PKC dissociates this protein from the membrane and from actin cross-linking to regulate motility and phagocytosis	down	↓ PKC downstream signalling altered motility, phagocytosis
PACSIN2	major PKC substrate when phosphorylated by casein kinase or PKC, links the actin cytoskeleton to vesicle formation as part of endocytosis (including of surface receptors)	absent	↓ PKC downstream signalling altered endocytosis
PGRMC2	surface progesterone steroid receptor high-dose progesterone (without altering cortisol levels) promotes random neutrophil motility and suppresses MPO-halide system-mediated iodination (without affecting phagocytic capacity or antibody-dependent cell-mediated cytotoxicity) [3] during pregnancy, progesterone also opposes NETosis [4]	absent	↑ directed motility ↑ MPO-halide system activity ↑ NETosis
WDFY1	PI3P-binding protein interacts with TICAM1 to enhance TLR-3- and 4-induced IRF3 and NFκB activation for expression of IFNβ and pro-inflammatory cytokines	unique	↑ TLR-4 signalling (possibly Mtb product-mediated) ↑ expression of type I IFN and pro-inflammatory cytokines
EEA1	binds PI3P-containing vesicles and is implicated in activated TLR-4 signalling	up	↑ TLR-4 signalling

Specifically regarding cell death, PRKCD (*down-regulated* in both classes) would normally (likely via activating AKT1) oppose TRAIL-induced (externally-initiated, e.g. via MAPK11/14 inhibiting AKT1) while favouring mitochondrial/ER/nuclear stress-induced (internally-initiated, including via mitochondrial BAX, or via DNA damage activating nuclear MAPK8/9) apoptosis. This suggests both classes are more susceptible to externally- but less susceptible to internally-initiated apoptosis. Furthermore, the CTRL0 class *uniquely* exhibits a programmed cell death protein (PDCD4) which *down-regulates* MAP4K1 expression and downstream pro-apoptotic JNK signalling (while also inhibiting EIF4A1-EIF4G interaction and protein translation), suggesting decreased susceptibility to stress-induced apoptosis. Similarly, *down-regulation* of mitochondrial death initiator BAX suggests enhanced neutrophil survival.

Regarding Ras activation, the CTRL0 class exhibits *down-regulated* m-Ras-activating RAPGEF6 (suggesting decreased Ras activation), while the IRIS0 class exhibits *absent* LYPLA1 (a lysophospholipase which may influence Ras localisation) and GNAS (activated by GPCRs – including β-adrenergic receptors – for example to inhibit AC activation or “to activate Ras via RAPGEF2”). The latter findings are difficult to interpret, but may also suggest decreased Ras (and thus PI3K-AKT and MAPK) activation.

Regarding MAPK (esp. p38 MAPK) and PI3K-AKT pathways, both neutrophil classes indeed exhibit changes (Table 7) suggestive of decreased PI3K-AKT and MAPK (especially MAPKAPK2-mediated p38 MAPK) signalling, perhaps due to restraint by up-regulated and possibly activated Lyn (as mentioned). Absent PIK3CG further supports decreased activity of PLC and PKC (supporting impaired neutrophil motility, extravasation, and respiratory burst capacity), as well as decreased endocytosis of the β-adrenergic receptor (possibly leading to increased cell sensitivity to – possibly lower levels of -adrenaline); PIK3CG inhibition is anti-inflammatory.

Table 7 Common Dysregulated Proteins Relevant to MAPK (esp. p38 MAPK) and PI3K-AKT Pathways

Protein	Function	Status	Hypothesised Outcome
MAPKAPK2	Ser/Thr protein kinase activated by stress-activated p38 MAPKs	absent	↓ MAPKAPK2 target phosphorylation
CSNK2A2	casein kinase II alpha subunit (catalytic)	absent	↓ casein kinase II target phosphorylation
PTPN11	phosphatase acting downstream of Tyr kinases; stimulates MAPK pathway	unique	response to ↓ MAPK signalling
PIK3CG	GPCR-linked PI3K, produces PIP3 (which recruits AKT and its activator PDK1)	absent	↓ PIP3 ↓ inflammation
VAC14	maintains normal PIP3 and PIP5 levels	unique	attempt to maintain PIP3 levels

The CTRL0 class exhibits additional unique changes (Table 8) supporting decreased PI3K-AKT signalling (decreased motility and/or anabolism, in agreement with Tables 10-12 results suggesting that AMPK dysfunction produces insufficient ATP). Perhaps in response to MAPK14 and AKT inhibition, there is *downstream* up-regulation of proteins enhancing MAPK14 activity and down-regulation of proteins that

inhibit AKT activity. The IRISO class exhibits unique changes (Table 9) suggestive of increased IFNAR signalling and insulin (anabolic) responsiveness as well as altered glucocorticoid signalling, but also strongly in support of already-noted decreased MAPK (especially MAPK14) stress signalling, with possible decreased signalling by MAPK14 target casein kinase (which normally phosphorylates multiple targets - including NFkB, STAT1, CREB1, and MYC - and interferes with various CASP8 activities). However, there are also indications of de-repressed MAPK8/JNK signalling; JNK is a growth factor/cytokine/stress-activated MAPK which (through phosphorylation) regulates AP-1 to inhibit (pro-survival) CREB/STAT-mediated signalling and/or activate pro-apoptotic pathways (including BAX-dependent cytC apoptosome formation and CASP9 activation). In combination with the above findings, this may suggest preferential signalling through the AP-1 rather than NFkB branch of stress response pathways, and possible priming for apoptosis, provided the correct stimuli are present.

Table 8 Control Group-specific Dysregulated Proteins Relevant to MAPK (esp. p38 MAPK) and PI3K-AKT Pathways

Protein	Function	Status	Hypothesised Outcome
DUSP3	phosphatase; enhances JNK and p38 MAPKα (MAPK14) activity	unique	response to ↓ MAPK14 signalling
FAM120A	scaffolding protein that activates SFKs and enables them to activate PI3K (critical component of oxidative stress-induced survival signalling)	absent	↓ PI3K activation ↓ PIP3 ? ↓ survival
AKT1	one of three Ser/Thr protein kinases all called AKT kinase; recruited by PIP3	absent	↓ PI3K-mediated AKT signalling ↓ anabolism, motility
STK4	caspase-activated pro-apoptotic kinase inhibited by AKT1 (and which reciprocally inhibits AKT1)	down	↓ AKT1 inhibition (response) ↑ survival
PPP2CA	phosphatase that deactivates AKT and opposes AKT signalling	down	↓ AKT inhibition (response)

Table 9 IRIS Group-specific Dysregulated Proteins Relevant to MAPK (esp. p38 MAPK) and PI3K-AKT Pathways

Protein	Function	Status	Hypothesised Outcome
MAP2K3 (MAPKK3)	usually activated by environmental stress, cytokines, adrenergic stimuli (via receptor ADRA1B), mitogens, and insulin (for GLUT expression) to phosphorylate (activate) p38α MAPK (MAPK14) implicated in the integrin pathway its inhibition "is involved in the pathogenesis of <i>Yersinia pseudotuberculosis</i> "	absent	↓ MAPK14 activation
TAOK3	activates MAPK14 cascade (e.g. in response to DNA damage), likely via activation of MAP2K3 and MAP2K6 inhibits MAPK8/JNK (e.g. activated in response to EGF) signalling	absent	↓ MAP2K3 and MAPK14 activation ↑ MAPK8/JNK signalling
LAMTOR2	MAPK pathway component adaptor that promotes MAPK cascade efficiency and thus MAPK2 activation a human mutation of LAMTOR2 is associated with primary immunodeficiency (possibly via a role in endosomal biogenesis)	absent	↓ MAPK2 activation
PTPN1	founding protein Tyr phosphatase inhibits growth factor and insulin signalling dephosphorylates JAK2 and TYK2 that transduce signals from the IFNα receptor likely also has a role in casein kinase II (and p60c-Src) signal transduction (ties in with CSNK2A2 absence)	absent	↑ responsiveness to insulin and IFNα receptor signalling ? ↓ casein kinase signalling
ACP1	Tyr phosphatase	absent	???
PPP2R2A	protein phosphatase 2 regulatory subunit this phosphatase is a major Ser/Thr phosphatase that inhibits cell growth	absent	↓ repression of anabolism
NEK9	Ser/Thr protein kinase whose targets include histones, MBP, and CSN2 related pathways include the glucocorticoid receptor regulatory network and signalling by ERBB4 (dexamethasone up-regulates secretion of CSN2 in certain human cells [5])	unique	altered glucocorticoid signalling?

Regarding the AMPK-mTOR pathway, both neutrophil classes exhibit changes (Table 10) suggestive of decreased (anti-inflammatory) AMPK activity (perhaps due to the previously-noted decrease in Ca²⁺ signalling), which would impair ATP generation (despite likely increased energy requirements), lysosome acidification, and mTORC2-mediated AKT1 activation, but would favour mTORC1-mediated pyrimidine/protein/lipid biosynthesis and mitochondrial biogenesis while suppressing stress-adaptive autophagy/mitophagy.

Table 10 Common Dysregulated Proteins Relevant to the AMPK-mTOR Pathway

Protein	Function	Status	Hypothesised Outcome
CAMKK2	activates AMPK (and Ca ²⁺ /calmodulin-dependent kinases)	absent	? due to ↓ Ca ²⁺ /calmodulin signalling ↓ AMPK activation
PRKAG1	AMP/ATP-binding subunit of AMPK (active state when ATP bound)	absent	↓ AMPK activity (thus ↓ mitochondrial biogenesis, ATP generation, lysosome acidification) ↑ mTORC1 activity (thus ↓ autophagy, ↑ mitochondrial biogenesis, pyrimidine/protein/lipid biosynthesis) ↓ mTORC2 activity (thus ↓ AKT1 activation, ? ↑ cytoskeletal dynamics)
ATP6V1A	component of vacuolar ATPase (V-ATPase) enzymatic complex that acidifies intracellular compartments (e.g. vacuoles, lysosomes, endosomes)	up	↑ vesicular compartment acidification

The CTRL0 class exhibits additional unique findings (Table 11) strongly supporting decreased CAMKK2 and AMPK activation, and the abovementioned downstream sequelae. The IRIS0 class exhibits additional unique findings (Table 12) supporting mTORC1 activation, yet absent LAMTOR2 suggests that the Ragulator complex cannot facilitate mTORC1 recruitment to the lysosome for activation, and there are indications of *increased* lysosome acidification. Although (as mentioned in Chapter 1) the baseline level of TGFβ has not yet been investigated in the context of TB-IRIS, the above findings may be due to higher TGFβ levels resulting from more advanced TB/HIV-coinfection in the IRIS class: TGFβ and AMPK oppose each other, with TGFβ1 facilitating FoxO1-mediated gluconeogenic gene expression [6]. Indeed, TGFβ is directly up-regulated (*unique*) in the IRIS class. Perhaps surprisingly, however, type I IFN (whose production is supported by AMPK) likely inactivates mTORC1 (favouring autophagy) while enhancing mTORC2/AKT activation (also inducing autophagy via PI3K/AKT inactivation causing FoxO3 degradation-mediated de-repression of both autophagy and IRF7-induced type I IFN production as well as ISG expression) [7].

Table 11 Control Group-specific Dysregulated Proteins Relevant to the AMPK-mTOR Pathway

Protein	Function	Status	Hypothesised Outcome
PPP1R7	Ser/Thr protein phosphatase 1 regulatory subunit involved in glycogen metabolism, protein synthesis, and HIV gene transcription dephosphorylates CAMKK2 and ATG16L1, opposing CAMKK2 function and autophagy	up	opposition of CAMKK2 activity ↓ autophagy
ATP6V1D	peripheral V1 V-ATPase complex subunit	absent	↓ lysosomal acidification?
LRRK2	kinase which activates CAMKK2/AMPK pathway via NADP receptor-mediated lysosomal alkalinisation and thus Ca ²⁺ efflux	down	↓ CAMKK2/AMPK activation ↑ mTORC1 activity ↓ mTORC2 activity ↓ autophagy

Table 12 IRIS Group-specific Dysregulated Proteins Relevant to the AMPK-mTOR Pathway

Protein	Function	Status	Hypothesised Outcome
LAMTOR2	already discussed above, but also a component of the Ragulator complex activated by amino acids (via a mechanisms involving lysosomal V-ATPase) to function as a Rag-activating GEF, thereby recruiting mTORC1 to the lysosomal membrane for activation LAMTOR2 is thus involved in amino acid sensing and mTORC1 activation to facilitate cell growth (when growth factors, amino acids, and energy levels are abundant)	absent	↓ mTORC1 activation (perhaps due to limiting amino acid levels)
ATP6V0A1	subunit of lysosomal V-ATPase required for its assembly and activity	unique	↑ lysosomal acidification capacity ↑ mTORC1 activation
ATP6AP1	non-lysosomal (e.g. granule) V-ATPase accessory subunit required for subcellular localisation of this acidifying V-ATPase may also play a role in V-type ATPase assembly under aerobic conditions activates Fe ²⁺ prolyl hydroxylase (PHD) enzymes (for iron homeostasis) as well as causing HIF1A degradation associated with immunodeficiency 47 and ficolin 3 deficiency	absent	↓ granule acidification altered iron homeostasis ↓ HIF1A degradation

Furthermore, hypoxic response transcription factor (TF) HIF-1α may be stabilised, leading to altered iron homeostasis, also consistent with hypotheses derived from first principles (Chapter 1). Indeed, HIF-1α inhibitor (**HIF1AN**) is *absent* from both classes, suggesting that HIF-1 is de-repressed to facilitate hypoxic response gene expression.

Regarding overall signal transduction-relevant proteomic changes (fig. 7), neutrophils from both classes appear prone to increased glycolysis and anabolism, but to decreased motility, extravasation, endocytosis, respiratory burst, and autophagy (including lysosome acidification) activity. Interestingly, they may also be more sensitive to cytokines signalling via JAK, as well as to adrenaline (perhaps suggesting lower levels of circulating adrenaline). IRIS0 findings support these (e.g. enhanced insulin sensitivity and thus anabolism, suppressed MAP2K3-activated MAPK14 and thus CKII signalling, and enhanced IFNAR and thus JAK-STAT signalling), but there are indications that although granule acidification may be decreased, lysosome acidification is actually increased (despite mTORC1 activation). In addition, IRIS0-specific findings include potential decreased sensitivity to anti-inflammatory progesterone (as well as altered glucocorticoid signalling), more prominent TLR-4 signalling (potentially with depressed TLR-9 sensitivity), enhanced MAPK8/JNK signalling, and likely increased HIF1A stability.

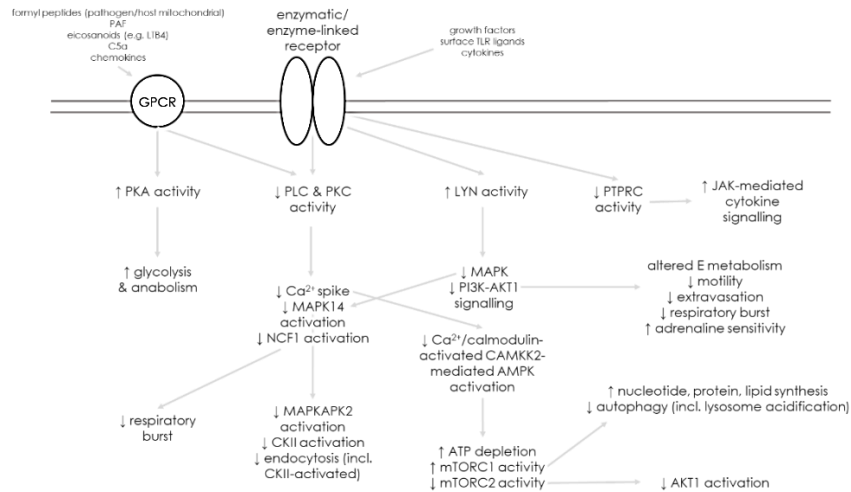


Figure 7 Schematic Summary of Signal Transduction-relevant Proteomic Changes. **PAF** platelet activating factor, **LTB4** leukotriene B4, **GPCR** G protein-coupled receptor, **TLR** toll-like receptor, **CKII** casein kinase II, and **mTORC** mammalian target of rapamycin complex.

3.2. Neutrophil Pattern Recognition and Cytokine Receptors

Both neutrophil classes exhibit changes (Table 13) suggestive of decreased TNF α (protective) but increased TGF β (pro-resolving, pro-healing) production and functions (e.g. T_{reg} induction, fibrosis).

Table 13 Common Dysregulated Proteins Relevant to TNF and TGF β Receptor Signalling

Protein	Function	Status	Hypothesised Outcome
ADAM10	produces mature soluble TNF α and FasL ectodomain shedding (producing transmembrane FasL APL remnant) also responsible for constitutive α -secretase cleavage of amyloid precursor, as well as for shedding of other cell surface proteins, including adhesion molecules (e.g. selectins, integrins)	absent	$\downarrow$ TNF α maturation
TGF β 1	precursor of immune regulatory/tissue-healing TGF β 1 which produces both active and regulatory (LAP) subunits; LAP ligation by integrins (ITGAV:ITGB6 or 8) releases active TGF β 1 ligation of TGFBR1/2 promotes T _H 17 (at low concentrations/when IL-6 and IL-22 are present) or T _{reg} (at high concentrations) and collagen production (via CREB3L1)	up	$\uparrow$ TGF β activity (e.g. fibrosis)

The CTRL0 class exhibits additional unique changes (Table 14) supporting decreased TNF α production (and TNF superfamily member, PGLYRP1 – a PDG-recognising PRR with bactericidal properties and a potential TREM-1 ligand – is *down-regulated*). The IRIS0 class exhibits additional unique changes (Table 15) supporting both decreased TNF α and increased TGF β signalling. It is possible that the overall decrease in protective anti-Mtb TNF α (and perhaps IL-12) signalling is due to inhibition of their production by type I IFN-mediated IL-10 induction, or (as hypothesised in Chapter 1) to HIV-mediated suppression of TLR/IL-1R-MyD88-NF κ B signalling.

Table 14 Control Group-specific Dysregulated Proteins Relevant to TNF and TGF β Receptor Signalling

Protein	Function	Status	Hypothesised Outcome
THEMIS2	myeloid inflammatory response regulatory point which promotes LPS-induced TNF production	down	$\downarrow$ TNF production

Table 15 IRIS Group-specific Dysregulated Proteins Relevant to TNF and TGF β Receptor Signalling

Protein	Function	Status	Hypothesised Outcome
STEAP4	TNF α -induced metalloendopeptidase associated with insulin resistance, obesity, and inflammatory arthritis	absent	$\downarrow$ TNF α stimulus
USP15	deubiquitinating enzyme with a role in enhancing TGF β signalling through inhibiting both TGFBR1 and SMAD degradation; additionally regulates NF κ B through de-ubiquitination of NFKBIA and VCP-bound substrates	unique	$\uparrow$ TGF β activity

In support of this hypothesis, a dominant feature represented by up-regulated proteins in both neutrophil classes is "cytokine signalling", with the IRIS0 class explicitly including "interferon signalling" as an additional dominant feature (Table 2). Both classes specifically exhibit IFN-inducible protein signatures (Tables 16-18), including STAT1 and 2, OASs, GBPs, IFITs, IFIs, TRIM22, and other ISGs. Indeed, searching the list of 225 proteins up-regulated under the CTRL0 condition against the Interferome v1.02 database (www.interferome.org, with *Homo sapiens* as the organism, and hematopoietic/immune as

the system) returns 114 interferon-regulated genes (51%). When restricting the search to type I IFN, 78 genes are returned, and when restricting the search to type II IFN, 88 genes are returned (with 55 genes overlapping between these). Searching the list of 176 proteins up-regulated under the IRIS0 condition against the Interferome database returns 128 interferon-regulated genes (73%; 89 genes for type I IFN, 97 genes for type II IFN, and 57 genes overlapping).

Most class-common ISG (Table 16) are concerned with cytoplasmic nucleic acid (NA) sensing (including GBP-mediated NLRP3 inflammasome assembly), increasing IFN/ISG expression (e.g. via STAT1/2-IRF3), and antiviral activity. Although RIG-I was not directly detected as a level-altered protein in this dataset, other ISGs positively regulating its function (e.g. DDX60) are, suggesting that it participates in cytoplasmic NA sensing. Furthermore, IFI16 is known to assist cytoplasmic viral dsDNA in recruiting STING, which may – in association with PYCARD – form an intranuclear inflammasome which activates CASP1, and yet inhibits AIM2 inflammasome activation.

Table 16 Common Dysregulated Proteins Relevant to IFN Signalling (encompasses entire CTRL Group-specific Dysregulated Protein Set)

Protein	Function	Status	Hypothesised Outcome
STAT1 and 2	transcription factors mediating IFN-induced gene transcription STAT2 also regulates mitochondrial fission via DNMI1 GTPase	unique	↑ transcription of IFN-induced gene modules
OAS2 and 3	dsRNA-activated enzymes which activate RNASEL for host and viral RNA degradation	unique	↑ host/viral RNA degradation
GBP1 and 5	antimicrobial guanylate-binding proteins GBP1 hydrolyzes GTP to GMP for oxidative killing and delivery of AMPs to autophagolysosomes GBP5 hydrolyzes GTP to non-GMP, activates NLRP3 inflammasome assembly in response to microbial and soluble agents	unique	↑ oxidative/AMP-mediated microbicidal activity ↑ NLRP3 inflammasome assembly
IFIT1 and 2	ssRNA-binding sensors which inhibit viral mRNA expression	unique	↓ viral gene expression
IFI16	viral dsDNA superstructure-binding transcriptional regulator also involved in energy-level-dependent AMPK activation and thus autophagy regulation	up	↓ viral gene expression
IFITM3	disrupts intracellular cholesterol homeostasis to prevent endosome-mediated viral entry newly-budded virions with a cholesterol-depleted membrane may be inactive known activity against HIV-1 critical role in structure and function of V-ATPase (e.g. required for phagosome acidification)	unique	↓ viral entry, activity ↑ V-ATPase-mediated phagosome acidification
TRIM22	ubiquitinates viral proteins; restricts HIV-1 replication	unique	↓ viral replication
ISG20	antiviral ssRNA exonuclease	unique	antiviral activity
ISG15 and UBE2L6	UBE2L6 conjugates ubiquitin-like protein ISG15 to targets (ISGylation) e.g. ubiquitinating enzymes (e.g. UBE2L6 itself), other ISGs (e.g. IFIT1, MX1, IRF3, FLNB), and cytoplasmic viral nucleic acid sensor *DDX58/RIG-I; ISGylation inhibits DDX58/RIG-I, prevents IRF3 degradation, inhibits FLNB-mediated IFNα-induced JNK signalling, prevention of host TSG101 interaction with HIV Gag	unique	↑ IRF3 signalling ↓ JNK signalling ↓ TSG101 interaction with Gag
TSG101	interacts with HIV Gag to support retrovirus budding	absent	↓ viral budding
E3 ubiquitin-protein ligase DTX3L and ADP-ribosyltransferase PARP9	E3 ubiquitin-protein ligase DTX3L co-functions with ADP-ribosyltransferase PARP9 in DNA damage repair (involving PARP1-dependent ubiquitination) and immune (incl. IFN-mediated antiviral) responses (e.g. proteasomal degradation of viral proteases, and histone ubiquitination-mediated enhancement of STAT1-dependent IFN-stimulated gene transcription, including pro-inflammatory cytokines)	unique	↑ viral protease degradation ↑ STAT1-dependent transcription of IFN-induced cytokines
PARP1	mediates glutamate and aspartate ADP-ribosylation of proteins (e.g. itself); key role in DNA repair (incl. being required to recruit DTX3L and PARP9 to DNA damage sites); with EE1A1 and TXK binds the IFNγ production to directly regulate its transcription; also involved in nuclear synthesis of ATP for extensive chromatin remodelling	absent	↓ IFNγ production that both PARP1 and ADPRHL2 (another transient, DNA damage-responder that protects cells from ADP-ribosylation-mediated parthanatos) are absent supports that DTX3L and PARP9 are not serving a DNA repair function in this context

The CTRL0 class indeed exhibits **up-regulated** PYCARD (not tabulated here; supporting hypothesised activation of the intranuclear inflammasome) as well as additional unique and notable ISG, e.g. iron-sulfur-binding RSAD2 (via IRAK1 ubiquitination promotes TLR-7/9-dependent IFNβ production), MATR3 (part of the complex facilitating IRF3 phosphorylation and cGAS-STING activation, although STING is **down-regulated** in this class), and myeloid inhibitory receptor CD300LF (inhibits MyD88- and TRIF-mediated TLR signalling via PTPN6/SHP-1 and PTPN11/SHP-2, both **up-regulated** in this class). Taken together with class-shared alterations, the CTRL0 class thus apparently favours STAT1/2-IRF3-type I IFN-ISG signalling, including those which oppose MyD88/TRIF-mediated TLR signalling and IFNγ expression while favouring NLRP3 (but not AIM2 or cGAS-STING)-mediated inflammasome assembly and various antiviral mechanisms. Another antiviral protein **unique** to the CTRL0 condition is SAMHD1, whose dNTPase activity suppresses cellular dNTP levels to prevent reverse transcription (though also regulating DNA precursor pool size) and promotes DNA degradation at stalled replication forks to prevent chronic type I IFN induction.

The IFN-dominated signature (including STAT1/2-IRF3-type I IFN-ISG, likely NLRP3 rather than AIM2 inflammasome activity, and antiviral mechanisms) is perhaps even more prominent in the IRIS0 class (Table 17). However, a major difference is up-regulation of TFs STAT3 and STAT6, suggesting simultaneous IL-4-mediated cytokine signalling not present in the CTRL0 class. It is known that STAT3 enhances the IFN response, induces an anti-inflammatory myeloid program (including suppression of IL-6, TNF α , IFN γ , MIP-1 β , and iNOS/NO production, while inducing ARG1 expression) and in T-cells opposes IL-6/IL-23-mediated induction of IL-17 production [8] [9] [10] [11]. STAT3 also enhances the acute-phase response, while inhibiting autophagy. Like STAT3, STAT6 enhances Th2-type signalling (e.g. expression of anti-apoptotic BCL2L1, not identified as dysregulated under either condition). During Mtb infection, two pathways regulate myeloid ARG1 expression: signalling via TLR-MyD88-STAT3 (induces G-CSF, IL-6, and IL-10, all of which favour ARG1 expression) and signalling via STAT6 (in alternately-activated myeloid cells) [8]. Interestingly, TGF β is known to up-regulate expression of both STAT3 [12] [13] and STAT6 [14], again favouring the hypothesis that this cytokine is elevated in the IRIS0 class. Cytokine IL-6, too, can signal via STAT1 and STAT3 [15], while IL-10 also signals via STAT3 [10]. When activated by IL-3, for example, STAT3 is also the major transducer of G-CSF signalling [16] (but IL-3 is known to be low at baseline in TB-IRIS developers). At least in tumour-associated macrophages, STAT3 and STAT6 (due to co-operative activation by IL-4 and IL-6 or IL-10) synergise to "potently up-regulate cathepsin secretion" by initiating the unfolded-protein response (UPR) [17], though the IRIS0 class exhibits no direct evidence of an activated UPR (*vide infra*).

Table 17 IRIS Group-specific Dysregulated Proteins Relevant to IFN Signalling

Protein	Function	Status	Hypothesised Outcome
OAS1	IFN-induced 2-5A synthesiser which is antiviral via RNase L activation and RNase L-independent mechanisms	unique	↑ antiviral activity
USP15	already discussed above: deubiquitinating enzyme with a role in enhancing TGF β signalling through inhibiting both TGFBR1 and SMAD degradation; additionally regulates NF κ B through de-ubiquitination of NF κ BIA and VCP-bound substrates	unique	↑ TGF β signalling ↑ NF κ B signalling
STAT3	phosphorylated in response to e.g. IFNs, IL-5, IL-6, IL-31, EGF, HGF, FGF, LIF, LEP, SCF, and BMP2 binds to IL-6-responsive elements in the promoters of various acute-phase reactants, but also regulates development of naïve Th into Th ₁₇ or T _{reg} , and inhibits autophagy (through inhibition of EIF2AK2/PKR)	up	↑ IFN response ↑ APR altered Th differentiation ↓ autophagy
STAT6	IL-4-induced involved in IL-3- and IL-4-mediated signalling, being key in executing IL-4-induced responses (e.g. expression of anti-apoptotic BCL2L1/BCL-XL, Th ₂ differentiation, and Ig class-switching)	unique	↑ Th2-type signalling

Intriguingly, regarding TFs apart from STATs, the master inflammation-regulating TF **NF κ B's subunit RELA** (p65) is **absent** from both classes (perhaps as an antiviral strategy, as it induces HIV LTR transcription [18]), while **MTFN** – which promotes NF κ B subunit dimerization and regulates NF κ B transcription factor activity – is **unique** in both classes, suggesting increased dimerization of non-RELA-containing (i.e. function-altered) NF κ B. Since I κ B-mediated NF κ B inhibition typically requires RELA, absence of the latter may suggest more constitutively-active NF κ B employing alternate subunits (or relative sensitisation to TNF α /DNA damage-induced apoptosis [18]). Mice lacking RELA undergo embryonic lethality (massive hepatocyte death due to being sensitised to TNFR1-mediated apoptosis [19], which is consistent with the above hypothesis that the IRIS0 class is more sensitive to externally-initiated cell death). *In vivo*, hematopoietic stem cells lacking RELA exhibit extreme dysfunction [20], likely due to the lack of RELA induction with serum response factor (SRF)-exhibiting genes (whose expression is required for myeloid chemotaxis, inside-out integrin activation, transmigration, phagocytosis, and cell survival [21] [22]).

3.3. Eicosanoid Cascade

Both neutrophil classes exhibit changes (Table 18) suggestive of decreased arachidonate provision (in agreement with Table 12 results) and LTB₄ production (usually required for normal neutrophil infiltration into inflammatory sites), perhaps due to impaired elicitation owing to hypothesised decreased TNF α signalling. Furthermore, sensitivity to LTB₄ also appears dampened.

Table 18 Common Dysregulated Proteins Relevant to Eicosanoid/Leukotriene Biosynthesis and Signalling

Protein	Function	Status	Hypothesised Outcome
ANXA1	Ca ²⁺ - and phospholipid-binding inhibits PLA2 "has anti-inflammatory/pro-resolving activity as an effector of glucocorticoid-mediated responses" also involved in granulocyte formyl peptide receptor activation and chemotaxis	up	↓ PLA2 activity ↓ arachidonate availability for downstream conversion to eicosanoids
LTA4H	Zn-dependent epoxide hydrolase which catalyzes the final step of proinflammatory LTB ₄ synthesis (i.e. conversion of LTA ₄ to LTB ₄ , a potent chemoattractant) also an aminopeptidase which degrades neutrophil chemoattractant proline-glycine-	down	↓ LTB ₄ production & resultant chemoattraction

	proline (PGP)		
LTB4R	LTB4- and extracellular ATP-responsive GPCR activates phosphatidylinositol-Ca2+ second-messenger system	down	↓ response to inflammatory leukotrienes/ATP

Additionally, an anticoagulant annexin, ANXA5 – which also inhibits PLA2 as well as PKC – is also up-regulated under both conditions. Another annexin, ANXA7, is simultaneously down-regulated under both conditions.

The CTRL0 class exhibits changes (not tabulated) additionally suggesting decreased leukotriene production: *down-regulated* ALOX5, which mediates the first step of pro-inflammatory leukotriene biosynthesis (a two-step conversion of arachidonate to LTA4). Furthermore, while arachidonate would normally stimulate p38 MAPKs to activate PLA₂, there appears to be decreased MAPKAPK2-mediated ALOX5-stimulation (Tables 7-9). Thus, CTRL0 neutrophils exhibit suppression of the eicosanoid cascade and specifically of the full molecular machinery for synthesis of and response to LTB₄ (perhaps with funnelling of limited residual arachidonate towards HETE/HPETE or prostanoid synthesis, though PGE₂ synthesis would be opposed by type I IFN). An additional enzyme (DHRS7) responsible for metabolizing various compounds (including prostaglandins, lipids, steroid hormones, retinoids, and xenobiotics) is *down-regulated* in the CTRL0 class, possibly suggesting decreased prostaglandin degradation. IRIS0 neutrophils also exhibit (Table 57) *down-regulated* ANXA6 (very similar to ANXA1, and implicated in prostaglandin synthesis regulation) and *up-regulated* ANXA11 (a Ca²⁺-dependent phospholipid- and calyculin-binding protein, recognised during autoimmune responses). The IRIS0 class may exhibit somewhat-preserved PLC-mediated minor arachidonate provision, and ALOX5 is not identified as level-altered (i.e. it is likely not down-regulated, although its activity-enhancing chaperone COTL1 is *down-regulated*). It is thus possible that the leukotriene, HETE/HPETE, and prostanoid arms of eicosanoid synthesis are somewhat preserved in this class. Recall from baseline literature (Chapter 1) that TB-IRIS developers exhibit elevated HETE production by the LOX arm of the eicosanoid cascade.

3.4. Neutrophil Inflammatory/Antimicrobial Response Inhibition/Activation

3.4.1. Neutrophil Inhibitory Receptors

A number of key inhibitory neutrophil receptors are *down-regulated* in or *absent* from both neutrophil classes (Table 19) suggesting de-repressed insulin signalling and fatty acid (FA) biosynthesis (CEACAM1) as well as an increased capacity for inflammatory responses (e.g. de-repressed TLR-4 signalling, IL-1β and ROS production, and NK/T-cell activation – although type I IFN would oppose TLR-4 and IL-1β activity) provided the correct stimuli (and once PTPN6/SHP-1 and PTPN11/SHP-2 stop opposing neutrophil-activating signalling cascades). Additionally suggested is increased sensitivity to pro-inflammatory angiotensin II, which induces NFκB-dependent production of cytokines such as G-CSF (which, recall, signals via STAT3), IL-8, and MIP-2, and p38 MAPK-dependent respiratory burst activity, as well as favouring NETosis and fibrosis [23] [24] [25] [26] [27]. Normally, hypotension stimulates conversion of angiotensinogen by renal renin to angiotensin I, which is converted by pulmonary ACE (and myeloid proteases) to angiotensin II (vasoconstrictive, increases cardiac sympathetic tone, and increases renal sodium and water reabsorption, but also induces NFκB-dependent cytokine production and p38 MAPK-dependent respiratory burst, NETosis, and fibrosis). Both classes exhibit *up-regulated* SERPINA3, preventing formation (and likely decreasing availability of) angiotensin II by myeloid proteases. During inflammation, the kallikrein system liberates bioactive (inflammatory, antimicrobial) peptide bradykinin from kininogen, and it is subsequently inactivated by three kininases: ACE, aminopeptidase P, and carboxypeptidase N (ACE inhibition produces bradykinin-induced bronchoconstriction, dry cough, and, in severe cases, angioedema). Both classes also exhibit *absent* immunoregulatory PECAM1, suggesting increased sensitivity of the bradykinin receptor BDKRB2 [28].

Table 19 Shared Dysregulated Neutrophil Inhibitory Receptors

Protein	Function	Status	Hypothesised Outcome
CEACAM1	co-inhibitory receptor in activated neutrophils, TLR4-SYK-CEACAM1 complex recruits PTPN6/SHP-1 for SYK dephosphorylation thereby suppressing ROS production, lysosome disruption, and inflammasome-mediated IL-1β production favours insulin degradation and suppression of FA synthesis promotes angiogenesis	down	↑ TLR-4 signalling ↑ ROS & IL-1β production ↑ facilitation of insulin-mediated anabolism
CD101	Immunoglobulin superfamily member inhibits activated T-cell IL-2 and IL-2RA expression	absent	↑ permissiveness to T-cell activation, proliferation
HLA-E	non-classical MHC-Ib; participates in self-tolerance with β2-microglobulin binds VL9 peptides from classical MHC-Ia complex forms inhibitory ligand for NK cell KLRD1-C1 during stress, HLA-E-β2M instead binds peptides derived from stress-induced chaperones and non-classical MHC-Ib HLA-G complex is then an activating ligand for NK cell KLRD1-C2 also presents pathogen peptides to unconventional CD8 ^α T-cells	absent	↑ potential for autoreactive NK cell responses ↑ overall potential for NK cell activation (perhaps part of antiviral strategy, also to prevent viral tolerance)

	viral infection often down-regulates MHC-I molecules while HLA-E presents viral peptides to NK cells, inducing tolerance		
AGTRAP	inhibits type-1 angiotensin II receptor-mediated signalling and gene transcription (favours receptor internalisation and desensitization)	absent	↑ responsiveness to angiotensin II

The CTRL0 class (Table 20) uniquely exhibits *up-regulation* of CD300LF (perhaps induced by higher levels of/sensitivity to vitamin D or another known inducer), again supporting *inhibition of TLR-MyD88/TRIF-NFκB signalling* even in CTRLs at baseline. Despite suggested STAT3 and STAT6 signalling, the IRIS0 class (Table 21) does not exhibit such up-regulated CD300LF levels. However, *absence* of PTPN1 and RETN *down-regulation* further support de-repressed growth factor/insulin signalling, while down-regulation of additional key neutrophil inhibitory receptors suggests even more *pronounced capacity for unrestrained inflammation* (phagocytosis, respiratory burst, NETosis), provided the correct stimuli (and once PTPN6/SHP-1 and PTPN11/SHP-2 stop opposing neutrophil-activating signalling cascades).

Table 20 Control Group-common Dysregulated Neutrophil Inhibitory Receptors

Protein	Function	Status	Hypothesised Outcome
CD300LF (CD300a- and f-related)	enhances uptake and clearance of phosphatidylserine-expressing apoptotic cells and negatively regulates MyD88/TRIF-mediated TLR signalling-mediated activation of NFκB through activation of PTPN6/SHP-1 and PTPN11/SHP-2 (protein phosphatases which negatively regulate signalling cascades; up under both conditions) may also be an IL-4 co-receptor enhancing IL-4 and IL-13-induced signalling [29] (in dendritic cells, active vitamin D, which programs them to become tolerogenic, induce increased CD300LF expression, among other genes [30]) (treatment with CD300LF ligands blocks TLR2, 3, 4, and 9-mediated expression of IL-8 and MMP-9, via inhibiting MyD88/TRIF and thus NFκB signalling through SHP-1 recruitment [31])	up	↓ TLR signalling ↑ IL-4 signalling ↑ resolution/skewing

Table 21 IRIS Group-common Dysregulated Neutrophil Inhibitory Receptors

Protein	Function	Status	Hypothesised Outcome
LILRB2 [32]	inhibits FcγRIIA- (but not FPR)-mediated neutrophil activation for early attenuation of ROS production major ligand for LILRB2 is soluble or cell-associated MHC-I (including cis-binding) ligation produces ITIM phosphorylation, SHP-1 recruitment, and Syk inhibition highest affinity for non-classical HLA-G (whose expression increases under pathological conditions), and avidity is highest for HLA-A, then -B, then -C ligation by "soluble HLA-G inhibits phagocytosis of opsonised bacterial particles"	down	de-repression of: - FcγRIIA-mediated ROS production - HLA-inhibited phagocytosis of opsonised particles - anti-inflammatory activities in response to unfolded proteins (provided these types of stimuli are indeed present)
SIGLEC-5 [32]	ligation by major ligand, sialic acid (ubiquitous in the ECM and on host cell surfaces, including cis-binding), causes ITIM phosphorylation, SHP-1/2 recruitment, and inhibition of signalling cascades provides baseline level of inflammatory inhibition, including of NETosis ligation of SIGLEC-5 and -9 are known to dampen NETosis	down	de-repression of: - neutrophil inflammation - NETosis

3.4.2. Neutrophil Chemotaxis, Adhesion, & Extravasation

Both neutrophil classes exhibit changes (Table 22) suggestive of neutrophils *not* predisposed to chemotaxis, adhesion, extravasation, or migration through the ECM, and less capable of taking up iC3b-opsonised particles. Alteration of certain protein levels (e.g. CEMIP2) suggest a more tissue-healing than inflammatory phenotype, in alignment with earlier results suggesting elevated TGFβ production (*vide supra*).

Table 22 Common Dysregulated Proteins Relevant to Neutrophil Chemotaxis, Adhesion, and Activation

Protein	Function	Status	Hypothesised Outcome
GLG1	binds E-selectin on activated endothelia also binds FGF	absent	↓ slow endothelial rolling
ITGB2	integrin beta-chain binds E-selectin on activated endothelia contributes to NK cell cytotoxicity beta-chain combines with different alpha-chains to form integrin e.g. ITGAM/ITGB2 (Mac-1/CR3) and ITGAX/ITGB2 (CR4) are receptors for iC3b and fibrinogen ITGAM/ITB2 binds fibrinogen gamma-chain, ICAM1, and factor X ITGAX/ITGB2 binds fibrinogen alpha-chain	down	↓ slow endothelial rolling
ITGAM	integrin alpha-chain + ITGB2: adherence to endothelial ICAM1, iC3b, fibrinogen, factor X phagocytosis of complement-opsonised particles	down	↓ adherence to ICAM1 (↓ arrest of rolling) and fibrinogen ↓ phagocytosis of iC3b-opsonised particles
ITGAX	integrin alpha-chain + ITGB2: neutrophil adherence to iC3b, fibrinogen for cell-cell adhesion (and chemotaxis) during inflammation neutrophil adherence to activated endothelium phagocytosis of complement-coated particles	absent	↓ adherence to fibrinogen ↓ chemotaxis and adhesion ↓ phagocytosis of iC3b-opsonised particles
PARVB	adapter protein with role in "integrin signaling via ILK" also contributes to GTPase (RAC1, CDC42) activation by GEFs (e.g. ARHGEF6) "role in cell adhesion, cell spreading, establishment or maintenance of cell polarity, and cell migration"	absent	↓ cell motility, adhesion, integrin signalling

LGALS3BP	favours integrin-mediated cell adhesion but likely also has antiviral/antitumour activity	unique	↑ antiviral activity
THBS1	glycoprotein mediating interaction with other cells/the ECM (e.g. heparin) CD36 ligand with anti-angiogenic effect	absent	↓ binding to other cells/matrix ↑ angiogenesis?
PECAM1	requisite cell adhesion molecule for transendothelial migration during inflammation interaction with CD177 contributes to neutrophil extravasation homophilic interaction with PECAM1 on neighbouring cells prevents phagocytosis of those cells (but favours uptake of apoptotic leukocytes)	absent	↓ extravasation
CEMIP2	surface hyaluronidase (cannot cleave ECM chondroitin or dermatan sulphate) regulator of angiogenesis	absent	↓ hyaluronan cleavage ? ↓ motility within ECM

Other cytoskeletal and signalling proteins with a role in cell morphology and motility (but which are also involved in various other processes) also exhibit level alterations (Table 57).

The CTRL0 class exhibits additional unique changes (Table 23) - although suggestive of preserved capacity for transient, rapid rolling via SELL – supporting neutrophils *not* predisposed to adhesion/arrest or (possibly) additional neutrophil recruitment. High levels of SELL may actually indicate neutrophils which have not been activated (i.e. are not actively shedding SELL). The IRIS0 class (Table 24) does not exhibit level-altered SELL, but may experience more pronounced alterations in integrin signalling (including priming for adhesion and transmigration). Changes unique to this class generally suggest decreased (B1 and B2) integrin activation, adhesion, and signalling (including via MAPK, Ras, and ARF6), but increased terminal arrest and transmigration effectors (vinculin, talin-1), perhaps in an effort to compensate for defective integrin activity. Although *non-integrin* adhesive receptors are up-regulated (e.g. for homotypic CEACAM interactions), ligands or mechanistic molecules required for adhesion via such receptors (e.g. ADGRE5, CD177) are absent (e.g. CD55, PECAM1) or down-regulated (e.g. ITGB2, ITGAM). Thus, CD177-mediated degranulation, respiratory burst, and TNFα activation are likely ineffective, and other indications (lack of inflammation-induced ADGRG3 up-regulation, and down-regulation of ARF6-mediated PLD activation) also suggest a decreased capacity for degranulation, phagocytosis, respiratory burst, hydrolytic enzyme activity, and overall bactericidal activity.

Table 24 Control Group-specific Dysregulated Proteins Relevant to Neutrophil Chemotaxis, Adhesion, and Activation

Protein	Function	Status	Hypothesised Outcome
SELL	L-selectin mediates Ca ²⁺ -dependent adhesion to neighbouring cell glycoproteins e.g. lymphocytes to endothelia	unique	↑ transient endothelial rolling/tethering
ICAM3	constitutively expressed by leukocytes key ligand for ITGAL/ITGB2 (LFA-1, which recognises ICAM1-4) → adhesion and signalling, also efferocytosis of apoptotic neutrophils	down	↓ adherence to other leukocytes ↓ contribution to local neutrophil recruitment/binding

Table 25 IRIS Group-specific Dysregulated Proteins Relevant to Neutrophil Chemotaxis, Adhesion, and Activation

Protein	Function	Status	Hypothesised Outcome
CEACAM8	surface glycoprotein which binds other CEACAMS (or neutrophil CEACAM8) Ca ²⁺ -dependent adhesion	up	↑ CEACAM-mediated adhesion capacity
ADGRE5	adhesive GPCR which mediates cell-cell and cell-ECM interactions likely roles in recruitment/migration, adhesion (incl. to CD55 and chondroitin sulphate), and activation esp. early adhesion signalling after activation	up	↑ CD55/ECM-mediated adhesion capacity (CD55 is absent under the IRIS0 condition)
ADGRG3/GPR97	adhesive GPCR abundant on terminally-differentiated and tissue-infiltrating neutrophils [33] unknown ligands regulates (at least endothelial) migration (via RhoA, CDC42) upregulated during inflammation; ligation increases bacterial phagocytosis, ROS production, proteolytic enzyme activity, and bactericidal activity, accompanied by increased MAPK activation and IκBα phosphorylation [33]	absent	? ↓ systemic inflammation ↓ capacity for activation of phagocytosis, respiratory burst, hydrolytic enzyme activity, bactericidal activity ↓ capacity for MAPK activation and IκBα phosphorylation
CD177	GPI-linked neutrophil surface glycoprotein roles in neutrophil adhesion/transmigration (e.g. via PECAM-1 binding, opposition of β2 integrin internalisation, and PRN3 surface display to enhance endothelial junction and vascular integrity) roles also in neutrophil activation (e.g. degranulation, ROS generation) and – in association with ITGAM/ITGB2 – neutrophil-mediated TNFα activation	unique	↑ capacity for adhesion and transmigration (if PECAM1 weren't absent, and ITGB2 weren't down-regulated) ↑ neutrophil degranulation and ROS generation (but down-regulation of both ITGAM and ITGB2 suggest not so much for TNFα activation)
MYLK	Ig superfamily member Ca ²⁺ /calmodulin-dependent kinase which phosphorylates myosin regulatory light chain to facilitate interaction with actin roles in vascular permeability, leukocyte motility and transmigration, apoptosis, and wound healing phosphorylates (activates) PTK2B/PYK2 to activate ITGB2 (essential for neutrophil transmigration during ALI)	absent	↓ ITGB2 activation, transmigration
MAP2K3	implicated in the integrin pathway	down	↓ integrin signalling?
TLN1	co-distributes with integrins to assist attachment of cells to other cells/ECM concentrated in sub-adhesive areas role in spreading and migration interacts with β1-integrin, VCL, actin, etc.	up	↑ arrest, adhesion, transmigration
VCL	vinculin associated with adhesive areas helps anchor F-actin to membrane for adhesion regulates surface E-cadherin expression and enhances its	up	↑ adhesion

	mechanosensing likely roles in morphology and locomotion		
CYTH1	cytohesin-1 membrane-associated protein that regulates intracellular trafficking "regulates adhesiveness of integrins at the leukocyte membrane": "CYTH1-deficient cells showed a reduced integrin β 1 activation response" promotes activation of ARF1, 5, and 6 through exchange of GDP with GTP a related pathway is PLD signalling	absent	$\downarrow$ β 1 integrin activation $\downarrow$ ARF6 activation
ARF6	part of Ras superfamily (i.e. GTPase) membrane-associated, regulates vesicle trafficking, membrane remodelling, and actin remodelling	absent	$\downarrow$ ARF6-mediated PLD activation and degranulation + oxidative burst
CIB1	Ca2+-binding protein that interacts with many partners (e.g. platelet integrin- α IIb β 3, FAK, PKD, etc.) regulates migration, angiogenesis, apoptosis, etc. negatively regulates α IIb β 3-integrin activation during thrombin stimulation, preventing platelet aggregation enhances FAK1 activity and is required for its targeting to focal adhesions enhances migration on fibronectin in a CDC42-dependent manner inhibits stress-activated MAPK signalling translocates sphingosine kinase (SPHK1) to membrane, preventing TNF α -induced apoptosis during ischemia-induced angiogenesis, activates PAK1 and ERK1/2 signalling	absent	$\downarrow$ FAK (?integrin)-mediated adhesion
RASGRP2	Ca2+-binding protein with GEF domain stimulated by DAG and Ca2+, can activate Ras, Rap1/Ras3, and possibly RRAS, NRAS, and KRAS (not HRAS) functions in neutrophil adhesion, likely via inside-out integrin activation	absent	$\downarrow$ (?integrin)-mediated adhesion

3.4.3. Activation & Microbicidalilty

Both neutrophil classes exhibit changes (Table 26) suggestive of increased neutrophil activation but suppression of Th1/cytotoxic/Th17-type and support of Th2/T_{reg}-type lymphocyte responses (provided that galactoside ligands for constitutive and TNF α /IL-8-inducible LGALS9 are present). Indeed, ARG1 (mentioned earlier to be up-regulated) can deplete extracellular arginine (thereby suppressing NK and T-cell activity while promoting ILC2-mediated Th2-type pulmonary inflammation) and intracellular arginine (thus competing with NOS and decreasing NO production). As hypothesised above, neutrophil motility/extravasation and respiratory burst capacity appear to be decreased, but there is increased lysosomal hydrolase (e.g. cathepsin protease) expression, and – concomitantly – expression of SERPIN anti-proteases (APRs). It is unclear whether lysozyme and cathelicidin are down-regulated due to not being elicited (e.g. the latter by IFN γ , TNF α , and vitamin D) or due to having been exocytosed (e.g. degranulation).

Table 26 Shared Dysregulated Proteins Relevant to Neutrophil Chemotaxis, Adhesion, and Activation

Protein	Function	Status	Hypothesised Outcome
LGALS9 [34] [35]	galactoside-specific helps activate macrophages (incl. IL-1 β secretion) induces ERK1/2 phosphorylation and thus myeloid IL-6, IL-8, IL-12, and CCL2 production induces DC maturation and migration also inhibits mast cell degranulation and induces their apoptosis inhibits NK cell function promotes stromal suppression of T-cell proliferation induces – via TIM3 ligation – Th1 death induces – via CD44 and SMAD3 – FOXP3 expression and T _{reg} expansion and function induces post-viral CD8 ⁺ T-cell apoptosis via PDIA1 (also up-regulated) ligation alters Th2 membrane redox state to enhance migration	unique	$\uparrow$ myeloid activation if ligands are present $\uparrow$ neutrophil/monocyte activation incl. IL-1 β , IL-6, IL-8, and IL-12 production $\downarrow$ NK, Th1, and CD8 ⁺ T activation $\uparrow$ Th2 migration
NCF1 and 2	subunits of neutrophil NADPH oxidase (for phagosomal superoxide burst) with cytochrome b558 are required for NADPH oxidase activation associated with chronic granulomatous disease	down	$\downarrow$ respiratory burst capacity
CTSB	cathepsin B lysosomal cysteine protease intracellular protein turnover (including for MHC-I antigen processing) role in proteolytic processing of amyloid precursor protein (APP)	unique	$\uparrow$ protease capacity (for pathogen or host proteins) $\uparrow$ CD8 ⁺ -stimulating antigen processing
CTSD	cathepsin D acid protease pepsin-like activity (including for MHC-I antigen processing) roles in protein turnover, proteolytic activation of hormones and growth factors, and APP processing	up	$\uparrow$ protease capacity (for pathogen or host proteins) $\uparrow$ CD8 ⁺ -stimulating antigen processing
CTSZ	cathepsin Z lysosomal cysteine proteinase roles in protein turnover and peptide hormone metabolism	unique	$\uparrow$ protease capacity (for pathogen or host proteins)
CD82	associates with CD4/CD8 to deliver co-stimulatory TCR/CD3 signals	absent	$\downarrow$ T-cell activation
SERPINA1	a.k.a. alpha-1-antitrypsin secreted serine protease (elastase, plasmin, thrombin, trypsin, chymotrypsin, plasminogen activator) inhibitor protects LRT from elastase-mediated damage (e.g. emphysema)	up	acute-phase reactant that negatively feeds back to help prevent inflammatory damage
SERPINA3	plasma protease inhibitor inhibits neutrophil CTSG and mast cell chymase (both of which convert angiotensin-I to active angiotensin-II)	up	acute-phase reactant that negatively feeds back to help prevent inflammatory damage
SERPINB9	serine protease inhibitor (e.g. of granzyme B) overexpression may interfere with cytotoxicity of CD8 ⁺ T-cells	unique	acute-phase reactant that negatively feeds back to help prevent inflammatory

			damage
S100A11	may function in motility, invasion, and tubulin polymerization	up	↓ motility and invasion
PSAP	saposin precursor, cleaved to generate lysosomal saposins A-D activate lysosomal hydrolases for degradation of glycosphingolipids [36]	up	↑ activation of lysosomal hydrolases ↑ sphingolipid catabolism
LYZ	antibacterial lysozyme, cleaves PDG	down	possibly down due to exocytosis, but otherwise ↓ antibacterial activity
CAMP	antimicrobial peptide cathelicidin, binds LPS in macrophages, expression is induced by IFN γ and TNF α recall that NOX2, apart from oxidant-based microbicidal activity and enhancement of granule protease functions, is required for TLR-2-dependent inflammation and vitamin D-mediated anti-Mtb activity (via cathelicidin expression)	down	possibly down due to exocytosis, but otherwise ↓ mycobactericidal activity

The CTRL0 class exhibits additional unique changes (Table 27) supporting increased activation by LGALS3 galactoside ligands or IgE (if present), decreased motility/extravasation and respiratory burst capacity, and uncertainty regarding lower granule protein levels being reflective of decreased production/stability or exocytosis. Perhaps due to IFN signalling, there may be decreased IL-6-mediated induction of some APRs. Although up-regulation of S100A9/calprotectin would normally suggest increased neutrophil activities, this would require intact PI3K-AKT and MAPK pathways and availability of arachidonate for NOX activation (all of which are suggested above to be inhibited in this context). Down-regulated S100A12 may de-repress MMP (including MMP-9) activity. The IRIS0 class exhibits additional unique changes (Table 28) supporting increased protease activity (with the notable exception of CTSS and lysosomal ceramidase, perhaps due to degranulation), but also decreased restraint of lectin-based complement activation, increased availability of neutrophil recruitment signals, and possible increased vimentin expression.

Table 27 Control Group-specific Dysregulated Proteins Relevant to Neutrophil Chemotaxis, Adhesion, and Activation

Protein	Function	Status	Hypothesised Outcome
ORM1	key circulating acute phase reactant transports hydrophilic ligands (including pharmaceutical drugs) modulates immune activity during acute inflammation	down	suggests participants are not acutely inflamed (APR mediated by IL-6, which may be down-regulated due to IFN signalling)
LGALS3	galactose-specific lectin (galectin-3) which binds IgE may contribute (with α -3/ β -1 integrin and CSPG4) to endothelial migration during acute inflammation is involved in "neutrophil activation and adhesion, monocyte recruitment, opsonization of apoptotic neutrophils, and activation of mast cells"	unique	↑ myeloid activation if ligands are present ↑ neutrophil activation/adhesion ↑ efferocytosis
CYBB	beta chain of cytochrome b245 critical component of membrane-bound NADPH oxidase	down	↓ respiratory burst capacity
PRG3	proteoglycan 3 cytotoxic/cytostimulatory effects resembling those of MBP/PRG2 stimulates neutrophils ROS and IL-8 production (and basophil histamine and LTC4 production)	absent	↓ cytotoxicity ↓ stimulation of neutrophil ROS and IL-8 production
S100A9	exists mainly as calprotectin (S100A8/A9 complex), various functions: may inhibit casein kinase antifungal, antibacterial (e.g. via chelating Zn ²⁺) regulates inflammation can induce neutrophil chemotaxis, adhesion, phagocytosis (via SYK, PI3K-AKT, ERK1/2), degranulation (via MAPK) intracellularly facilitates arachidonic acid trafficking and metabolism, activation of neutrophil NADPH oxidase (via facilitating its assembly at the membrane and providing arachidonate – an essential cofactor) extracellularly (secreted) is proinflammatory, antimicrobial, oxidant-scavenging, apoptosis-inducing (e.g. leukocyte recruitment, favouring chemokine/cytokine production, regulating adhesion and migration, alarmin/DAMP that binds TLR-4 and AGER to activate MAPK and NF κ B, can induce autophagy and apoptosis via ROS-mediated cross-talk between mitochondria and lysosomes, but opposes neutrophil apoptosis) transnitrosylase activity: oxLDL-insudes S-nitrosylation of GAPDH iNOS-calprotectin transnitrosylation complex is proposed to direct inflammatory stimulus-dependent S-nitrosylation of various targets (e.g. ANXA5, EZR, MSN, VIM)	up	↓ casein kinase activity ↑ microbicidal activity ↑ neutrophil chemotaxis, adhesion, phagocytosis, degranulation (though mediating PI3K-AKT and MAPK pathways are likely suppressed in these cells) ↑ calprotectin functions (e.g. NADPH oxidase activation when arachidonate is available)
S100A12	likely involved in Ca ²⁺ -dependent signal transduction regulates cytoskeleton and thus neutrophil activity proinflammatory: recruits leukocytes, promotes chemokine/cytokine production, and regulates adhesion and migration includes an antimicrobial peptide binds Ca ²⁺ , Zn ²⁺ , and Cu ²⁺ (Zn ²⁺ chelation inhibits MMP2, 3, and 9 activity) acts as an extracellular alarmin/DAMP and binds AGER to activate MAPK and NF κ B signalling pathways (for cytokine production and up-regulation of adhesion molecules ICAM1 and VCAM1) can stimulate mast cell degranulation and activation to generate additional proinflammatory mediators	down	↓ microbicidal activity ↓ neutrophil recruitment, adhesion, and activation (incl. chemokine/cytokine production) de-repression of MMP activity

Table 28 IRIS Group-specific Dysregulated Proteins Relevant to Neutrophil Chemotaxis, Adhesion, and Activation

Protein	Function	Status	Hypothesised Outcome
CTSC	lysosomal cysteine proteinase activates many leukocyte serine proteinases (e.g. elastase, CTSG, granzymes A and B), neuraminidase, and factor XIII	up	↑ hydrolase activity, esp. in combination with lysosomal acidification findings above ↑ factor XIII cleavage
CTSS	lysosomal cysteine proteinase (can function as an elastase) may process peptides for MHC-I presentation (also responsible for "removal of the invariant chain from MHC-II)	absent	↓ proteinase activity ↓ antigen presentation

	"involved in collagen chain trimerization"		
CD55	surface glycoprotein accelerates decay of bound complement proteins – e.g. C3b, C4b - to prevent host damage also interferes with the ability of C3b and C4b to convert C2 and factor B to active C2a and Bb, preventing formation of C3bBb and C4b2a (amplification convertases, i.e. C3 and C5 convertase) also exhibits antigens which are part of the Cromer blood group system, is exploited as a receptor for certain coxsackie- and echoviruses, and is implicated in the lectin-activated complement pathway	absent	absence (perhaps due to overall cellular antiviral strategy) suggests a reduced capacity of these neutrophils to restrain lectin-based complement activation
MCEMP1	2005-identified membrane protein thought to be involved in mast cell degranulation	absent	↓ degranulation
DDT	D-dopachrome decarboxylase homologue of the cytokine macrophage inhibitory factor (MIF), with which it synergises to increase neutrophil recruitment [37] at least in cervical cancer, inactivity of DDT and MIF restores E-cadherin expression while suppressing expression of vimentin and phosphorylation of AKT and GSK3-β [38]	up	↑ neutrophil recruitment ↓ E-cadherin expression ↑ vimentin expression ↑ phosphorylation of AKT and GSK3-β
ASAH1	lysosomal acid ceramidase which hydrolyses ceramides to produce sphingosine and free FA, and thus sphingosine-1-phosphate proteolytic processing of the ASAH1 preproprotein generates subunits which dimerize to form the mature enzyme mutations in this gene are associated with lysosomal storage disorder	absent	↓ capacity to degrade exogenous and endogenous ceramides

In agreement with TB-IRIS literature (Chapter 1), various indicators of altered iron metabolism are evident at baseline. Iron availability is typically decreased via sequestration during the acute-phase response (as well as during chronic inflammation) to restrain microbial growth. Both classes exhibit *down-regulated* NGAL (likely due to exocytosis), which sequesters iron-containing microbially-produced siderophores to prevent their uptake into microbes. The levels of two other iron transporting proteins are also altered in both classes: lactotransferrin (LTF, found in neutrophil secondary granules) is *down-regulated* and serotransferrin (TF) is *absent* (again, these findings may be due to exocytosis). LTF contributes iron-sequestering antimicrobial function to body secretions while TF carries iron from the GIT, RES, and liver to other body cells (i.e. "from sites of absorption and heme recycling to sites of storage and utilisation"). Therefore, if decreased intracellular levels of these proteins are due to exocytosis, this suggests increased microbistatic iron sequestration in both classes, as well as increased iron transport from sites of heme recycling (e.g. myeloid cells) to sites of storage and utilisation. Only the CTRL0 class exhibits *unique* heavy chain of ferritin (FTH1), which stores intracellular iron in a soluble, non-toxic, readily-available form. No additional general iron-related protein alterations are noted in the IRIS0 class.

3.5. Carbon-Energy Metabolism (including protein homeostasis and integrated nitrogen-sulfur-B-vitamin and redox/xenobiotic/glyoxal metabolism)

3.5.1. Glycogen Metabolism

Both classes exhibit changes (not tabulated) suggesting simultaneously decreased glycogen synthesis (*down-regulated* PGM1) and catabolism (*down-regulated* PHKG2 and PYGL), although the former may be in service of preserving glucose for entry into glycolysis/the pentose phosphate pathway (PPP). The CTRL0 class exhibits additional unique changes supporting decreased glycogen synthesis (*down-regulated* GYS1) and catabolism (*down-regulated* AGL). The IRIS0 class exhibits no additional changes directly relevant to glycogen metabolism.

3.5.2. Glycolysis, the PPP, and Nucleotide Salvage/Synthesis (results not tabulated)

Both classes exhibit a surprising major change suggesting inhibition of the first committing step of glycolysis (*absent* PKFM, a key regulatory enzyme), perhaps suggesting avoidance of its initial (energy-consuming) phase, or *diversion of glucose into the PPP. However, the subsequent glycolytic step (production of G-3-P + DHAP) appears somehow enhanced in the CTRL0 class (*up-regulated* ALDOC), which would require pre-existing build-up of the PKFM product (F-1,6-BP), which would indeed inhibit PKFM activity. Indeed, both classes exhibit enhancement of G-3-P and DHAP inter-isomerisation (*up-regulated* TPIS) and of post-DHAP/G-3-P glycolytic steps: formation of PEP (*up-regulated* ENOA, also anti-thrombotic and immunoglobulin-eliciting) and its conversion to pyruvate + ATP (*up-regulated* KPVM). Given suppression of the initial glycolytic steps, substrates for G-3-P synthesis (and the latter half of glycolysis) may instead derive from ribose-5-P, a product of the PPP (and ultimately of pyrimidine nucleoside breakdown product ribose-1-P). Indeed, in both classes, the enzyme converting ribose-1-P to ribose-5-P (PGM2) is *up-regulated*.

It is only in the CTRL0 class that LDHA is *up-regulated*, suggesting conversion of pyruvate + NADH to lactate + NAD under HIF-1 α -activating anaerobic conditions (or another limiting factor preventing oxphos from meeting ATP requirements of rapidly-metabolising cells). Indeed, HIF-1 α would also induce expression of glycolytic enzymes and pro-inflammatory genes (e.g. IL-1 β). Recall that regeneration of NAD (usually by the electron transport chain) is also required to maintain glycolytic flux: this is usually achieved by the mitochondrial respiratory chain complex III FAD-dependent re-oxidation of G-3-P to DHAP, but can also be achieved by reducing pyruvate to lactate). It is unclear why the IRIS0 class would not also be experiencing hypoxic HIF-1 α -mediated LDHA induction, unless CTRL0 class myeloid cells are relatively more activated and therefore create a more hypoxic inflammatory environment.

*In both classes, however, changes suggest decreased entry of G-6-P into the PPP (*down-regulated* G6PD, also the major mechanism for regenerating NADPH, without which glutathione cannot be maintained in its reduced state (GSH), and cellular vulnerability to oxidative stress becomes significantly increased). And yet, similarly to the glycolytic situation, *subsequent* steps of the PPP, such as formation of ribulose-5-P + NADPH, are enhanced (*up-regulated* 6PGD), perhaps to compensate for NADPH regeneration. Again, this would require substrate provision, but clearly not G-6-P, and likely not from glycolytic intermediates (e.g. G-3-P), which under these circumstances would be required to maintain glycolytic flux. Indeed, suppression of G-3-P conversion to PPP intermediate ribose-5-P is suggested (*down-regulated* vitamin B1-dependent TKT, which also performs many substrate interconversions within the PPP).

It is notable that the interconvertible nature of G-3-P, ribulose-5-P, and ribose-5-P links glycolysis, the PPP, and nucleotide synthesis/catabolism. Ribulose-5-P can convert to PRPP for **purine** or NAMPT-mediated NAD biosynthesis (from tryptophan), or to ribose-5-P for **pyrimidine** biosynthesis (and/or funnelling into glycolysis/the PPP). Thus, glycolytic and PPP intermediates *could* derive from nucleotide breakdown products (or vice versa). At least in the IRIS0 class, however, conversion of ribose-5-P intermediates to G-3-P is suppressed (*down-regulated* TALDO1). This may suggest a tension between maintaining glycolytic/PPP flux and pyrimidine synthesis, perhaps due to oxidative DNA damage at the same time as increased cellular ATP/NADPH requirements. At least in the CTRL0 class, NAD biosynthesis from tryptophan is enhanced (*up-regulated* NAMPT), which is *unlikely* a consequence of IFN-mediated signalling (which is also strongly active in IRIS0 neutrophils). Rather, the IRIS0 class may experience tryptophan depletion, or somehow already have sufficient NAD reserve despite no obvious reason for a decrease in NAD demand (e.g. generated by the electron transport chain, although this seems unlikely given findings below).

Adenine and guanine are purine nucleotide bases, while thymine, cytosine, and uracil are pyrimidine nucleotide bases. Most susceptible to oxidation is guanine (which has a lower redox potential): 8-oxoguanine is the most frequent DNA lesion observed under oxidative stress [39]. IFN-induced GBP5 uses GMP for oxidative elimination of microbes. Indeed, both classes exhibit changes suggestive of enhanced *de novo* guanine synthesis (*up-regulated* NAD-dependent and rate-limiting IMPDH2 and PUR9/ATIC, which catalyses the final two steps). Similarly, both classes exhibit *absent* adenine metabolism enzymes (also involved in energy metabolism): BPNT1 (converts PAPS to AMP) and AK1 (reversibly transfers phosphate between ATP and AMP). The CTRL0 class exhibits changes suggestive of favoured AMP synthesis and conservation: *down-regulated* AMPD (converts AMP to IMP, a highly-regulated branch-point in adenylate metabolism) and *up-regulated* AK2 (reversibly transfers phosphate groups among adenine nucleotides; required for AMP *de novo* biosynthesis). The IRIS0 class exhibits changes perhaps suggestive of conservation of AMP precursors (e.g. adenosine, Ap4A), perhaps for adenine nucleotide synthesis or to boost adenosine levels for signalling purposes: *absent* ADA2 (adenosine deaminase 2; lowers high adenosine levels, including extracellularly – when secreted), ADK (highly-conserved, major intracellular route of steady-state adenosine metabolism; transfers phosphate from ATP to adenosine to produce AMP and ADP; also regulates intracellular adenine nucleotide and extracellular adenosine levels), and NUDT2 (pyrophosphatase which hydrolyzes Ap4A to produce AMP and ATP, thereby regulating intracellular levels of dinucleotide Ap4A). Since increased extracellular adenosine is anti-inflammatory, its conservation by IRIS0 neutrophils may have an anti-inflammatory effect if made available extracellularly.

Regarding pyrimidine nucleotides: both classes exhibit *up-regulated* CMPK2 (phosphorylates UMP, CMP, and monophosphates of pyrimidine nucleoside analogs as part of mitochondrial nucleotide salvage) and *unique* NT5C3A (dephosphorylates CMP over 7-methylGMP), suggesting preferential catabolism of pyrimidine rather than purine intermediates, perhaps due to oxidative guanine damage (perhaps specifically of mitochondrial DNA – *vide infra*) in combination with adenine funnelling into ATP synthesis.

3.5.3. The Krebs/TCA Cycle

Above, we hypothesised that neutrophils may be more responsive to insulin and adrenergic agents. These would favour pyruvate dehydrogenase-mediated conversion of pyruvate into acetyl-CoA for entry into the TCA cycle or lipid synthesis.

Both classes exhibit changes suggestive of increased conversion of isocitrate + glutamate to α -KG + NADH (*up-regulated* IDHC and IDHP) but decreased subsequent conversion of α -KG to succinyl-CoA (*down-regulated* thiamine-dependent OGDH), which would result in build-up of α -KG that can also feed into other metabolic pathways (e.g. providing carbon skeletons for amino acid and nucleotide synthesis). High α -KG levels can result e.g. during exercise or nitrogen starvation, and actually inhibit ATP synthase function as well as regulating arginine and FA metabolism. Despite apparently favouring conversion of pyruvate to lactate (*vide supra*), the CTRL0 class, however, exhibits *unique* DLD, which is a component of three complexes: pyruvate dehydrogenase (in agreement with the suggested enhanced latter half of glycolysis and subsequent acetyl-CoA production), α -KG dehydrogenase (which would suggest improved capacity relative to the IRIS0 class to convert built up α -KG to succinyl-CoA), and branched-chain amino acid-dehydrogenase (for catabolism of valine and leucine).

Both classes also exhibit *up-regulated* ME2, which converts malate to pyruvate + NAD(P)H. Although the steady-state functions of malic enzymes are poorly understood, ME2 may contribute to pyruvate provision for TCA flux maintenance "when glucose is limiting" [40]. If malate derives from the TCA cycle, however, it is unclear at which point carbons potentially exiting the TCA cycle (in the form of malate and α -KG) are replenished, but it would have to be prior to malate conversion, at the succinate/fumarate/malate stage. Substrates other than pyruvate which can replenish the TCA include: aspartate (aspartate transaminase generates oxaloacetate), glutamate (glutamate dehydrogenase generates α -KG itself), adenylosuccinate (adenylosuccinate lyase generates fumarate), and FA (β -oxidation generates succinyl-CoA). Since aspartate-derived oxaloacetate is a step ***too* early to account for lost α -KG, adenylosuccinate-derived fumarate would be more useful, and indeed, adenylosuccinate arises during interconversion of IMP and AMP during purine nucleotide metabolism (ribose-5-P $\rightarrow$ PRPP $\rightarrow$ IMP, the precursor for both AMP and GMP) [41]. Level-altered amino acid metabolism-relevant enzymes are discussed in Section 3.5.7. Briefly, both classes appear to conserve glutamate (perhaps for α -KG generation). Although the above suggests the potential for competition between purine nucleotide synthesis and the TCA cycle for adenylosuccinate, the IRIS0 class additionally ***does* exhibit enhanced aspartate generation (perhaps to provide oxaloacetate) and *inhibited* conversion of adenylosuccinate to fumarate.

3.5.4. Neutrophil-specific Oxphos [42], Mitochondrial Dysfunction, and Oxidative Stress

Neutrophils (including under glucose starvation and/or hypoxic conditions) are thought to rely more on glycogen metabolism and glycolysis (with higher lactate production) than on oxidative phosphorylation (oxphos) to maintain ATP production (based on reducing equivalents derived from both the TCA and fatty-acid oxidation) and even mitochondrial membrane potential (which, in neutrophils, is apparently less coupled to ADP phosphorylation).

Indeed, in neutrophils (in general): respiratory complex I (accepts NADH electrons derived from glycolysis/malate) has *relatively lower* activity (though dysfunction does increase H_2O_2 and lactate production), complex II (accepts FADH₂ electrons derived from succinate) has *relatively normal* activity (dysfunction negatively impacts neutrophils), complex III (accepts CytQ electrons from complex II) has *relatively lower* activity in neutrophils but is *required to maintain mitochondrial membrane potential* despite fewer proton-motive force-generating steps – perhaps in part through acceptance of electrons directly from G-3-P rather than from complex II (dysfunction significantly increases lactate production), *relatively lower* levels of CytC in neutrophil mitochondria result in *relatively inefficient* electron transfer from complex III to IV, complex IV has *only residual* activity in neutrophils - electrons are instead transferred directly from complex III to O₂, generating superoxide (though complex IV dysfunction during glucose starvation can decrease ATP production), and complex V has *relatively normal* activity but is not required to maintain mitochondrial membrane potential except during respiratory chain dysfunction (e.g. perhaps when G-3-P is limiting or complex III is dysfunctional), during which it can co-operate with the adenine nucleotide importer (ANT1/SLC25A4) to maintain net negative charge influx and thus polarity (F1 dysfunction does increase lactate production, and dysfunction during failure of preceding respiratory chain components will compromise mitochondrial membrane potential). There can even be net ATP *consumption* in an attempt to maintain mitochondrial membrane potential. The F1-ATPase converts ATP to ADP, and resulting ADP³⁻ (adenosine 5'-diphosphate) is exchanged for adenosine tetraphosphate (Ap₄; which may explain the likely preservation of Ap₄A noted above). The

enzyme ACS2 (a short-chain acyl-CoA synthetase, which activates acetate for lipid synthesis by converting it to acetyl-CoA) also catalyzes the synthesis of Ap4 from ATP + and tri/tetrapolyphosphate, and apparently Ap4 can also contribute to osmotic pressure maintenance.

Although mitochondrial inhibition does not affect glucose-starved neutrophil ATP levels as profoundly as it would those of PBMC, it does eventually lead to mitochondrial depolarisation and/or activation of mitochondrially-initiated apoptotic pathways. Mitochondria also contribute significantly to iron metabolism (sensing and regulating iron status) as well as to conserved biosynthesis of critical iron-sulfur (Fe-S)-containing proteins (e.g. aconitase, respiratory complexes, DNA polymerases) [43].

Regarding the current dataset, above indications actually suggest relatively *decreased* glycogen metabolic flux in both classes. However, as is often the case during inflammation/cachexia, the latter parts of glycolysis do appear elevated beyond the likely already-high neutrophil glycolytic rate, perhaps due to hypoxia-induced HIF-1 α (with lactate production supporting ATP and NAD generation) in the CTRL0 class. As noted above, both classes exhibit changes also suggestive of up-regulated PPP and nucleotide (including adenosine) metabolic flux, as well as TCA-mediated generation of α -KG. Furthermore, also as noted above, while apparently-active mTORC1 would enhance glycolysis, protein/lipid/pyrimidine synthesis, and mitochondrial biogenesis, it would also suppress autophagy/mitophagy (which would exacerbate any potential metabolic – e.g. mitochondrial – stresses).

Indeed, regarding mitochondrial oxphos, both classes exhibit changes (Table 29) suggestive of respiratory chain failure at already-dysfunctional complex IV, but a concomitant increase in complex V activity, perhaps as a compensatory attempt to maintain mitochondrial membrane potential.

Table 29 Shared Dysregulated Proteins Relevant to Oxidative Phosphorylation

Protein	Function	Status	Hypothesised Outcome
NDUFA4	complex IV (cytochrome C oxidase) component transfers electrons to O ₂ , translocates protons required for complex maintenance	absent	electron transport chain interruption at complex IV (terminal respiratory chain complex) ↓ proton-motive force generation ?↑ mtROS production
ATP5F1A, F1B, and PD	ATP synthase subunits ATP5F1A and B are catalytic core components ATP5PD is part of the proton channel and peripheral stalk	up	downstream attempt to ↑ ATP generation

The CTRL0 class additionally exhibits changes (Table 30) suggestive of increased complex I activity, perhaps due to increased NADH provision by glycolysis or (albeit less likely) the TCA cycle. The IRIS0 class, however, exhibits changes (Table 31) suggestive of *significant mitochondrial dysfunction*: complex III (required to maintain mitochondrial membrane potential), is *absent*, while components of complex II, IV, and V are up-regulated (IV obviously in opposition to shared class findings), perhaps in a compensatory attempt. Although ANT/SLC25A4 is not detected as level-altered, there is increased IRIS0 class expression of VDAC3, a voltage-gated ion channel facilitating gradient-dependent diffusion of adenine nucleotides (ADP/ATP) which may thus assist complex V-mediated mitochondrial membrane potential maintenance.

Table 30 Control Condition-specific Dysregulated Proteins Relevant to Oxidative Phosphorylation

Protein	Function	Status	Hypothesised Outcome
NDUFS1	complex I largest subunit (part of Fe-S group) transfers electrons from NADH to coenzyme Q	up	↑ transfer of electrons to respiratory chain ?↑ mtROS production

Table 31 IRIS Condition-specific Dysregulated Proteins Relevant to Oxidative Phosphorylation

Protein	Function	Status	Hypothesised Outcome
VDAC3	voltage-dependent mitochondrial outer membrane anion channel conducts ATP and small hydrophilic metabolites (including adenine nucleotides) may form part of mPTP	unique	?↑ export of ATP/import of ADP (with attrition of proton-motive force) ↑ adenine nucleotide transport into/out of mitochondrion (?due to mtDNA damage/depletion) ?↑ capacity for mPTP opening and MoM permeabilisation
SDHB	complex II subunit (part of Fe-S group) transfers electrons from FADH ₂ to coenzyme Q	unique	↑ transfer of electrons to respiratory chain no impact on proton translocation ?↑ mtROS production
CYC1	complex III subunit (part of Fe-S group) transfers electrons from coenzyme Q to cytochrome C	absent	additional respiratory chain interruption at complex III ?↓ proton translocation

			±↑ mtROS production
MT-CO3	complex IV functional core subunit mitochondrially-encoded	unique	↑ transfer of electrons from cytochrome C to oxygen
COX6B1	complex IV subunit 6B1	unique	↑ proton translocation
			±↑ mtROS production
ATP5PO	ATPase subunit (part of stalk)	absent	↓ ATP production

Dysfunctional mitochondria can be a significant source of mtROS, further exacerbating respiratory chain dysfunction. Indeed, it is known that TGFβ increases complex III mtROS production, which feeds forward to "enhance TGFβ signaling and promote fibrosis" in a manner dependent on transfer of electrons by the Rieske Fe-S protein to cytQ (prior to complex III) [44]. This may be relevant given above hypotheses suggesting increased TGFβ production/activity in the IRIS0 class: stressors such as ROS or Ca²⁺ overload can open the mitochondrial permeability transition pore (mPTP), a common final cell death pathway among various pathologies, and opposed by adenine nucleotides. The VDAC3 anion channel up-regulated in the IRIS0 class is also believed to be part of the mPTP, and may increase the likelihood of mitochondrial permeability transition and thus cell death. Furthermore, recall that various inner mitochondrial membrane transporters (for pyruvate, malate, aspartate, glutamate, and adenine nucleotides) all rely on the proton-motive force, and it is interesting to note that these substrates are all hypothesised above to be dysregulated in some way, perhaps suggesting that mitochondrial respiratory dysfunction is responsible. Such dysfunction could result from unopposed mtROS-mediated mtDNA and respiratory complex damage (perhaps because of positive feedback cycles set up by higher levels of type I IFN, TGFβ, and ROS in the IRIS0 class).

Additional findings regarding mitochondrial stress indicators do appear to support this. Both classes exhibit changes (Table 32) suggestive of increased Fe-S cluster synthesis, ATP-alternative energy production through inorganic pyrophosphate hydrolysis (especially in the CTRL0 class; Table 33), and increased capacity for mitochondrial membrane permeability transition, but also increased opposition to *intrinsic* pathway apoptosis despite mitochondrial stress (and inhibition of NFκB by EFHD2).

Table 32 Shared Dysregulated Proteins Relevant to Mitochondrial Stress

Protein	Function	Status	Hypothesised Outcome
HSPA9/GRP75	Hsp70 family chaperone mainly mitochondrial, but also found in the ER role in stress response and mitochondrial maintenance	up	↑ Fe-S cluster biogenesis attempt to maintain mitochondrial homeostasis
VDAC1	forms channels through the mitochondrial outer and plasma membranes MoM channel facilitates diffusion of small hydrophilic molecules plasma membrane channel is involved in cell volume regulation and apoptosis open (weak anion selectivity) at lower membrane potential, closed (cation-selective) at higher potentials may contribute to mitochondrial permeability transition pore complex (PTPC) and apoptosis triggering	unique	↑ hydrophilic molecule diffusion across membranes ↑ capacity for mitochondrial membrane permeabilisation
PPA1	inorganic pyrophosphatase (hydrolyses inorganic pyrophosphates) inorganic pyrophosphate (PPi) results from ATP hydrolysis and drives an alternate means of cellular energy generation: energy resulting from highly exothermic hydrolysis of PPi by PPA1 can in some contexts help compensate for a lack of ATP [45]	unique	↑ hydrolysis of inorganic pyrophosphates in the mitochondrion ↑ ATP-alternative energy production
BAX	member of BCL2 family of apoptosis regulators; forms heterodimer with BCL2 to activate apoptosis; also promotes opening of mitochondrial VDAC, leads to depolarisation and cytochrome c release, promotes activation of casp3	down	attempt to ↓ mitochondrially- initiated cell death?
EFHD2	controls spontaneous apoptosis via regulating BCL2L1 abundance also inhibits canonical NFκB activation	up	↓ apoptosis ↓ NFκB activation

Only the CTRL0 class exhibits *absent* DDI2 (proteolytically releases ubiquitinated NRF1 from the ER membrane), suggesting increased NRF1 translocation to the nucleus. Apart from inducing transcription of mitochondrial genes, NRF1 is also a key sensor of ER cholesterol overload, in response inhibiting "CD36-driven inflammatory signaling and de-repressing LXR activity" [46]. The IRIS0 class also exhibits unique additional changes (Table 34) suggestive of mitochondrial stress, including decreased ATP out/ADP in exchange via SLC25A24 (consistent with above hypotheses of altered adenine metabolism perhaps due to ATP deficiency, or an attempt by complex V to maintain mitochondrial membrane potential), decreased defense against oxidative stress, and decreased autophagy/mitophagy (consistent with above hypotheses concerning mTORC1 activation), but also opposition to mitochondrially-mediated cell death despite mitochondrial stress.

Table 33 Control Group-specific Dysregulated Proteins Relevant to Mitochondrial Stress

Protein	Function	Status	Hypothesised Outcome
PPA2	inorganic pyrophosphatases (hydrolyse inorganic pyrophosphates) PPA2 is mitochondrial and essential for maintenance of mitochondrial membrane potential, organisation, and function	unique	↑ hydrolysis of inorganic pyrophosphates in the mitochondrion attempt to maintain mitochondrial and possibly energy homeostasis

Table 34 IRIS Group-specific Dysregulated Proteins Relevant to Mitochondrial Stress/Cell Death

Protein	Function	Status	Hypothesised Outcome
SLC25A24	Ca ²⁺ -dependent mitochondrial solute exchanger (Mg-ATP/ADP for phosphate) catalyzes net uptake or efflux of adenine nucleotides across mitochondrial inner membrane inactive when cytosolic Ca ²⁺ is low, activated for nucleotide transport when cytosolic Ca ²⁺ rises may protect against oxidative stress-induced cell death (promoting formation of calcium-phosphate precipitates in the mitochondrial matrix probably buffers mitochondrial Ca ²⁺ levels)	absent	↓ export into cytosol of ATP and import into mitochondrion of ADP and phosphate ↓ capacity for protection against oxidative stress/Ca ²⁺ -induced mitochondrial cell death
HEBP2	enhances mitochondrial inner and outer membrane permeabilisation, especially during oxidative stress involved in mitochondrial membrane depolarisation prior to necrosis	absent	↓ susceptibility to necrosis
SH3GLB1	interacts with pro-apoptotic BAX, promoting caspase-mediated apoptosis also contributes to caspase-independent apoptosis under starvation conditions helps regulate autophagy: activates PIK3C3, which may regulate ATG9A trafficking for autophagosome formation may contribute to maintaining mitochondrial architecture and be required for normal MoM dynamics	absent	↓ susceptibility to mitochondrially-initiated apoptosis ↓ autophagy ↓ maintenance of mitochondrial homeostasis
CASP1	as part of the inflammasome, proteolytically matures IL-1β during DNA (but not RNA) viral infection, also cleaves (inactivates) cGAS to restrain inflammation also proteolytically activates SREBPs and thus cholesterol and FA biosynthesis can promote apoptosis	absent	↓ IL-1β and IL-18 production ↓ restraint of cGAS ↓ SREBP activation ↓ susceptibility to apoptosis

Given that levels of IL-1β and IL-18 are known to be higher at baseline in TB-IRIS developers, a surprising finding in the IRIS0 class is **absent** CASP1 (while neither NLRP3 nor ASC are detected to have altered levels). However, this is likely due either to inhibition of cholesterol synthesis as an antiviral strategy, or to the hypothesised relative inhibition of NFκB at baseline in TB-IRIS developers, since it is this transcription factor which is responsible for inducing procaspase-1 expression [47] (type I IFN, too, oppose NLRP3 inflammasome-mediated IL-1β production, i.e. that which is induced by host cell stress). In fact, high levels of ROS themselves can inhibit NFκB activation (actually decreasing LPS-induced pulmonary injury) [48]. Taken together, this suggests that it may instead be type I IFN-induced expression of CASP4/5 and their NLRP1-dependent activation (rather than NLRP3 or AIM2 inflammasome-mediated activation of CASP1) that results in maturation of IL-1β and IL-18 (with possible contributions towards pyroptosis) at baseline. Usually, CASP1 is also involved in maintenance of mitochondrial homeostasis and pyroptosis, as well as SREBP activation (i.e. increased FA and cholesterol synthesis) and restraint of cGAS. It is tempting, then, to speculate that other intracellular PRR (e.g. RLR, cGAS-STING) must also be responding to cytosolic NA. Recall (based on current data) that RIG-I is indeed likely active, but that STING is down-regulated in both classes.

Alterations to levels of multiple redox/iron (including Fe-S) homeostasis-relevant proteins (also relevant to mitochondrial stress) are discussed in detail in Section 3.5.7.

3.5.5. Lipid Metabolism

3.5.5.1. Mitochondrial & Peroxisomal FA β-oxidation

Both classes exhibit changes (Table 35) suggestive of decreased cellular long-chain FA and mitochondrial fatty acyl-CoA uptake, at first glance suggesting decrease FA β-oxidation, but which may actually be due to cellular/mitochondrial repletion with FA (despite ongoing FA oxidation [FAO]; e.g. high malonyl-CoA/low carnitine levels inhibit CPT1-mediated mitochondrial FA uptake). Indeed, there is enhanced transfer of early FAO FADH₂ electrons to the likely-dysfunctional respiratory chain, which would exacerbate mtROS production.

Table 35 Shared Dysregulated Proteins Relevant to FA β -oxidation

Protein	Function	Status	Hypothesised Outcome
CPT1A	part of CPT1 also important in TG metabolism	absent	↓ transport of fatty acyl-CoA into mitochondria
ETFB	component of ETF dehydrogenase required for normal mitochondrial FA oxidation and normal amino acid metabolism	unique	↑ transfer of step 1 electrons to the respiratory chain
SLC27A3/FATP4	FA transport protein (FATP) family member highly expressed in lung [49] acyl-CoA ligase activity for long- and very-long-chain FA	absent	↓ FA uptake and synthesis?

Similarly to mitochondrial function findings, the CTRL0 and IRIS0 classes appear to differ significantly with regards to FA β -oxidation. The CTRL0 class exhibits additional unique changes (Table 36) supporting up-regulation of the mitochondrial FA β -oxidation steps producing FADH₂ and NADH (for electron transfer to the respiratory chain), as well as of peroxisomal β -oxidation (especially steps generating 3-ketoacyl-CoA), either to assist (possibly dysfunctional) mitochondria in the context of very high FA levels, or because of the nature of available FAs (e.g. possibly very-long-chain). **Unique** NAD-dependent mitochondrial HSD17B10 may also suggest altered isoleucine/FA/steroid hormone (e.g. 17- β -oestradiol, allopregnenolone)/bile acid/xenobiotic metabolism, and/or mitochondrial tRNA transcript processing [50]. Curiously, despite major CTRL0 enhancements of FA β -oxidation (perhaps facilitated by CTRL0 class-specific NRF1 translocation to the nucleus to inhibit ACC expression), *no additional IRIS0 class-specific alterations are detected* (perhaps due to more complete inhibition of AMPK/PGC-1 α /NRF signalling in this class).

Table 36 Control Group-specific Dysregulated Proteins Relevant to FA β -oxidation

Protein	Function	Status	Hypothesised Outcome
ACADM	β -oxidation step 1 enzyme specific for medium-chain (4-16-carbon) FAs	unique	↑ initiation of medium-chain FA oxidation and production of FADH ₂
HSD17B10	enzyme which performs the same function as step 3 HADHA essential for mitochondrial structural and functional integrity of mitochondria also catalyzes β -oxidation at carbon 17 of androgens and estrogens (including 3- α -hydroxysteroid dehydrogenase activity on androsterone) exhibits 20- β -OH and 21-OH dehydrogenase activities with C21 steroids interacts with intracellular amyloid- β	unique	↑ step 3 execution and production of NADH ?attempt to restore mitochondrial homeostasis ?altered steroid hormone metabolism
DHB4	peroxisomal FA β -oxidation enzyme also catalyzes formation of 3-ketoacyl-CoA intermediates from FAs	up	↑ formation of 3-ketoacyl-CoA
HSD17B4	peroxisomal FA β -oxidation enzyme catalyzes formation of 3-ketoacyl-CoA intermediates	up	↑ formation of 3-ketoacyl-CoA

3.5.5.2. FA, Cholesterol, and Steroid Hormone Biosynthesis

Despite the above indications of decreased FA uptake and increased FA oxidation, both classes also exhibit **unique** FASN and RALY (Table 37), suggesting (respectively) simultaneous *increased* FA synthesis (from acetyl- and malonyl-CoA) and cholesterol biosynthesis. Indeed, here there is also evidence of decreased triglyceride uptake at the same time as increased glycerol synthesis and mevalonate/isoprenoid/sterol synthesis pathway alteration. Apparently increased mevalonate synthesis (early in the pathway) from acetyl-CoA is followed by IFN-induced RSAD2-mediated inhibition of FPPS (i.e. synthesis of downstream FPP). This suggests build-up of intermediates in the pathway between mevalonate $\rightarrow$ IPP $\rightarrow$ GPP, due to decreased GPP conversion to FPP. This would also impair protein farnesylation, and, indeed, expression of an enzyme required for C-terminal cleavage of farnesylated proteins is **decreased**. It is notable that both GPP and FPP have been shown to be anti-inflammatory in an LPS stimulation setting. A bottleneck at this early stage of the pathway would also ultimately impair oxysterol, cholesterol, and steroid hormone synthesis (low oxysterol/cholesterol levels may be responsible for increased RALY levels in a fruitless effort to generate downstream cholesterol precursors).

Table 37 Shared Dysregulated Proteins Relevant to FA, Cholesterol, and Steroid Hormone Synthesis

Protein	Function	Status	Hypothesised Outcome
FASN	FA synthesis from acetyl-CoA, malonyl-CoA, and NADPH	unique	↑ FA synthesis
RALY	required for expression of genes for cholesterol biosynthesis	unique	↑ cholesterol synthesis
AKR1A1	catalyses reduction of mevaldate to mevalonate (and of glyceraldehyde to glycerol)	up	↑ mevalonate and glycerol synthesis
RSAD2	expression induced by IFN signalling impedes viral budding by disturbing lipid rafts, at least in part via inhibiting enzyme farnesyl diphosphate synthase – FPPS – essential for the biosynthesis of isoprenoid-derived lipids	unique	↓ FPPS activity and isoprenoid-derived lipid biosynthesis
ZMPSTE24	cleaves C-terminal residues from farnesylated proteins	down	↓ requirement for C-terminal cleavage of farnesylated proteins
APOBR	receptor which mediates uptake of triglyceride-rich lipoproteins – including rapid high-affinity uptake of chylomicrons and HTG-VLDL – and may be involved in foam cell formation	down	↓ TG uptake

The CTRL0 class exhibits additional changes (Table 38) suggesting additional IFN-induced IFITM3-mediated cholesterol homeostasis disruption, decreased uptake of pro-inflammatory oxLDL (and AGEs), de-repression of steroid hormone synthesis as well as decreased steroid hormone degradation, and an attempt to increase protein farnesylation and geranylgeranylation (fruitless if indeed FPP and thus GGPP are not being synthesised). The IRIS0 class, on the other hand, exhibits changes (Table 39) suggesting increased cholesterol export via HDL, possible attempts to decrease mevalonate synthesis via ER-associated protein degradation (ERAD) of HMGcR, and decreased steroidogenesis. Simultaneously, anabolic insulin signalling appears to be de-repressed, with increased provision of acetyl-CoA for lipid synthesis, and increased conversion of G-3-P to phosphatidic acid for TAG/glycerolipid synthesis. The *absence* of DBI - which mediates corticotropin-induced adrenal steroidogenesis - from the IRIS0 class is interesting in light of various β -adrenergic stimulus-relevant proteins being *absent* in the IRIS0 class. For example, PIK3CG absence in both classes may decrease β -adrenergic receptor (e.g. ADRA1B) endocytosis, increasing neutrophil sensitivity to adrenaline. And yet, absence of both GNAS and MAP2K3 suggest decreased signalling downstream of stress stimuli such as adrenaline. Furthermore, COPS6 - *absent* in the IRIS0 class - is involved in the response to glucocorticoid receptor-mediated signalling. Finally, expression of a pyruvate dehydrogenase subunit (when the enzyme complex is activated by both insulin and adrenaline) is *up-regulated* only in the CTRL0 class.

Table 38 Control Group-specific Dysregulated Proteins Relevant to FA, Cholesterol, and Steroid Hormone Synthesis

Protein	Function	Status	Hypothesised Outcome
IFITM3	expression induced by IFN signalling known to disrupt intracellular cholesterol homeostasis	unique	↑ disruption of cholesterol homeostasis
OLR1	oxidised LDL receptor, initiating internalisation, degradation, ROS production-mediated NFkB activation, decreased NO release, and possibly apoptosis cAMP-regulated expression also acts as a receptor for Hsp70, AGEs, apoptotic cells, and possibly as a scavenger receptor	down	↓ oxLDL uptake and NFkB activation
HSD17B11	short-chain dehydrogenase which metabolizes secondary alcohols and ketones can convert androstan-3- α , 17- β -diol to adrosterone in vitro, suggesting its involvement in androgen metabolism during steroidogenesis may metabolize compounds stimulating steroid synthesis, or generate metabolites that inhibit it	down	↑ steroid hormone synthesis
HSD17B10	alternate enzyme mediating step 3 of mitochondrial FA β -oxidation (and required for mitochondrial maintenance), but which also metabolises steroid hormone intermediates	down	↓ breakdown of steroid hormone biosynthesis intermediates
DHRS7	dehydrogenase/reductase enzyme that metabolizes steroid hormones, prostaglandins, retinoids, lipids, and xenobiotics	down	↓ lipid, steroid hormone, and prostaglandin degradation
FNTA	essential subunit of the farnesyltransferase and geranylgeranyltransferase complexes which transfer farnesyl or geranylgeranyl moieties from diphosphates to target proteins may also help prenylate (activate) Rac1	unique	↑ protein farnesylation and geranylgeranylation
NME1- NME2	protein resulting from a naturally-occurring read-through transcript unknown function, but NME1 has a key role in synthesis of non-ATP nucleoside triphosphates (e.g. NTP) while also acting as a Ser/Thr protein kinase and geranyl and farnesyl pyrophosphate kinase can enhance DNA damage and thus cell death	up	↑ synthesis of mevalonate pathway intermediates

Table 39 IRIS Group-specific Dysregulated Proteins Relevant to Lipid Biosynthesis

Protein	Function	Status	Hypothesised Outcome
RETN	insulin effect-suppressing (i.e. anti-adipogenic) hormone possibly decreased in response to gamma-chain family cytokine signalling	down	↑ anabolic processes such as lipid biosynthesis (supporting initial results regarding PKA signalling)
ERLIN2	ER lipid raft-associated protein which mediates ERAD of activated IP3 receptors (to regulate IP3 signalling) & HMGcR (sterol-accelerated ERAD, probably involving GP78) also regulates cholesterol homeostasis via insulin-induced gene 1 protein (INSIG1)-mediated SREBP signalling regulation: - regulates activation of SREBP1c (key regulator of <i>de novo</i> lipogenesis) e.g. under stress conditions - negatively regulate SREBP activation when cholesterol levels are high [51]	unique	↓ IP3 signalling ↓ HMGcR-mediated cholesterol synthesis ↑/↓ SREBP activation (and thus lipid biosynthesis), depending on cholesterol levels
ACLY	ATP citrate lyase main enzyme which synthesises cytosolic acetyl-CoA (and oxaloacetate) from citrate and CoA acetyl-CoA is then used in lipogenesis and cholesterologenesis in tumour cells, is a key regulator reprogramming metabolism to high rates of aerobic glycolysis and <i>de novo</i> lipid synthesis	up	↑ availability of acetyl-CoA for lipid biosynthesis ↑ aerobic glycolysis
GPAT3	converts G-3-P to lysophosphatidic (and thus phosphatidic) acid for TAG synthesis transfers acyl-CoA acyl group to G-3-P, an essential step in glycerolipid biosynthesis an associated pathway is PPAR α -regulated	unique	↑ TAG/glycerolipid biosynthesis (with consumption of G-3-P)
DBI	conserved, hormone-regulated diazepam-binding inhibitor binds medium- and long-chain acyl-CoA esters with very high affinity may be an intracellular carrier of these involved in lipid metabolism displaces β -carbolines and benzodiazepines from type A GABA receptors, possibly as a neuropeptide mediates CCK release and feedback-based pancreatic secretion regulation mediates corticotropin-induced adrenal steroidogenesis an associated disease includes Abdominal Obesity-Metabolic Syndrome associated pathways include PPAR α pathway and regulation of lipid metabolism by PPAR α	absent	↓ steroidogenesis
APOA1	apolipoprotein A-I major protein component of circulating HDL	unique	↑ cholesterol export to liver via HDL ↑ plasma cholesterol ester levels

	proteolytically-generated mature form transports cholesterol from tissues to liver cofactor for LCAT, which enzymatically forms the majority of plasma cholesterol esters		

Thus, both classes apparently engage in elevated FAO, lipid, and mevalonate synthesis, but while the CTRL0 class attempts to compensate for decreased cholesterol/steroid hormone availability, the IRIS0 class attempts to suppress further mevalonate/steroid hormone synthesis as well as to export cholesterol and further increase lipid (especially triglyceride) synthesis. Pilot datasets within the current study initially implicated neutrophil mevalonate/isoprenoid synthesis pathway dysregulation in IRIS manifestation (data not shown). Evidence also tentatively suggests that the IRIS0 class at baseline engages to a lesser degree in corticotropin-induced responses (production of adrenaline and adrenal glucocorticosteroids) to stressors such as inflammation (in which context these hormones normally have anti-inflammatory and pro-resolving – respectively – effects on neutrophils [52] [53] [54] [55]), either because there is little inflammation to oppose, or due to adrenal exhaustion by chronic inflammation, or altered cholesterol/steroid hormone metabolism.

3.5.6. Protein Homeostasis

Increased secretory protein expression and transit (or protein mis-folding due to ATP depletion), increased ROS, lipid overload, and xenobiotic detoxification all individually induce ER stress. *Mycobacterium tuberculosis* (*Mtb*) antigens also induce ER stress (via TLR-2/4 and ROS/Ca²⁺). However, no direct protein indicators of ER stress – nor UPR sensors or effectors – are detected to be dysregulated in the current dataset.

It is possible that both classes (Table 40) are experiencing some degree of ER architecture compromise, with decreased quality control of secreted protein folding (although this may yet reflect overall decreased expression of secreted proteins at baseline). Various prior protein level alterations (e.g. CAMKK2, CKII, etc.) have also hinted at some degree of altered Ca²⁺ signalling. Here, findings suggest that the CTRL0 and IRIS0 classes, respectively, exhibit increased and decreased pumping of cytosolic Ca²⁺ back into the ER, reflecting either opposing cytosolic Ca²⁺ levels, or differential regulation/functional capacity of the ER. Recall that Ca²⁺ exits the ER via IP3R (when Ca²⁺ levels are high) and the ryanodine receptor (whose conductance is regulated by calmodulin/calsequestrin as a proxy for Ca²⁺ levels, FKBP – *up-regulated* in both classes, and redox conditions – which may differ between classes).

Table 40 Dysregulated Proteins Relevant to ER Architecture and Ca²⁺ Homeostasis Regulation

Class	Protein	Function	Status	Hypothesised Outcome
both	LNPK	contributes to ER network tubular junction morphology	absent	↓ maintenance of ER tubular junctions
	BCAP31	chaperone with roles in exporting secreted proteins from the ER, and one of the most abundant ER proteins recognises abnormally-folded proteins and targets them to ERAD "may be involved in caspase-8-mediated apoptosis"	absent	↓ export of secreted proteins from ER ↓ targeting of misfolded proteins to ERAD
CTRL0	ATP2A2	ER ATP-dependent Ca ²⁺ pump	unique	suggesting either a response to high cytosolic Ca ²⁺ levels, or that such levels are being kept low (perhaps to prevent neutrophil hyper-reactivity)
IRIS0	ATP2A3	high-affinity ER ATP-dependent Ca ²⁺ pump pumps cytosolic Ca ²⁺ into the endoplasmic reticulum to reinstate a low cytoplasmic Ca ²⁺ concentration	down	decreased need for pumping Ca ²⁺ back into the ER, or ER Ca ²⁺ -regulating dysfunction

Both classes (Table 41) exhibit *increased* expression of chaperones and chaperonins, (including one which selectively represses transcription of the IFN-induced antiviral PKR-mediated stress response [56]), which should enhance protein folding (although this is often ATP-dependent). Up-regulation of chaperones and chaperonins likely reflects a general neutrophil stress response to partially-treated co-infection. The CTRL0 class also exhibits *increased* expression of a mitochondrial-specific chaperonin, suggesting increased mitochondrial protein import. *Increased* expression of additional chaperones (especially HSPH1) and chaperonins in the IRIS0 class suggests relatively more pronounced/prolonged metabolic stress, possibly with significant ATP depletion. However, one of these chaperones is a major repressor of the UPR (though at least the ERAD component may remain functional, as suggested by ERLIN2). If this class truly experiences inhibition of AMPK, autophagy, the PKR-mediated stress response, and the UPR, this would severely compromise metabolic stress-adaptive mechanisms.

Table 15 Dysregulated Proteins Relevant to Chaperones, Chaperonins, and Co-chaperones

Class	Protein	Function	Status	Hypothesised Outcome
both	CCT3, 4, 5, 6A, 7	chaperonins, part of CCT complex	up	↑ CCT-mediated protein folding (provided ATP is available)
	TCP1	part of CCT complex	up	
	NPM1 HSPA8 HSP90AB1 HSP90B1	chaperones phosphoprotein NPM1 helps shuttle ribosomal proteins and histones from the nucleus to the cytoplasm (and negatively regulates activation of IFN-inducible EIF2AK2/PKR) both HSPA8 (member of Hsp70 family) and HSP90AB1 (cytosolic member of Hsp90 family) are constitutively-expressed and facilitate correct protein folding HSPA8 is particularly pivotal in protein quality control and protection against proteome stress ("ensuring correct protein folding, re-folding of misfolded proteins, controlling targeting of proteins for degradation, participating in ERAD in conjunction with co-chaperones and the E3 ligase STUB1, and acting as a repressor of transcriptional activation") HSPA8 also binds LPS and mediated LPS-induced inflammation (including TNF secretion) HSP90AB1 also has a role in inflammation HSP90B1 is specifically located in the ER, "functions in processing and transport of secreted proteins", is – along with CNPY3 – required for appropriate TLR folding, and has a role in ERAD	up	↑ protein-folding quality-control ↓ transcription ↑ processing and transport of secreted proteins ?↑ LPS binding and LPS-induced TNF secretion ?↑ TLR folding
	FKBP5	protein-folding co-chaperone immunophilin and prolyl isomerase regulates conductance of ryanodine receptor (as does calmodulin, calsequestrin, and redox conditions) and thus Ca ²⁺ exit from the ER) also regulates basic cellular processes and immunity via modulation of AKT phosphorylation and NFκB activation [57]	up	↑ protein-folding
CTRL0	HSPD1	mitochondrial chaperonin essential for folding and assembly of proteins imported into mitochondrion may also promote proper refolding and assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix may function as a signalling molecule in innate immunity	up	↑ mitochondrial protein-folding during stress conditions
IRISO	CCT8	chaperonin, part of CCT complex	up	↑ CCT-mediated protein folding (provided ATP is available)
	HSPA5 HSPB1 HSPH1	chaperones HSPA5 assists in transport of secretory proteins through the ER, and is a major repressor of the IRE1-mediated UPR, and ER misfolded protein accumulation facilitates HSPA5 release of IRE1 to initiated the UPR (discussed below) HSPB1 responds to environmental stress by functioning as a cytoplasmic and nuclear chaperone promoting correct protein folding; likely "maintains denatured proteins in a folding-competent state" HSPH1 is a nucleotide-exchange factor (NEF) for other chaperones (e.g. HSPA1A and B), and prevents aggregation of denatured proteins during severe stress (which markedly decrease ATP levels)	up	↑ protein-folding under stress conditions ↓ UPR

Regarding the ER protein-folding quality control cycle, both classes (Table 42) exhibit **decreased** glucosidase II of the CANX/CALR cycle (perhaps suggesting decreased flux of proteins through this ER cycle, or suggesting ER dysfunction), despite simultaneous attempts to enhance glycosylation of nascent proteins translated into the ER (perhaps due to a shortage of glycol-moiety donors). The CTRL0 class exhibits additional changes suggestive of increased N-linked glycosylation. The IRISO class additionally exhibits **decreased** glucosidase I of the CANX/CALR cycle, decreased cleavage of signal peptides from incoming nascent proteins (again: either decreased protein translation or ER dysfunction), and decreased O-linked glycosylation flux (interesting, since the majority of human protein glycosylation is N-linked).

Table 43 Dysregulated Proteins Relevant to ER-mediated Folding of Glycoproteins

Class	Protein	Function	Status	Hypothesised Outcome
both	PRKCSH	regulatory subunit of glucosidase II known PKC substrate likely stabilises the catalytic subunit and increases N-glycan processing speed [58]	down	↓ exposure of lectin-binding sites of nascent N-linked glycoproteins entering the ER, and/or: ↓ liberation of ER-folded proteins from the CANX/CALR cycle
	STT3A	catalytic subunit of the oligosaccharyl transferase (OST) complex which catalyzes the first step of co-translational N-glycosylation (glycan transfer from a lipid carrier to Asn) as nascent proteins enter the ER	unique	↑ N-glycosylation of nascent (probably secretory) proteins entering the ER
	GFPT1	rate-limiting enzyme of hexosamine biosynthesis pathway regulating glucose flux into the pathway controls availability of precursors required for protein (N- and O-linked) and lipid glycosylation [59]	unique	↑ availability of precursors for protein glycosylation (provided glucose is available)
CTRL0	RPN1	rough ER integral membrane protein which is also part of an OST complex "that links high-mannose oligosaccharides to nascent peptide Asn residues" also part of the 26S proteasome regulatory subunit	up	↑ N-linked glycosylation
IRISO	MOGS	glucosidase I	absent	↓ exposure of lectin-binding sites of nascent N-

				linked glycoproteins entering the ER
	OGA	both adds and removes O-GlcNAc to/from protein Ser/Thr residues as part of O-glycosylation [Gao 2001]	absent	↓ O-glycosylation flux
	SPC3	component of the complex which cleaves signal and other N-terminal peptides from proteins translated into the ER	absent	↓ nascent protein signal peptide cleavage (due to ER dysfunction, since it appears there is increased passage of glycosylated proteins through the ER?)

Additional enzymes enhancing protein folding via promotion of correct disulphide bond formation are PDIs. Both conditions (Table 44) exhibit *increased* expression of various PDIs, but simultaneous *decreased* TMX-mediated disulphide bond formation assistance (perhaps reflecting this class of enzymes belonging to an independently-controlled transcriptional cassette) and *decreased* QSOX1-mediated protein-folding (which also produces hydrogen peroxide). Potentially dysregulated redox homeostasis is discussed in detail in Section 3.5.7. The CTRL0 class exhibits *increased* expression of an additional PDI (which may also contribute to transport of S-nitrosothiol-bound NO and - with ligand LGALS9 at the cell surface - may alter membrane redox status). No additional PDI-relevant protein level alterations are noted in the IRIS0 class.

Table 44 Dysregulated ER Protein Disulfide Isomerases (PDIs)

Class	Protein	Function	Status	Hypothesised Outcome
both	PDIA3	especially involved in CANX/CALR cycle glycoprotein quality control helps prevent protein aggregation	up	↑ PDI-mediated combating of improper protein folding and aggregation
	PDIA4	with cyclophilin B also enhances IgG inter-chain disulphide bond formation	up	
	PDIA6	helps prevent protein aggregation	up	
	TXNDC5	protein with thioredoxin (antioxidant) and PDI activity	unique	↓ TMX-mediated enhancement of disulphide bond formation and protein folding
	TMX3	a.k.a. TXNDC10 oxidises dithiol groups participates in folding of proteins containing disulphide bonds	absent	
	TMX4	participates in protein folding and thiol-disulfide interchange	absent	
CTRL0	QSOX1	oxidises thiols during protein folding, reducing oxygen to hydrogen peroxide [60] also oxidises peptide sulfhydryl groups	absent	↓ thiol oxidation-based protein folding and hydrogen peroxide production
	PDIA1	at high concentrations also inhibits aggregation of misfolded proteins role in transport of S-nitrosothiol-bound NO also a subunit of prolyl 4-hydroxylase (which hydroxylates procollagen prolyl residues) and microsomal triacylglycerol transfer protein (MTTP) at the cell surface acts as an LGALS9 receptor (leading to cleavage of surface protein disulphide bonds to e.g. enhance Th2 migration); recall that LGALS9 is unique to the CTRL0 condition	up	↑ combating of improper protein folding and aggregation ↑ capacity for NO transport with LGALS9 ↑ alteration of surface redox status
IRIS0	none	N/A	N/A	N/A

Examining effectors of protein elongation unfortunately does not provide much additional information to support/oppose the hypothesis of generally inhibited protein expression. While both classes exhibit level-alterations of many proteins involved in gene expression/protein synthesis (e.g. chromatin architecture, transcriptional regulation, RNA processing, and translation) – although not master controller EIF2AK3 – the general increase in expression of elongation factors (Table 45) is not very informative, except for EIF6 being responsible for increased expression of insulin/growth factors and thus expression of anabolic (including glycolysis- and adipogenesis-regulating) transcription factors and mtROS-generating respiratory chain proteins. *Down-regulation* of a CTRL0 class elongation factor may be an antiviral strategy. The IRIS0 class exhibits *increased* EEF1D expression, which may account for increased heat-shock protein (chaperone) expression, and a *decreased* eIF-3 complex component may decrease susceptibility to apoptosis (e.g. may account for decreased expression of BAX).

Table 45 Dysregulated Proteins Relevant to Gene Expression/Protein Synthesis

Class	Protein	Function	Status	Hypothesised Outcome
both	EEF1A1P5	elongation factors, no particularly notable functions	up	↑ protein expression
	EEF2		up	
	EIF4A1		up	
	EIF4H		up	
	EIF6	stimulates expression of insulin/growth factors RACK1-dependent PKC activity releases EIF6 from 60S ribosomal subunits; in insulin-responsive tissues, it thereby regulates expression of adipogenic transcription factors (CEBPB, CEBPD, ATF4) and in so doing regulates glycolysis and fatty acid synthesis also likely "controls the expression of mitochondrial respiratory chain genes involved in reactive oxygen species (ROS) synthesis"	unique	↑ expression of anabolic (including glycolysis-regulating and adipogenic) proteins ?↑ expression of mitochondrial respiratory chain proteins which produce mtROS
CTRL0	EIF4A2	no particularly notable function	up	↑ protein expression
	EIF2S3	subunit of eIF2 and associated with viral mRNA translation	down	↓ viral protein expression
IRIS0	EEF1D	subunit of eIF-1 complex which interacts with HIV Tat and "regulates induction of heat-shock-responsive genes"	up	↑ heat-shock protein expression
	EIF5A	cofactor of HIV Rev and regulates TNFα-mediated apoptosis	up	↑ protein (including viral) expression
	EIF3E	involved in early protein synthesis as part of the eIF-3 complex this complex specifically translates genes involved in cell proliferation and apoptosis	down	?↓ expression of apoptotic proteins

Specifically regarding the UPR, both classes exhibit *up-regulated* PDIA6, which *inhibits* the UPR through binding sensors ERN1 and possibly EIF2AK3. However, recall that the IRIS0 class exhibits unique STAT3 and STAT6; IL-4 in combination with IL-6 or IL-10 allows these transcription factors to synergise in initiating the UPR (also incidentally “potently up-regulating cathepsin secretion”) [17]. In this class, there is also some indication of cellular protein damage (e.g. *up-regulation* of carboxyl methyltransferase PCMT1, which helps repair/degrade damaged proteins – e.g. EIF4EBP2, calreticulin, clathrin, etc. - by correcting spontaneously-deamidated iso/aspartyl residues). These findings suggest the UPR may be more likely/beneficial in the IRIS0 class, and indeed an additional relevant protein (PTPN1, which inactivates EIF2AK3/PERK) is *absent*.

Regarding the UPS, both classes (Table 46) exhibit *increased* expression of multiple ubiquitinating enzymes, but also *absence* of many E3 ligases (perhaps due to negative regulation by IFN, given their occasional role in viral reproduction). Thus, there is likely both increased and decreased UPS-mediated degradation of selected (presumably differential) targets. Degradation targets include certain histones (enhancing STAT1-mediated IFN signalling), but simultaneously also IFNAR1 (decreasing surface expression). Stabilisation targets include certain histones, and proteins whose stabilisation de-represses growth factor/anabolic and TNF α signalling. Findings also indicate increased ISGylation of selected targets, and down-modulation of mTORC1 downstream signalling (perhaps in response to upstream mTORC1 activation). The CTRL0 class exhibits additional changes suggestive of increased UPS-mediated degradation of selected targets, but increased rescue of inappropriate targets (provided ADRM1-activated UCHL5 and PSMD14 are present; ADRM1 is indeed *up-regulated* in the CTRL0 class, while UCHL5 is *absent* only in the IRIS0 class). The IRIS0 class also exhibits additional changes suggestive of increased UPS-mediated degradation of selected targets (though absence of UCHL5 suggests decreased rescue of inappropriate targets), but with simultaneous stabilisation of (presumably differential) targets. For example, there appears to be decreased movement of ER misfolded/fusion proteins to the cytoplasm for UPS-mediated degradation, as well as (via *absent* UFD1, normally complexed with VCP) stabilisation of DDX58/RIG-I-mediated type I IFN production and (via *unique* USP15, possibly induced by TGF β [61]) of pro-inflammatory NF κ B and autophagy- and aggresome formation-relevant cargo receptor SQSTM1. Integrated VCP findings and functions are discussed in greater detail below.

Table 46 Dysregulated Proteins Relevant to Protein Ubiquitination

Class	Protein	Function	Status	Hypothesised Outcome
both	UBA1	catalyses first step in ubiquitination to flag proteins for degradation	up	↑ UPS-mediated protein degradation
	UBE2L6	involved in covalent attachment of ubiquitin or ISG15 to target proteins	unique	↑ UPS-mediated protein degradation or ISGylation-mediated alteration in protein function
	DTX3L	E3 ubiquitin ligase involved in IFN-mediated antiviral response ubiquitinates histones (e.g. H4) for DNA damage repair ubiquitination of H2B promotes chromatin remodelling to positively regulate STAT1-dependent IFN-stimulated gene transcription for viral control also promotes sorting of CXCR4 from early endosome to the lysosome after CXCL12 stimulation	unique	↑ ubiquitination of histones (e.g. to enhance IFN signalling)
	FBXO7	substrate-recognition component of E3 ubiquitin ligase complex	down	↓ ubiquitination of selected targets
	CUL4B	core architectural component of E3 ubiquitin ligase complexes (substrate specificity depends on substrate-recognition subunit) targets CD11 and histones via interaction with MLST8 – regulates mTOR signalling mTORC1 signalling requires 26S proteasomal function, facilitated by interaction of Raptor with CUL4B [Ghosh 2008]	down	↓ ubiquitination of selected targets down-modulation of mTORC1 downstream (RPS6KB1, EIF4EBP1) signalling [Ghosh 2008]
	UBAC1	non-catalytic component of E3 ubiquitin ligase complex	down	↓ ubiquitination of selected targets
	NEDD4	ubiquitinates Lys63 of targets (e.g. RAPGEF2, ERBB4, BRAT1) favours growth factor receptor (e.g. EGFR, FGFR1, and IFG1R, as well as TNF α) degradation also involved in viral budding	absent	↓ ubiquitination of selected targets (including de-repressed growth factor/anabolic signalling)
	RAD23A	delivers polyubiquitinated proteins to the 26S proteasome involved in Vpr-dependent HIV replication	absent	↓ delivery of ubiquitinated proteins to 26S proteasome ↓ HIV replication; absence likely reflects antiviral responses
CTRL0	BRCC3	metalloprotease which cleaves Lys63-linked polyUb e.g. of IFNAR1 (enhancing its stability and surface expression, and suppressing cell responsiveness to LPS)	absent	↓ de-ubiquitination of selected targets (incl. IFNAR1)
	SUGT1	role in ubiquitination and proteasomal degradation of target proteins	up	↑ UPS-mediated target protein degradation
IRIS0	ADRM1	ubiquitin receptor subunit of 26S proteasome activates deubiquitinases UCHL5 (19S-associated) and PSMD14 (19S subunit)	up	↑ UPS-mediated target protein degradation, but rescue of incorrect targets
	USP15	de-ubiquitinates target proteins (e.g. NFKBIA, SQSTM1) ubiquitinated SQSTM1 restrains cognate vesicles perinuclearly instead of releasing them to the periphery	unique	↑ de-ubiquitination of selected targets

		already discussed: enhances TGF β -induced signalling and target gene activation		
	UBA2	E1 ligase with SAE2 activates SUMO1-4 for protein sumoylation	down	↓ protein SUMOylation
	COPS4, 6, and 8	subunits of conserved COP9 signalosome complex regulates various pathways essential regulator of ubiquitin conjugation pathway: mediates de-neddylation of cullin and non-cullin E3 ligase complex subunits phosphorylates - possibly with casein kinase II and PKD - p53/TP53, c-jun/JUN, I κ B α /NF κ B α , and IRF8/ICSBP TP53 and JUN phosphorylation enhance and inhibit (respectively) UPS-mediated degradation COPS6 inhibits COP1 degradation, is somewhat responsive to glucocorticoid receptor activation, and is likely a co-factor for HIV Vpr	absent	↑ neddylation of E3 ligase complex subunits ?↓ p53 degradation ?↑ JUN degradation
	UCHL5	19S-associated de-ubiquitinating enzyme activated by transient interaction – via ADRM1 – of INO80 complex with 26S proteasome (ADRM1 is only detected as up-regulated under the CTRL0 condition, however)	absent	↓ de-ubiquitination of selected targets (↓ rescue of inappropriate targets)
	UFD1	part of complex with VCP and NPLOC4 required to move misfolded proteins from ER to cytoplasm and for degradation of ubiquitinated (incl. fusion) proteins complex negatively regulates type I IFN generation (promotes DDX58/RIG-I degradation)	absent	↓ movement of ER misfolded proteins to cytoplasm for UPS-mediated degradation (incl. fusion proteins) ↓ DDX58/RIG-I degradation and suppression of type I IFN production
	FAM105A/OTULINL	de-ubiquitinating enzyme	absent	↓ de-ubiquitination of selected targets

While AMPK activation (likely inhibited here) would decrease SQSTM1 expression, oxidative stress (likely enhanced here) would increase NRF2-dependent SQSTM1 expression (as well as decreasing SUMOylation, increasing NEDDylation of E3 ligases, and possibly decreasing p53 but increasing JUN degradation) [62]. For example, damaged mitochondria become ubiquitinated, which recruits SQSTM1 and other autophagy-specific proteins. Although USP15 is a component of the COP9 signalosome, *absence* of three other COP9 signalosome subunits in the IRIS0 class suggests that USP15 may be acting independently of the signalosome. Furthermore, USP15 inhibits mitophagy by counteracting PRKN-mediated UPS degradation of mitochondrial fusion-favouring MFN2, while FBXO7 (*down-regulated* in both classes) targets mitochondrial fusion-favouring MFN1 for UPS-mediated degradation while targeting PRKN to depolarised mitochondria (downstream of PINK1), thereby favouring mitophagy *when it is present*. These findings further support defective adaptive mechanisms attempting to oppose mitochondrial stress.

Regarding proteasomes, specifically, it is unsurprising that many proteasomal proteins are identified and quantitated (Table 47), given high cellular proteasomal abundance. Overall, proteasomal findings reflect *increased* levels of housekeeping 20S and UPS-mediating 26S proteasomal components, as well as an *increase* in two IFN γ -inducible immunoproteasome (involved in the UPS and MHC-I antigen presentation) components. Recall that 20S subunits are common to both the 26S proteasome and immunoproteasome. Proteasome subunit level changes are likely partially in response to type I IFN (especially the IRIS0 class) and IFN γ (both classes), but – given baseline literature data - not TNF α (both conditions). Altogether findings suggest increased protein turnover, including that mediated by an activated UPS, and perhaps increased MHC-I (but decreased MHC-II) antigen presentation.

Table 47 Dysregulated Proteins Relevant to Protein Ubiquitination

Class	Protein	Function	Status	Hypothesised Outcome
both	PSMA5	20S core alpha subunit	absent	differential regulation of 20S alpha subunits
	PSMA6	20S core alpha subunit	up	
	PSMB2, 3	26S core beta subunits	up	↑ 26S beta subunit
	PSMC4	26S protein-unfolding ATPase subunit part of the 19S regulator with role in proteasome assembly	up	↑ unfolding of proteins entering 26S
	PSMD2	19S regulator non-ATPase subunit also interacts with TNFR1 to participate in TNF signalling	up	differential regulation of 26S's 19S regulator subunits
	PSMD4	19S regulator non-ATPase subunit selects ubiquitinated targets for degradation	absent	?↑ TNF signalling ?↓ selection of ubiquitinated targets
	PSME1, 2	IFN γ -induced beta subunits of 11S immunoproteasome regulator required for efficient antigen processing to generate MHC-I-presented peptides	up	?↑ IFN γ signalling ↑ immunoproteasome-mediated MHC-I antigen processing
CTRL0	PSMA2	20S core alpha subunit	up	↑ 20S alpha subunit
	PSMD9	19S regulator non-ATPase subunit role in 26S assembly (acts as chaperone during assembly)	up	↑ 26S assembly capacity
	ADRM1	discussed above ubiquitin receptor subunit of the 26S proteasome engages and activates deubiquitinases UCHL5 (19S-associated) and PSMD14 (19S subunit)	up	↑ differentiation between appropriate and inappropriate 26S proteasome substrates
	PSMC2	26S protein-folding ATPase subunit	down	↓ unfolding of proteins entering 26S
	TPP2	neutral pH peptidase that cleaves N-terminal tripeptides (part of the proteolytic cascade downstream of the ubiquitin-26S proteasome pathway)	up	↑ post-26S MHC-I antigen processing

		essential for certain MHC-I antigen presentation activities may complement 26S proteasome function when the latter is inhibited		
IRIS0	PSMC3	26S protein-folding ATPase subunit	up	↑ unfolding of proteins entering 26S
	PSMD11	19S regulator non-ATPase subunit required for proteasome assembly phosphorylated by AMPK	up	↑ 26S assembly capacity
	ANPEP	aminopeptidase involved in peptide hormone (e.g. angiotensin III and IV), chemokine, and MHC-II peptide processing also exploited as a receptor by certain viruses may favour cholesterol crystal precipitation	down	↓ MHC-II antigen processing (down-regulation may be an antiviral strategy)

Regarding the above indications of inhibited autophagy, both classes exhibit *absent* ATG3 (a major E2 conjugating enzyme *required* for cytoplasm-to-vacuole transport, autophagy, and mitochondrial homeostasis – also further supporting mitochondrial dysfunction). Only the CTRL0 class exhibits *up-regulated* ATG7, an E1-like activating enzyme also *required* for cytoplasm-to-vacuole transport and autophagy, including mitophagy and CASP-8 inhibition-induced autophagic cell death. Cells from this may respond more adaptively to mitochondrial stress (e.g. with mitophagy and/or autophagic cell death).

Membranous organellar subsystems such as the ER, mitochondrion, and lysosomes display significant functional inter-linking. For example, each acts as a cellular hub integrating survival and stress cues (e.g. nutrient depletion, oxidative stress, Ca²⁺ imbalance, protein aggregation, etc.); stress of each can produce stress in the others (e.g. via ROS); while each induces adaptive general stress responses, supra-maximal/prolonged stimuli can lead such responses to become maladaptive; and each can initiate cell death pathways often involving the others. For example, severe ER stress actually maladaptively *inhibits* autophagic flux, and ER stress-induced cytosolic Ca²⁺/ROS/protein aggregate overload induces mitochondrial and lysosomal stress. Because import of ~1500 nuclear-encoded proteins into the double-membrane-enclosed mitochondria involves unfolding and refolding and stoichiometric matching of nuclear- and mitochondrially-encoded proteins for electron transport chain complex assembly, mitochondria are particularly vulnerable to proteotoxic stress [63] and require mitochondrion-specific protein quality control systems. Although induction mechanisms are still unclear, misfolded proteins trigger the mtUPR, consisting of a system of mitochondrial proteases (which are responsible for degrading “the majority of mitochondrial proteins” [64]) and chaperones [63].

Further supporting the mitochondrial dysfunction hypothesis, the CTRL0 class exhibits *up-regulated* ATPase VCP – crucial for translocation to the cytoplasm “of stressed/damaged mitochondrial outer-membrane proteins” prior to proteasomal degradation. VCP is part of a complex that extracts ubiquitinated proteins from other complexes or from lipid membranes for clearance (it is required for autophagy-mediated clearance of these, for example), and is also part of the VCP-GP78 complex contributing to end-stage sterol-mediated ERAD of HMGcR, suggesting that – provided sterol levels are high and ERAD is functional – there may be increased HMGcR degradation in the CTRL0 class, with attendant effects of inhibited mevalonate generation on isoprenoid and sterol biosynthesis (recall that in the IRIS0 class *unique* ERLIN2 may mediate sterol-accelerated ERAD of HMGcR in a manner involving GP78), perhaps in an attempt to combat mevalonate/GPP build-up. Furthermore, recall that VCP-co-operating proteins USP15 and UFD1 are *unique* and *absent* (respectively) in the IRIS0 class. USP15 de-ubiquitinates VCP-bound substrates, suggesting increased rescue (perhaps including DDX58/RIG-I) from degradation, while UFD1 (with VCP) enhances cytoplasmic UPS-mediated degradation of misfolded ER proteins while inhibiting type I IFN production. Its absence thus suggests a lower capacity for misfolded ER protein degradation, but a higher capacity for type I IFN production

3.5.7 Integrated Amino Acid, Vitamin, H₂S/Sulfate, Glutathione, Redox, Glyoxylate, Xenobiotic, and Fe-S Protein Metabolism

3.5.7.1. Amino Acids, Including the Methionine-SAMe-cysteine-GSH Continuum

Both classes exhibit changes (Table 48) suggesting: decreased (PLP-dependent) production of glutamine from glutamate (perhaps reserved for serine/proline synthesis or import of cysteine), increased production of serine (perhaps for NA, phospholipid, glycine, or cysteine – with methionine’s functional group - synthesis) from G-3-P’s conjugate acid + glutamate (or perhaps production of α-KG/oxaloacetate from 2-hydroxyglutarate/malate), decreased (PLP-dependent) lysine degradation (perhaps reserving it for carnitine synthesis to facilitate exchange-based mitochondrial FA import via

CTP1), and increased ARG1-mediated conversion of arginine to ornithine + urea (perhaps as part of the urea cycle, and perhaps driven by Th2/T_{reg}-type cytokines inducing myeloid ARG1 expression).

Typically, nitrogen for amino acid synthesis is sourced from glutamate or serine, both of whose levels are increased in this context. Depleted intracellular arginine impairs NOS-mediated NO synthesis (and, along with depleted leucine, should restrain mTORC1 activity, although this appears not to be the case here), and depleted extracellular arginine impairs NK and T-cell activity while promoting ILC2-mediated type 2 inflammation. Ornithine metabolites (e.g. proline) are required for collagen synthesis/fibrosis, perhaps driven by TGF β in this context. It is also possible to synthesise proline from glutamate/glutamine, however. Decreased lysine degradation may reflect limiting PLP, or dysfunction of the (largely somatic) mitochondrial saccharopine or (largely neuronal) peroxisomal pipecolate lysine catabolic pathways, of which the former converts lysine to saccharopine with concomitant conversion of α -KG to glutamate [65].

Table 48 Shared Dysregulated Proteins Relevant to Amino Acid Metabolism

Protein	Function	Status	Hypothesised Outcome
GLUL	glutamine synthetase, PLP-dependent catalyses ATP-dependent conversion of glutamate and ammonia to glutamine either for detoxification or to generate the amino acid also regulates RhoJ activity and thus motility	absent	↓ glutamine production ↑ glutamate availability
PHGDH	catalyses first step of phosphorylated L-serine biosynthesis (reversible oxidation of 3-phospho-D-glycerate to 3-phosphonoxypropylate) also catalyses reversible oxidation of 2-hydroxyglutarate to α -KG and reversible oxidation of malate to oxaloacetate	unique	↓ 2-hydroxyglutarate, malate ↑ serine, α -KG, oxaloacetate
PHYKPL	catalyses PLP-dependent breakdown of 5-phosphohydroxy- L-lysine , producing ammonia, inorganic phosphate, and 2-aminoadipate semialdehyde	absent	↓ lysine breakdown
ARG1	converts L-arginine to L-ornithine and urea L-ornithine is metabolized into L-proline and polyamides that drive collagen synthesis and bioenergetic pathways	Up	↓ arginine availability ↓ NO synthesis via iNOS ↑ ornithine ? ↑ capacity for collagen synthesis/fibrosis (linked to TGF- β)

The CTRL0 class exhibits additional changes (Table 49) suggesting: preservation of cytosolic serine (rather than entry into the mitochondrion for conversion to glycine and formate, suggesting decreased glycine – and thus perhaps NA/GSH - production and decreased donation of formate methyl groups to recharge folates) as well as increased ATP- and vitamin B3 (NAD)-dependent synthesis of universal methyl group donor SAME from methionine. Once SAME has donated its methyl group, resulting homocysteine can either – when methionine is lacking, and in the absence of oxidative stress – be remethylated by methyltetrahydrofolate (MTHF) and B12 to methionine OR – when methionine is replete – undergo PLP-dependent trans-sulfuration to cysteine, facilitating GSH synthesis or PLP-dependent H₂S synthesis (the latter consuming α -KG and GSH).

Thus, methionine and cysteine levels exist in balance, both drawing from the homocysteine pool, which becomes elevated when there is deficiency of NADPH/B9/12 (to facilitate methionine regeneration) or PLP (to make cysteine and thus GSH/H₂S). Trans-sulfuration provides approximately half of the cysteine used for GSH synthesis. Especially, under oxidative stress (which inhibits regeneration of methionine from homocysteine but favours the initial step of trans-sulfuration), PLP is a key factor determining whether homocysteine is driven back into one-carbon metabolism or forward into trans-sulfuration. Low plasma PLP is strongly associated with high homocysteine levels (especially for the 677T genotype of NADPH-dependent MTHFR, which generates MTHF - if NADPH is indeed lacking in both classes, decreased methyltetrahydrofolate generation would decrease recycling of homocysteine back to methionine). Deficiencies of two other key B-vitamins – folate/B9 and B12 - participating in homocysteine metabolism are also associated with hyperhomocysteinemia.

High homocysteine levels trigger increased oxidative stress, ER stress, mTOR activation, inhibited autophagy, lysosomal dysfunction, and inflammation (including elevated CRP, IL-6, and TNF α) – somewhat relieved by vitamin B supplementation or rapamycin. Hyperhomocysteinemia also worsens oxidative/hypoglycemic/hypoxic-reperfusion cellular injury. Independently, both low plasma vitamin B6 levels and elevated plasma homocysteine are a risk factor for cardiovascular disease (low vitamin B6 is also associated with other inflammatory disorders such as rheumatoid arthritis and independently with higher CRP). Although vitamin D stimulates trans-sulfuration, plasma levels are not associated with inflammatory markers (CRP, SAA, IL-6, TNF α , IL-10), at least in acute-phase coronary syndrome patients. Interestingly, mtDNA depletion → decreased oxphos → decreased NAD → decreased SAME production and methionine salvage → altered DNA methylation → changes in gene transcription (including TCA cycle, serine/folate metabolism, and trans-sulfuration. Thus, increased methionine-to-

SAME conversion occurring only in the CTRL0 class may suggest inhibition by higher levels of mtDNA depletion in the IRIS0 class.

Table 49 Control Condition-specific Dysregulated Proteins Relevant to Amino Acid Metabolism

Protein	Function	Status	Hypothesised Outcome
SFXN1	transports D-/L-serine into mitochondria for conversion to glycine and formate formate exits to the cytosol to produce charged folates that act as one-carbon donors also transports other amino acids (e.g. alanine), and possibly a component required for iron utilisation	absent	↑ cytosolic serine ↓ charged folate production ↓ glycine production
DLD	component of glycine cleavage system and of three different α-ketoacid dehydrogenase complexes (pyruvate-, α-KG-, and branched-chain amino acid-dehydrogenases) α-KG-dehydrogenase operates mainly in the mitochondrion, but in the nucleus is required for histone Lys succinylation (provides succinyl-CoA to histone-modifying KAT2A) pyruvate dehydrogenase converts pyruvate into acetyl-CoA on its own may also be a serine protease	unique	↓ glycine ↑ conversion of pyruvate to acetyl-CoA some ↑ conversion of α-KG to succinyl-CoA?
MAT2B	kinetics-regulatory subunit of S-adenosylmethionine synthetase 2, which produces S-adenosyl-L-methionine (SAM-e, cysteine precursor) from essential amino acid L-methionine and ATP (step 1 of SAM-e synthesis from L-methionine) subunit increases MAT2A affinity for L-methionine	unique	↓ methionine ↑ SAM-e and cysteine

The IRIS0 class exhibits additional changes (Table 50) suggesting: increased asparaginase-mediated conversion of asparagine to aspartate (which can contribute to ATP, NA, or asparagine synthesis) + ammonia (which can enter the ATP-dependent urea cycle or PLP-dependently combine with glutamate to form glutamine, which is cytotoxic at high levels). However, both classes exhibit decreased PLP-dependent conversion of glutamate to glutamine, and the IRIS0 class also exhibit a decrease in the arginosuccinate lyase-mediated step of the urea cycle: conversion of arginosuccinate to arginine + fumarate. This would further deplete arginine, and may also suggest ammonia toxicity in this class. Excess amino acid levels lead to their oxidative degradation in mitochondria, producing TCA cycle intermediates and urea. Recall also that an imbalance in uncharged tRNAs triggers the GCN2/ATF4 system to enhance amino acid uptake and synthesis, especially of asparagine (from glutamine and aspartate); it is unclear whether this system is active in this context.

Table 50 IRIS Condition-specific Dysregulated Proteins Relevant to Amino Acid Metabolism

Protein	Function	Status	Hypothesised Outcome
ASPG	asparaginase converts L-asparagine to L-aspartate and ammonia also has lysophospholipase, transacylase, and PAF acetylhydrolase activities including conversion of glycerol-phosphocholines to FAs and other glycerol-phosphocholines various asparaginase and asparaginase pathway polymorphisms are known	unique	↓ asparagine ↑ aspartate ?↑ PAF inactivation ↑ ammonia ↑ FAs
ASL	arginosuccinate lyase converts arginosuccinate into arginine and fumarate, for example to detoxify ammonia as part of the hepatic urea cycle	absent	↑ arginosuccinate ↑ ammonia ↓ arginine ↓ fumarate

Although little can be directly gleaned from the dataset regarding essential amino acid tryptophan, it is worth mentioning that in both classes, levels of IDO1 (which would consume tryptophan – including 5-HT/serotonin - required by T-cells and bacteria for proliferation, to generate the anti-inflammatory kynurenine cascade, inducing Treg) are not detected to be altered, but in the CTRL0 class only, NAD biosynthesis from tryptophan (and PPP product PPRP) is enhanced.

3.5.7.2. B-vitamins (results not tabulated)

Many of the amino acid metabolic enzymes noted to have altered levels require B-vitamin co-factors. For example, in both classes: **absent** GLUL (B6 as PLP, B7), **unique** PHGDH (B3 as NAD/NADH), and **absent** PHYKPC (B6 as PLP). In the CTRL0 class: **unique** DLD (B2 as FAD) and **unique** MAT2B (B3 as NADP/NADPH). In the IRIS0 class: none are obviously known to require B-vitamin co-factors. Furthermore, TKT (**down-regulated** in both classes) is also vitamin B1-dependent, and enhanced NAD biosynthesis in the CTRL0 class is clearly relevant to vitamin B3 metabolism.

Vitamin B6 is converted *in vivo* to active co-factors (e.g. PLP). In both classes, the enzyme (PNPO) catalysing the first step of PLP biosynthesis is **absent**, perhaps suggesting either pre-existing depletion or resulting deficiency of this cofactor. As part of ATT, INH is known to interfere with vitamin B6 function, necessitating routine pyridoxine supplementation in order to prevent peripheral neuropathy [66]. In the CTRL0 class only, an important enzyme in the synthesis of vitamin B5 (and cysteamine) is also **absent**: VNN1, which recycles D-pantetheine back to vitamin B5. This suggests decreased production of vitamin B5 (pantothenate), which would decrease cellular antioxidant capacity, innate- and adaptive-mediated Mtb control [67], and coenzyme A (CoA) synthesis (also interlinked with thiamine and folate

pathways). Indeed, CoA synthesis from vitamin B5 is dysregulated: PPCS is *absent* from both classes (suggesting decreased early-stage conversion of vitamin B5 to CoA, perhaps due to vitamin B5 deficiency), while COASY is *unique* only in the CTRL0 class (suggesting increased compensatory late-stage CoA synthesis enhancement). Given above indications of elevated nucleotide synthesis in these neutrophils, folate levels may be limiting (perhaps also for erythrocyte synthesis, linking back to the potentially low baseline haemoglobin levels in IRIS developers noted in Chapter 1).

3.5.7.3. H₂S Metabolism (results not tabulated)

As mentioned, H₂S is generated from homocysteine and cysteine (whose levels typically increase under pathological conditions), with all biosynthetic steps being PLP-dependent. Step 1 (CSE-mediated conversion of cysteine to pyruvate) is activated by Ca²⁺/calmodulin (e.g. under hypoxic conditions), step 2 (CBS-mediated joining of homocysteine to cysteine to produce serine) is activated by high SAME levels, step 3 (CAT-mediated transamination of cysteine using α -KG to produce 3-mercaptopyruvate) is enhanced by aspartate, and step 4 (3-MST-mediated conversion of 3-mercaptopyruvate to persulfides, which react with GSH/thioredoxin/dihydrolipoic acid thiol groups to generate H₂S) is inhibited by Ca²⁺ but enhanced by high thioredoxin and dihydrolipoic acid levels. Alternatively, 3-MST converts sulfate to thiosulfate anions, then reduced by TST to H₂S. TST also converts thiosulfate to sulfite (which can then be reconverted to sulfate), using cyanide (respiratory complex IV inhibitor) as the thiol acceptor (which is in fact the major pathway of endogenous cyanide detoxification, generating thiocyanate for renal excretion (although it can also be modified by MPO as described in Chapter 3); the hydroxycobalamin form of vitamin B12 can also rapidly enter the mitochondrion to bind oxphos-inhibiting cyanide, generating non-toxic cyanocobalamin for renal excretion). Low-level endogenous hydrogen cyanide is produced by leukocytes as a microbicidal mechanism, and thus may be modulated by muscarinic cholinergic signalling (cigarette smoke also contains hydrogen cyanide), providing a link back to the hypothesis that smoking may induce immunometabolic adaptations protective against TB-IRIS (Chapter 3).

In both classes (Ca²⁺/calmodulin signalling may be dampened), step 1 of synthesis may be suppressed while step 4 is de-repressed (there are already suggestions above that GSH may be limiting in these cells, and - as we will see below - increased levels of thioredoxin activity occur in both classes, although it is simultaneously decreased in the IRIS0 class). Furthermore, the CTRL0 class likely exhibits elevated SAME production (activating step 2), while the IRIS0 class likely exhibits elevated aspartate production (activating step 3 - indeed, α -KG production is likely elevated in both classes). Overall, this appears to suggest increased post-step 1 H₂S synthesis in both classes. Only the CTRL0 class exhibits *unique* TST, suggesting increased conversion of thiosulfate to H₂S or to sulfite (and thus thiocyanate, or back to thiosulfate).

Various H₂S functions include: targeting G-3-P production by GAPDH, targeting CytC, activating K⁺ ATPases while inhibiting Ca²⁺ channels, inducing adaptive stress responses (e.g. inducing HIF-1 α and SIRT1 [68], stimulating nuclear translocation of NRF2 to activate mitochondrial biogenesis and antioxidant response - including GSH and thioredoxin - pathways) in an anti-apoptotic manner. It is also vasodilatory and alters insulin signalling and glucose utilisation. Although H₂S induces eNOS, it inhibits iNOS and NF κ B expression, thereby suppressing NO and pro-inflammatory cytokine production. Physiological H₂S levels are thus protective in many settings, e.g. attenuating ischemia-reperfusion injury. However, high H₂S levels can also be toxic, via inhibiting respiratory complex IV, as well as being a neurotoxin and lung irritant. In solution, H₂S exists in equilibrium with HS⁻, partially transforming into the sulphide anion (S²⁻); high sulphide levels are cytotoxic, inhibiting respiratory complexes II and IV, and inducing oxidative DNA damage, mitochondrial depolarisation, and apoptosis.

Many of the H₂S biosynthetic enzymes have both cytosolic and mitochondrial forms. Enzymatic catabolism occurs in the mitochondrion: SQOR/SQRDL-mediated conversion of HS⁻ to molecular sulfur donor thiosulfate (S₂O₃²⁻), TSTD1-mediated reversible transfer of sulfur from this to a thiophilic receptor (e.g. GSH) to form persulfide (e.g. glutathione persulfide), and ETHE1/SDO-mediated irreversible transfer of sulfur from this unstable intermediate to oxygen to form sulfite (SO₃²⁻, subsequently converted by SUOX to sulfate: SO₄²⁻) to prevent build-up of H₂S to CytC-toxic levels. Various S-methyltransferases may also methylate H₂S to form methanethiol and dimethyl sulfide. Non-enzymatically, H₂S can also dissociate to HS⁻/S²⁻, or can interact with haemoglobin (to form sulfhaemoglobin) or mitochondrial haem proteins (CytC/complex IV) to form molecular sulfur + a reactive thiol radical (HS[•]). Both HS⁻ and HS[•] can interact with RNS (e.g. NO[•], ONOO⁻) to create HSNO, which reacts with thiols to reform H₂S and nitrosothiols (RSNO), a cellular reserve form of NO.

Here, both classes exhibit *up-regulated* SQOR/SQRDL, suggesting increased quinone-dependent catabolism of (perhaps elevated levels of) H₂S to thiosulfate, and thus glutathione persulfide formation (but only provided sufficient GSH – or another sulfur accepting thiol - is available). Only the IRIS0 class exhibits *absent* TSTD1, suggesting a bottleneck in H₂S catabolism resulting in build-up of thiosulfate, perhaps due to a deficiency in GSH to which to transfer thiosulfate's molecular sulfur group. However, recall from above that only the CTRL0 class exhibits *unique* TST, which can instead convert thiosulfate back to H₂S or to sulphite for conjugation to cyanide. An elevation of H₂S in the CTRL0 class (but thiosulfate in the IRIS0 class) may contribute to the suggested altered NFκB, iNOS, G-3-P, oxphos, HIF-1α, SIRT1, NRF2, thioredoxin, and other activities in the context of TB-IRIS.

3.5.7.4. Glutathione and Redox Homeostasis, with Links to Amino Acids, B Vitamins, and Tuberculosis

GSH is key to cellular antioxidant defence, as well as participating in various other functions (e.g. purinergic P2X7 receptor activation, the NO cycle, leukotriene synthesis, protein folding, H₂S catabolism, iron metabolism, and detoxification of xenobiotics and methylglyoxal). Typically, such functions convert GSH to GSSG (oxidised glutathione), which is reduced back to GSH by FAD/vitamin B2- and NADPH/vitamin B3-dependent enzyme GSR (depletion of these vitamins, or of G6PD-mediated NADPH generation, can impair GSH recycling). Similarly, depleted vitamin B5 or B6 can impair *de novo* synthesis of GSH, typically from cysteine (supplementable as NAC or SAME; the latter likely being elevated in the CTRL0 class), but also from glutamate (likely up-regulated in both classes) or glycine (likely down-regulated in the CTRL0 class). This proceeds via intermediate GGC, which is converted by GSS to GSH. In the reverse process, GGT1 and 5 can convert GSH metabolism intermediates back into glutamyl derivatives, glutamate, and alanyl/cysteinylglycine; ANPEP releases alanine/cysteine + glycine from the latter.

In both classes, down-regulated G6PD suggests decreased NADPH generation (although up-regulated 6PGD may partially compensate) while PLP (vitamin B6) biosynthesis is also likely decreased, both suggesting decreased recycling of GSSG back to GSH (in the CTRL0 class, decreased recycling of D-pantetheine back to vitamin B5 suggests the same). Furthermore, in both classes, *up-regulated* peroxisomal GSTK1 suggests increased GSH conjugation to hydrophobic substrates for elimination from the cell, perhaps contributing to GSH depletion. No CTRL0 class-specific changes to GSH metabolism-related enzymes is noted. The IRIS0 class exhibits *down-regulated* ANPEP, suggesting decreased availability/use of GSH intermediates for amino acid synthesis.

Cells can generate ROS/RNS via the NOX-mediated respiratory burst, but ROS actually mainly arise via (especially dysfunctional) mitochondrial oxphos and peroxisomal FA β-oxidation. Thus, cells employ a protective system of anti-oxidant enzymes, mitophagy, and (in response to supramaximal oxidative stress) apoptosis. Major cellular (and pulmonary) anti-oxidant enzyme systems include SOD, CAT, GSH-dependent Se-GPx, and NADPH-dependent Se-Trx reductase (although Prx, GSH- and NADPH-dependent Glrx, and HMOX1 also contribute).

Methionine (and thus homocysteine and cysteine) exists in two forms: sulfur (S) and seleno (Se). Cysteine is a precursor not only for PLP-dependent GSH/H₂S synthesis, but also for synthesis of many proteins of the cysteine proteome (including anti-oxidant enzymes such as GPx, Trx reductase, and PRDXs). The Se-cysteine form is required for synthesis of GPx and Trx reductase, whose active site Se-cysteines have particularly high reductive capacity. The enzyme SCLY acts on Se-cysteine to release toxic selenide which must be immediately detoxified via SAME-mediated methylation, or phosphorylation for reattachment to a carbon skeleton (e.g. PLP-dependent incorporation into tRNA Se-cysteinyl, for synthesis of Se-proteins, such as Se-dependent GPx). Only the CTRL0 class exhibits *absent* selenium-binding SELENBP1 (which also senses reactive xenobiotics and oxidises methanethiol, an organosulfur compound produced e.g. by gut bacteria), perhaps suggesting decreased selenium metabolism (perhaps due to PLP deficiency). Since trans-sulfuration of homocysteine through GSH synthesis requires only two PLP-dependent reactions, while conversion of Se-homocysteine to Se-GPx requires five PLP-dependent reactions, the impact of PLP deficiency will be more pronounced for the latter. Porcine evidence suggest that higher dietary vitamin B6 levels favour SCLY-dependent Se-GPx synthesis. Oxidative stress favouring trans-sulfuration/Se-GPx/Se-Trx reductase synthesis over methionine regeneration/one-carbon metabolism (which may impair nucleic acid and neuronal/erythrocyte synthesis) would clearly exacerbate the sequelae of PLP deficiency, as homocysteine would continue to build up (further exacerbating oxidative stress) and anti-oxidant GSH and anti-oxidant enzymes would fail to synthesise.

Indeed, various above indications suggest oxidative stress, during which depletion of NADPH, GSH/GPx, and inhibition of mitophagy/apoptosis would significantly increase susceptibility to oxidative damage.

Both classes exhibit redox-relevant protein level alterations (Table 51) suggesting: increased redox-sensitive chaperone activity and increased thioredoxin (TXN)/Se-Trx reductase-mediated rescue of oxidised macromolecules (provided sufficient NADPH is available), but decreased GSH-dependent Se-GPx-mediated opposition of membrane/lipoprotein phospholipid peroxidation (and thus of ferroptosis resulting from iron-dependent accumulation of oxidised lipids) and decreased thiol-specific peroxidase (PRDX4)-mediated peroxide sensing and detoxification. This suggests greater susceptibility to iron-induced lipid peroxidation and ferroptosis.

Table 51 Shared Dysregulated Proteins Relevant to Redox Homeostasis

Protein	Function	Status	Hypothesised Outcome
ATOX1	delivers copper to cytosolic ATPases and may be important in antioxidant defence	unique	↑ copper delivery to ATPases response to redox stress?
PARK7	peptidase which positively regulates androgen-receptor-dependent transcription and functions as an oxidative stress sensor as well as protecting against oxidative stress, e.g. through functioning as a redox-sensitive chaperone	up	response to redox stress?
PRDX4	thiol-specific peroxidase which converts hydrogen peroxides and organic hydroperoxides to water and alcohols, respectively also acts as a sensor of hydrogen peroxide-mediated signalling events	down	↓ sensing of peroxide signalling ↓ detoxification of peroxides
GPX4	essential antioxidant peroxidase that prevents membrane lipid peroxidation, reduces phospholipid hydroperoxides, including those incorporated into membranes and lipoproteins, and prevents ferroptosis resulting from iron-dependent accumulation of lipid-ROS	down	↑ lipid peroxidation
TXNDC5	thioredoxin (TXN) which – via TRX reductase – accepts electrons from NADPH (which may be lacking in these cells) in order to reduce oxidised proteins [69]	unique	↑ rescue of oxidised macromolecules (provided NADPH is available)
TXNIP	inhibits thioredoxin activity/availability	down	↑ thioredoxin activity/availability

The CTRL0 class exhibits additional changes (Table 52) suggesting: paradoxically increased PRDX3-mediated peroxide detoxification, but decreased rescue or degradation of oxidatively-damaged proteins. The IRIS0 class exhibits additional changes (Table 53) suggesting: decreased NADPH-dependent TXN/Se-Trx reductase-mediated rescue of oxidised macromolecules, but increased GSH/GSH reductase- and NADPH-dependent Glrx-mediated reduction of disulfides (and possibly increased S-glutathionylation of signalling molecules, provided sufficient GSH is available).

Table 52 Control Group-specific Dysregulated Proteins Relevant to Redox Homeostasis

Protein	Function	Status	Hypothesised Outcome
PRDX3	thiol-specific peroxidase which converts hydrogen peroxides and organic hydroperoxides to water and alcohols, respectively	up	↑ detoxification of peroxides (incl. to alcohols)
APEH	protein-rescuing acylpeptide hydrolase which is important in degradation of oxidatively-damaged proteins in living cells	down	↓ rescue/degradation of oxidatively-damaged proteins

Table 53 IRIS Group-specific Dysregulated Proteins Relevant to Redox Homeostasis

Protein	Function	Status	Hypothesised Outcome
TXNL1	thioredoxin with redox potential ~250mV	down	↓ thioredoxin activity
GLRX	reversibly reduces glutathione-protein mixed disulfides in the presence of NADPH and glutathione reductase, as a key part of antioxidant defense also impacts signalling pathways by regulating the S-glutathionylation status of signalling molecules	up	attempted ↑ reduction of disulfides for antioxidant defence (but NADPH may be limiting) attempted ↑ S-glutathionylation of signalling molecules (but glutathione may be limiting)

Opposing simultaneous findings within an anti-oxidant enzyme class are not easy to interpret without additional investigations, but suggest differential control as part of transcriptional cassettes unrelated to redox homeostasis (e.g. as part of antiviral mechanisms or hypoxic responses). For example, hypoxia-responsive HIF-1α induces PRDX1 (which feeds forward to further enhance HIF-1α expression) but suppresses PRDX3, and nuclear translocation of PRDX4 triggered by prolonged hypoxia inhibits HIF-1/2 induction of target genes [70]. Thus, simultaneously increased expression of PRDX3 (mainly mitochondrial) but decreased expression of PRDX4 (mainly cytoplasmic) in the CTRL0 class could - for example - suggest an enhanced mitochondrial stress response, or decreased HIF-1α signalling. Because different TXNs are simultaneously up- and down-regulated in the IRIS0 class, this is likely not a consequence of Se-cysteine/PLP/NADPH in/sufficiency. Expression of TXNDC5 occurs in response to hypoxia and ER stress [71], and it is also induced by TGFβ (including via ER stress), downstream of which it favours fibroblast activation, ECM protein folding, and fibrosis (including pulmonary) [72]. Expression of TXNL1, on the other hand, is induced by glucose deprivation (but not oxidative stress) [73] and decreased CTSD levels [74] (though these are in fact up-regulated in both classes here), and favours 26S UPS-mediated degradation of DNA excision-repair protein XRCC1 [75]. Thus, simultaneous increased expression of TXNDC5 (mainly ER and lysosomal) but decreased expression of TXNL1 (mainly cytoplasmic and nuclear) in the IRIS0 class could – for example – suggest induction of the former by TGFβ/ER stress and simultaneous inhibition of the latter in favour of maintaining the DNA damage response.

3.5.7.5. The Glyoxalase System (results not tabulated)

Apart from ROS and RNS, a third and longer-lived/more systemically-acting group of highly-reactive species are the reactive carbonyl species (RCS, which can also generate AGEs that can cause irreversible damage). All the above reactive species damage biological macromolecules such as NA, phospholipids, and proteins. Damaging methylglyoxal, in particular, is generated during times of increased glycolytic flux, due to non-enzymatic degradation of G-3-P and DHAP. Detoxification of glyoxalates occurs via GLO1 and HAGH, which convert methylglyoxal via lactoylglutathione to GSH and lactate (lactate can induce HIF-1 α , thereby further enhancing glycolysis and LDHA-mediated lactate production). Altered levels of these enzymes are not detectable in either class, but both classes exhibit *up-regulated* protein and nucleotide deglycase PARK7, which deglycates early glycation intermediates (Maillard adducts of reactive glyoxals and nucleotide/protein amino groups) to repair (methyl)glyoxal-glycated molecules, liberating glycine or lactate (respectively) and preventing formation of AGEs. This suggests a response to increased reactive carbonyl stress, supported by the above-observed increase in glycolytic flux. No CTRL0 class-specific glyoxal-relevant changes are noted. Only the IRIS0 class exhibits *down-regulated* GRHPR, which - as part of nitrogen and glyoxylate metabolism - converts glycerate to hydroxypyruvate and glycolate to glyoxalate. This may suggest an additional attempt to decrease glyoxalate production.

3.5.7.6. Xenobiotic Metabolism

The aryl hydrocarbon receptor (AhR, induced by NF κ B) responds to ligands (including indoles, kynurenine, tryptamine, 5-HT, bilirubin, biliverdin, LXA4A, PGG2, 12-HETE, etc.) by inducing phase I and II xenobiotic metabolizing enzymes (e.g. cytochrome P450s such as CYP1A1, CYP1A2, CYP1B1, as well as NADPH-quinone oxidoreductase) and interacting transcription factors (e.g. NF κ B, ATRA, and the estrogen receptor) as part of immune-regulatory feedback loops (e.g. regulation of NF κ B and induction of T_{reg}) [76]. The AhR-interacting protein (AIP) enhances AhR signalling, and in both classes is *unique*, suggesting enhancement of the xenobiotic detoxification response (perhaps in response to ATT or products of co-infection).

Indeed, various xenobiotic detoxification-relevant proteins exhibit level alterations in the current context (some already discussed above under other functional banners). Both classes also exhibit changes (Table 54) suggestive of decreased ATP-dependent broad-specificity drug efflux from cells (but increased NSAID-specific efflux), but increased intracellular xenobiotic detoxification/degradation (including GSTK1-mediated conjugation to GSH), including increased inactivation of catechol hormones.

Table 54 Shared Dysregulated Proteins Relevant to Xenobiotic Metabolism

Protein	Function	Status	Hypothesised Outcome
ABCB1	ATP-dependent multi-drug efflux pump	down	↑ xenobiotic accumulation in cells
GSTK1	conjugates glutathione to hydrophobic substrates for their elimination from the cell	up	↑ xenobiotic elimination (provided glutathione is available)
AKR1A1	aldo/ketoreductase that converts aromatic and aliphatic aldehydes to alcohols and plays a role in xenobiotic – e.g. anthracycline - metabolism, as well as activating polycyclic aromatic hydrocarbon trans-dihydrodiols	up	↑ aldehyde detoxification (increased alcohol production) ↑ xenobiotic detoxification ↑ AhR activation by activated polycyclic aromatic hydrocarbon alcohols
MFSD10	may act as an efflux pump to confer cellular resistance to apoptosis induced by NSAIDs	unique	↑ selected xenobiotic efflux from cells
COMT	inactivates catechol hormones and shortens the half-life of drugs including L-DOPA and isoproterenol	unique	↓ catechol hormone action ↑ catabolism of certain xenobiotics

The CTRL0 class exhibits additional changes (Table 55) suggestive of altered xenobiotic metabolism, including increased circulatory transport of hydrophobic ligands (including xenobiotics). Only the IRIS0 class (results not tabulated) exhibits *absent* HMOX2, which converts heme to biliverdin (for conversion to bilirubin), suggesting altered iron metabolism, including decreased conversion of heme to AhR ligands (perhaps to preserve heme groups for incorporation into mitochondrial respiratory chain protein prosthetic groups for electron acceptance/donation). Recall that mitochondria contribute significantly to iron status. Recall also the above indications of altered iron transport and storage, suggesting increased sequestration of extracellular iron (if decreased protein levels are due to exocytosis) in both classes, and increased ferritin-mediated intracellular iron storage in the CTRL0 class. This may also have relevance to the noted potential increased susceptibility to ferroptosis.

Table 55 Control Group-specific Dysregulated Proteins Relevant to Xenobiotic Metabolism

Protein	Function	Status	Hypothesised Outcome
ORM2	circulatory carrier for hydrophobic ligands including synthetic drugs	unique	↑ transport (and ?uptake) of hydrophobic xenobiotics
SELENBP	senses circulating reactive xenobiotics	absent	↓ sensing and response to reactive xenobiotics
DHRS7	responsible for metabolizing various compounds (including prostaglandins, lipids, steroid hormones, retinoids, and xenobiotics)	down	↓ metabolism of xenobiotics
HSD17B11	metabolizes secondary alcohols and ketones may participate in androgen metabolism during steroidogenesis	down	↓ alcohol and ketone metabolism
HSD17B10	NAD-dependent enzyme involved in isoleucine, lipid, and steroid metabolism also acts on bile acids and xenobiotics	unique	↑ xenobiotic metabolism

3.5.7.7. Fe-S Cluster & Fe-S-Containing Protein Biosynthesis

Both mitochondria and mitosomes (pared-down mitochondrial derivatives incapable of carbon metabolism) contribute significantly to conserved biosynthesis of critical Fe-S-containing proteins (including respiratory complexes). Via multi-protein iron-sulfur cluster (ISC) assembly complexes (e.g. FDX2, GRP75, GLRX5, and BOLA3) they mature Fe-S clusters for incorporation into target apoproteins (a GSH-dependent process). Post-translationally, iron-regulatory proteins IRP1 and 2 alter cellular iron requirements, linking iron-sulfur protein biogenesis to iron supply and metabolism.

It was noted above that sulfur metabolism is likely altered (e.g. SQOR may increase availability of molecular sulfur). Both classes also exhibit changes (Table 56) in levels of Fe-S metabolism proteins suggestive of Fe-S cluster sequestration by antiviral IFN-induced RSAD2 and increased GRP75- and GLRX3-mediated synthesis of Fe-S clusters and Fe-S-containing proteins, respectively (the latter requiring sufficient GSH availability). This may suggest increased mitochondrial respiratory complex synthesis. Recall that the CTRL0 class also exhibits *unique* TST, which also contributes to Fe-S complex formation, supporting increased Fe-S complex synthesis. However, no additional IRIS0 class-specific changes relevant to Fe-S-containing protein biosynthesis are noted.

Table 56 Shared Dysregulated Proteins Relevant to Sulphur Metabolism

Protein	Function	Status	Hypothesised Outcome
RSAD2	IFN-induced antiviral protein binds Fe-S clusters	unique	↑ Fe-S cluster binding
HSPA9/GRP75	mitochondrial (and ER) chaperone important role in mitochondrial Fe-S cluster biogenesis interacts with and stabilises such cluster assembly proteins (e.g. FXN, NFU1, NFS1, ISCU)	up	↑ mitochondrial Fe-S cluster synthesis
GLRX3	co-operates with BOLA2 as an Fe-S cluster assembly factor facilitates cluster insertion into a subset of cytosolic proteins incorporation of Fe-S clusters into target apoproteins involves glutathione [77]	unique	↑ biosynthesis of Fe-S complex-containing proteins (provided BOLA2 is present and glutathione is available)

3.6. Miscellaneous Additional Dysregulated Pathways

Various additional neutrophil proteins whose levels are altered in the CTRL0 and IRIS0 classes belong to pathways modulating cytoskeletal dynamics and vesicle trafficking (including exocytosis/degranulation) (Table 57), as well as chromatin architecture, mRNA/ncRNA processing, and protein translation (Table 58). Indeed, CTRL0 versus IRIS0 changes to these pathways appear quite distinct from each other. However, given their incredible complexity, details of these pathways are beyond the scope of this thesis and will be addressed in a subsequent research paper in order to maximise data utility.

Table 57 Dysregulated Proteins Relevant to Cytoskeletal Dynamics and Vesicle Trafficking

Unique to Class		Up-regulated in Class		Absent from Class		Down-regulated in Class	
CTRL0	IRIS0	CTRL0	IRIS0	CTRL0	IRIS0	CTRL0	IRIS0
ANKFY1	RDX	ACAP1	ACTA1	CKAP5	ARF6	ACTR3	ACTR2
AP2S1	VPS11	ARCN1	ACTR1B	CLIP1	DOP1B	ANXA7	ANXA6
AP3M1		CORO7	ANXA11	CYTH4	MEMO1	AP2A1	ARPC2
ARHGAP26		PFN1	AP1B1	DOCK11	PITPNB	ARHGAP1	MAPRE1
CAPNS1		SURF4	CAP1	DSTN	PRPF40A	ARHGAP9	MICAL1
CCDC93		TUBB4B	CAPG	EHD4	RAB35	ARPC1B	PLEC
COPS2		TWF2	CFL1	EXOC2	RAB5C	CAPZB	RALB
CORO1B			DCTN2	EXOC7	RAB8A	DCTN1	VPS26A
DYNLRB1			FLOT2	GOLPH3	RUFY1	DNM2	

FRYL			NAPA	GRIPAP1	SACM1L	DPYSL2	
HIP1			OLA1	LMNB2	SAR1A	DYSF	
IGF2R			TLN1	MYO5A	SNX27	GDI1	
MAPRE2			TUBA1B	NUP155	VPS26C	IQGAP1	
PSTPIP2			TUBA4A	RAP1GAP2	VPS29	IQGAP2	
RANGAP1			TUBB	RAPGEF6	VPS35L	LSP1	
SEC23A			VCL	RIPOR2		MYL6	
SNX6				SH3GL1		MYO1F	
STX11				SIPA1		NCKAP1L	
STXBP3				SNF8		PDCD6IP	
TMED4				TBC1D9B		RAB11B	
TMPO				TMOD1		RAB14	
UTRN				TSG101		RAB2A	
						RAB44	
						ROCK1	
						TPM3	
						VAT1	

Table 58 Dysregulated Proteins Relevant to Chromatin Architecture, RNA Processing, & Translation

Unique to Class		Up-regulated in Class		Absent from Class		Down-regulated in Class	
CTRL0	IRIS0	CTRL0	IRIS0	CTRL0	IRIS0	CTRL0	IRIS0
CELF2	RBM12	DDX17	EEF1A1P5	HNRNPH2	EIF3E	EIF2S3	HIST1H1B
EDC4		EEF2	EEF1D	HNRNPUL1	FARS8	HIST1H2BL	
EIF4A2		EIFA3	EIF5A	MECP2	HCFC1	HNRNPU	
EIF4H		EIF4A1	HNRNPM	RBBP7	LARS	HP1BP3	
EIF6		HMG2	PCBP2	RPRD1B	LUC7L2	SMC1A	
FASRA		HNRNPA2B1	RPL10A	RPS15A	NAP1L1	TSN	
GARS		HNRNPD	YARS	SART3	NELFB		
HARS		HNRNPF		UPF1	PRPF19		
NOP56		HNRNPH1			RPL13		
QARS		HNRNPK			RPL23A		
RPL10		HNRNPL			RPS20		
RPS12		HNRNPR			RPS24		
RPS4X		NME1-NME2			RPS24		
RPS5		ROA2			RPS9		
RTRAF		SMCHD1			SMARCE1		
SF1		WARS			SNRNP70		
SF3B2					SRSF2		
SNRNP1					STAG2		
SRRT							

3.7. Head-to-head Comparison Reveals Additional Differences

Interestingly, a head-to-head CTRL0 versus IRIS0 comparison highlights SDE proteins and differences (Table 59) not detectable when - but supporting findings of - comparing CTRL0 versus IRIS0 differences from HV. For example, IRIS0 neutrophils exhibit relatively: down-regulated PLC, PKC, PI3K-AKT1, and MAPK signalling; up-regulated response to Th2 cytokines; down-regulated activation and antimicrobial activity; and a decreased capacity for ER-mediated protein-folding quality control.

In addition, relative up-regulation in IRIS0 neutrophils of the calpain 1 large subunit suggests an increased capacity for necrosis (and pathogen/antigen release) in response to micromolar increases in cytosolic Ca²⁺ levels (e.g. during dysfunction of ER and/or mitochondrial Ca²⁺ uptake/buffering). Recall that there is some indication that IRIS0 neutrophils are less inclined to pump cytoplasmic Ca²⁺ back into the ER, and we also see here relative down-regulation of calreticulin, the major ER lumen Ca²⁺ buffering protein.

Lyn (up-regulated) phosphorylates (activates) Syk, but Syk is down-regulated here. Both activated Lyn and Syk normally phosphorylate (activate) BTK, but BTK is absent in the IRIS0 class, and therefore cannot stimulate PLC and PKC activity. At least in B-cells, BTK activation is required for nucleic acid-stimulated TLR-9-mediated type I IFN production. Indeed, when BTK is absent, TLR-9 stimulation instead promotes IL-12 and decreases IL-10 production, which suggests that eventual ARV therapy may induce provision of Mtb/HIV nucleic acids that ligate TLR-9 (whose expression may be up-regulated due to higher ammonia levels deriving from IRIS-specific metabolic changes) in cells lacking BTK, thereby inducing release of IL-12 (although, based on literature, IL-10 also remains high) to favour Th1/cytotoxic T-cell responses.

Table 59 Additional Between-class SDE Identified During a Head-to-Head Comparison

Status	Protein	Function	Likely Hypothesis
relatively up-regulated in IRIS0	LAP3	leucine aminopeptidase involved in N-terminal amino acid cleavage from peptides (e.g. as part of glutathione metabolism and general protein turnover)	↑ protein turnover
	SAMHD1	deoxynucleoside triphosphate restriction factor up-regulated in response to viral (incl. HIV) infection may regulate innate response (e.g. mediating TNFα pro-inflammatory response)	↑ antiviral activity ↑ degradation of abnormal host DNA fragments

		promotes DNA degradation at stalled replication forks to prevent unnecessary type I IFN induction, thereby preventing chronic inflammation	
	LYN	non-receptor Src-family Tyr kinase for immunoreceptor ITIMs (recruits PTPN6 and 11) also transmits signals from integrins, TLR, cytokine/chemokine receptors, and TXA2 receptors acts downstream of TLR-2 and -4 and receptors for CXCR4, IL-3, IL-5, and CSF2 phosphorylates e.g. BTK, LILRB3, SYK, etc. regulates PI3K-AKT1 and MAPK signalling cascade important role in regulation of immune responses (e.g. hematopoiesis, response to cytokines and growth factors) also involved in the DNA damage response	↓ PI3K-AKT1 and MAPK signalling ↑ response to Th2 cytokines
	PTPN6	SHP-1	↑ inhibition of signalling pathways downstream of immunoreceptors
	CAPN1	subunit of calpain 1, a Ca2+-activated non-lysosomal cysteine protease catalyses proteolysis of substrates involved in signal transduction and cytoskeletal remodelling also mediates Ca2+-dependent necrosis: ER release of Ca2+ resulting in altered mitochondrial Ca2+ homeostasis which can initiate necrosis through activation of Ca2+-activated calpains (calpain 1 is activated by only micromolar Ca2+ concentrations to undergo auto-cleavage for translocation from the cytosol to intracellular membranes where they cleave various substrates) [78] necrosis can facilitate intracellular pathogen spread, including Mtb and viruses a related pathway is beta-adrenergic signalling	↑ capacity for Ca2+-initiated necrosis and pathogen release
relatively down-regulated in IRIS0	BPI	LPS-binding antibacterial protein associated with neutrophil granules activity against Gram-negative bacteria by inducing permeability	↓ antibacterial activity
	PPIA	accelerates protein folding may contribute to cyclosporine-mediated immunosuppression interacts with HIV proteins for virion formation	↓ protein-folding capacity (down-regulation is likely an anti-viral strategy)
	SYK	non-receptor RTK for immunoreceptor ITAMs e.g. phosphorylates (activates) PLCG1 and PKC signalling (also phosphorylates BTK, also activates MAPK cascade) crucial in innate response to pathogens including for integrin-/CEACAM20-mediated adhesion, inflammasome activation, degranulation, phagocytosis, ROS production, cytokine expression (e.g. IL-8)	↓ PLC, PKC, MAPK signalling ↓ neutrophil antimicrobial activity
	CALR	major Ca2+ storage and protein-folding quality control protein of the ER lumen prevents glucocorticoid, androgen, and retinoic acid receptors from transcribing their target genes	↓ ER lumen Ca2+ buffering ↓ CANX/CALR protein-folding quality control cycle capacity

4. Integrated Models of CTRL0 & IRIS0 Neutrophil Immunometabolic Differences

Current study findings (as detailed above) are integrated with Chapter 1 literature to generate neutrophil immunometabolic functional profile predictions (fig.s 8 and 9).

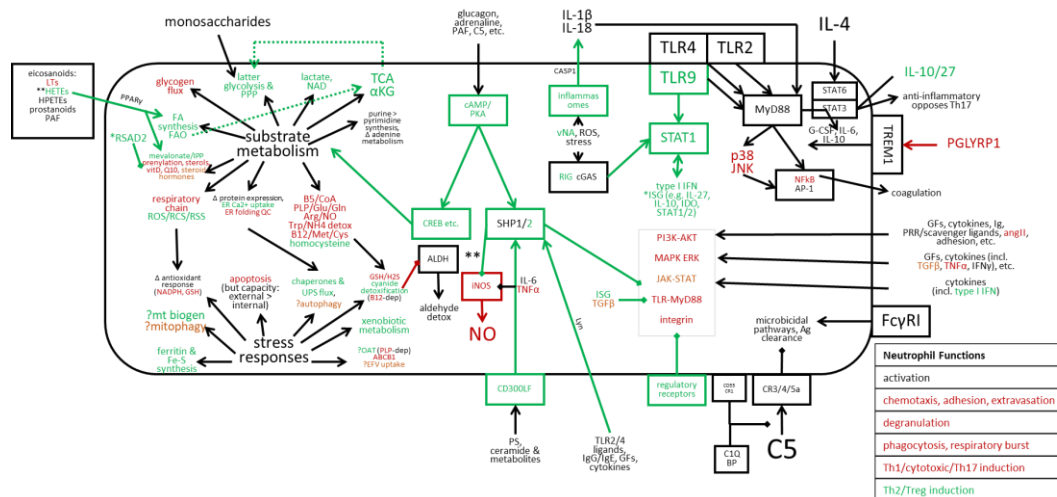


Figure 8 CTRL0 Neutrophil Integrated Functional Profile. Green indicates predicted up-regulation; red indicates predicted down-regulation. Abbreviations: **LTs** leukotrienes, **HETEs** hydroxyeicosatetraenoic acids, **HPETEs** hydroxyperoxyeicosatetraenoic acids, **PAF** platelet activating factor, **TCA** tricarboxylic acid cycle, **FA** fatty acid, **FAO** fatty acid oxidation, **vNA** viral nucleic acids, **viD** vitamin D, **ROS** reactive oxygen species, **RCS** reactive carbonyl species, **RSS** reactive sulphur species, **ISG** interferon-stimulated genes, **Gfs** growth factors, **Ig** immunoglobulin, **PRR** pattern recognition receptor, **angII** angiotensin II, **GSH** glutathione, **UPS** ubiquitin-proteasome system, **Ag** antigen, **PLP** pyridoxal phosphate, **EFV** efavirenz, **NO** nitric oxide, **PS** phosphatidylserine. All remaining abbreviations are gene/protein names or accepted amino acid or metabolic intermediate/reducing equivalent abbreviations. Double asterisks indicate data deriving from literature rather than from the current study. The single asterisk indicates that RSAD2 is an anti-viral ISG.

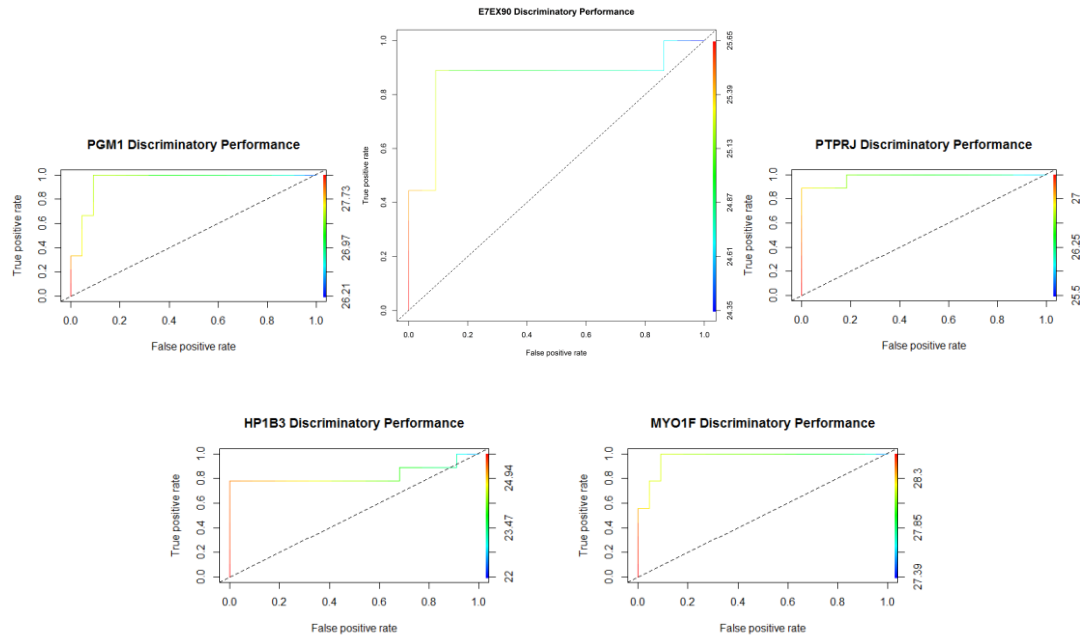


Figure 11 ROC Curves for Top Five Candidate Biomarkers

Table 60 Sensitivity and Specificity of Top Five Candidate Biomarkers

Candidate (UniProt ID)	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
PGM1	100	91	27.47
DCTN1 (E7EX90)	89	91	25.24
PTPRJ	89	100	26.93
HP1BP3 (HP1B3)	78	100	25.18
MYO1F	100	91	28.19

Table 61 Candidate Suitability as Biomarkers

Candidate (UniProt ID)	Tissue-specificity	Available in Blood/ Urine	Association with Other Diseases	Suitable?
PGM1	widely-expressed	both	myocardial infarction, spinal cord injury	(Y)
DCTN1 (E7EX90)	widely-expressed	blood	n/a	Y
PTPRJ	widely-expressed	both	cancer, obesity	(Y)
HP1BP3 (HP1B3)	widely-expressed	blood	5-FU resistance, maternal post-partum resilience	Y
MYO1F	widely-expressed	blood (RNA only)	coronary artery disease	N

As noted above, HV and IRIS0 neutrophil proteomes also differ significantly. The RandomForest algorithm identifies a number of SDE able to discriminate well between these classes in this cohort (fig. 12). Again, these do not represent ISG, which are among the detectable/undetectable rather than SDE proteins. Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. Receiver-operator characteristic-mediated performance evaluation for the top five candidate biomarkers (Table 62) is shown in fig. 13 and their suitability as biomarkers is evaluated in Table 63.

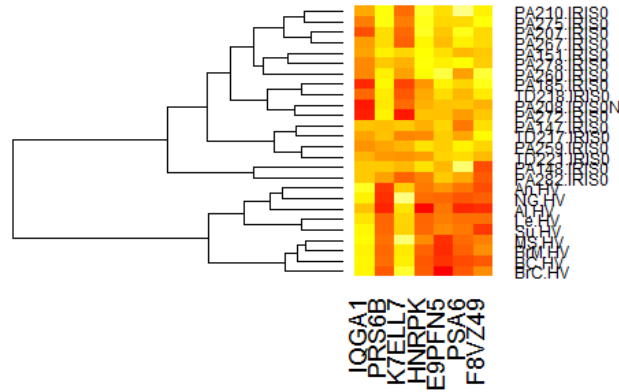


Figure 12 Heatmap Demonstrating Class Stratification on the Basis of Candidate Biomarkers

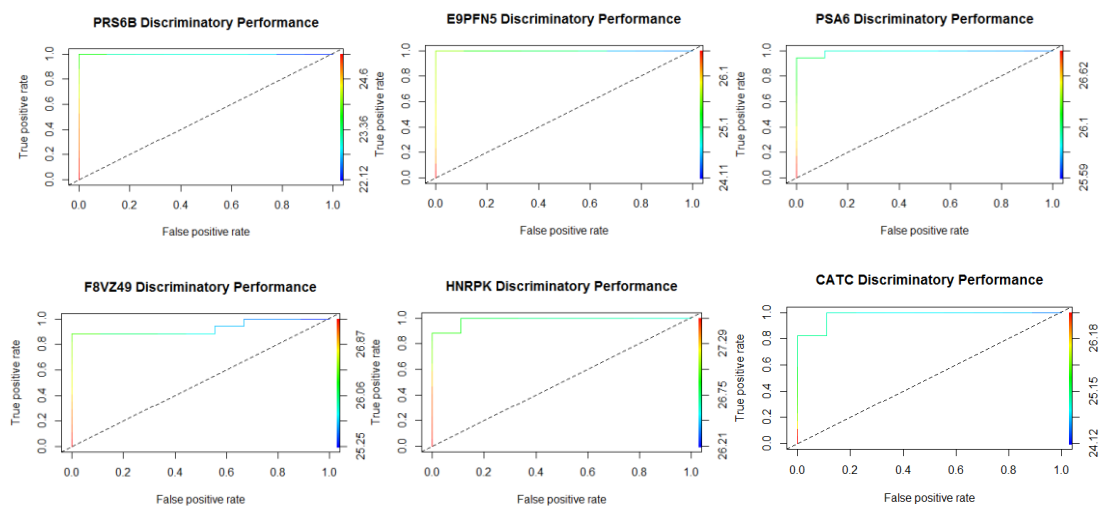


Figure 13 ROC Curves for Top Six Candidate Biomarkers

Table 62 Sensitivity and Specificity of Top Six Candidate Biomarkers

Candidate (UniProt ID)	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
PRS6B	100	100	23.67
GSTK1 (E9PFN5)	100	100	25.71
PSA6	94	100	26.15
HNRNPA1/ROA1 (F8VZ49)	88	100	26.38
HNRPK/HNRPK	100	89	26.82
CATC	100	89	24.92

Table 63 Candidate Suitability as Biomarkers

Candidate (UniProt ID)	Tissue-specificity	Available in Blood/ Urine	Association with Other Diseases	Suitable?
PRS6B/PSMC4	widely-expressed	urine	male infertility, Parkinson's disease, cancer	(Y)
GSTK1 (E9PFN5)	widely-expressed	urine	cardiomyopathy, type II diabetes mellitus, ischemic stroke, age-related infertility, cancer, Alzheimer's disease, etc. (associated with mt dysfunction and circulating mtDNA [79])	N
PSMA6/PSA6	widely-expressed	both	diabetic nephropathy, cancer, ischemia	N
HNRNPA1/ROA1 (F8VZ49)	widely-expressed	urine	cancer, Alzheimer's disease	(Y)
HNRNPK/HNRPK	widely-expressed	urine	cancer	(Y)
CTSC/CATC	widely-expressed	both	cancer, thromboembolism, neutrophilic pulmonary inflammation	N

5.2. Distinguishing Between Partially ATT-treated Co-infected Patients Who Do and Do Not Develop TB-IRIS

As noted above, CTRL0 and IRIS0 neutrophil proteomes share many similarities (fig. 14), largely due to domination by the IFN-induced signature.

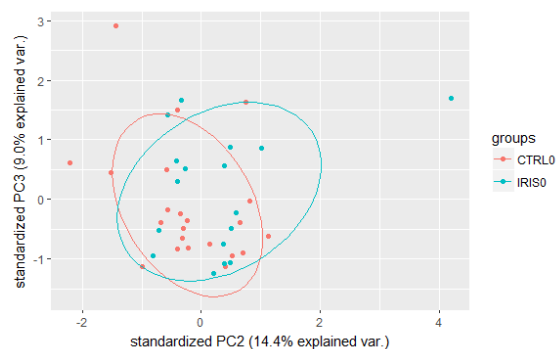


Figure 14 PCA Biplot Demonstrating a Large Degree of Between-class Overlap

Nonetheless, some differences do exist, and the RandomForest algorithm identifies a number of SDE able to discriminate reasonably well between these classes in this cohort (fig. 15). Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. Receiver-operator characteristic-mediated performance evaluation for the top five candidate biomarkers (Table 64) is shown in fig. 16 and their suitability as biomarkers is evaluated in Table 65.

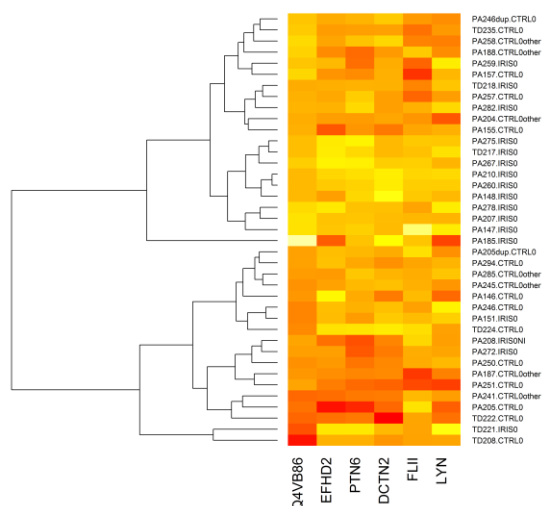


Figure 15 Heatmap Demonstrating Class Stratification on the Basis of Candidate Biomarkers

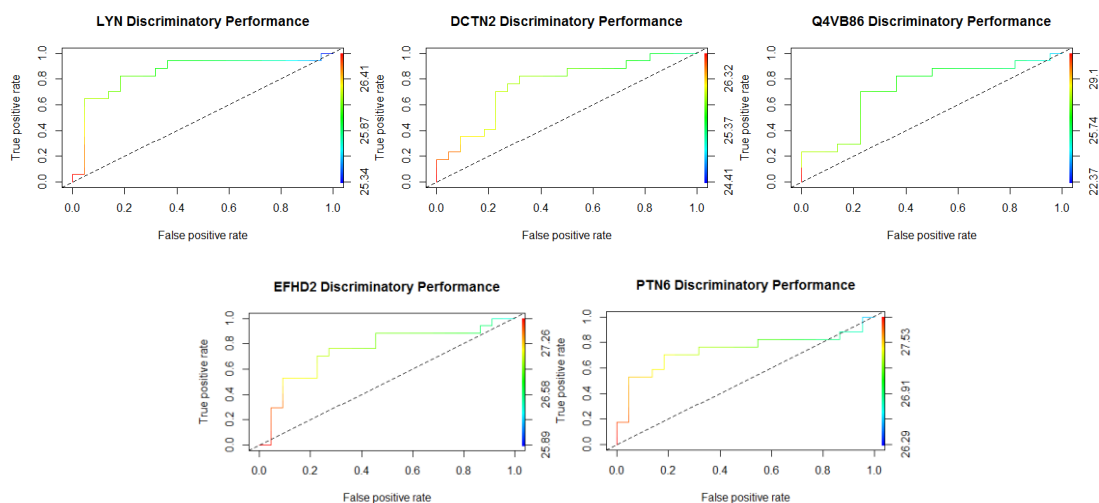


Figure 16 ROC Curves for Top Five Candidate Biomarkers

Table 64 Sensitivity and Specificity of Top Five Candidate Biomarkers

Candidate	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
LYN	82	82	26.13
DCTN2	76	73	26.00
Q4VB86 (EPB41)	71	77	26.96
EFHD2	76	73	27.03
PTPN6	71	82	27.44

Table 65 Candidate Suitability as Biomarkers

Candidate	Tissue Specificity	Accessible in Blood/Urine?	Association with Other Diseases	Suitable?
LYN	widely-expressed non-receptor Tyr kinase for immunoreceptor ITIMs also acts downstream of TLR-2 and -4 and receptors for IL-3, IL-5, and CSF-2	urine	cancer, mercury exposure, steroid-induced osteonecrosis	(Y)
DCTN2	widely-expressed	blood (RNA only)	cancer	(Y)
EPB41	widely-expressed	urine	ALS, cancer	(Y)
EFHD2	widely-expressed	urine	neurodegenerative disease, cancer	(Y)
PTPN6	widely-expressed	blood (RNA only)	neutrophilic disorders	(Y)

All above biomarker candidates take into account only *quantifiable* proteins identifiable between the classes being compared. However, differentially *detectable/undetectable* proteins between classes may represent a more powerful way than SDE to discriminate between classes. Furthermore, candidate biomarkers which discriminate between the CTRL0 and IRIS0 classes in a head-to-head comparison may be less useful for risk stratification than the differences arising between HV and CTRL0 or IRIS0, given apparently superior ability of the latter to discriminate classes.

Appendix J Per-comparison Estimated Protein Fold-changes and Statistical Significance

Table 1 CTRL0 Changes Relative to HVs

Protein	logFC	FC	Unadj. p-value	Adj. p-value
M0QXS5	0,606217	1,522262	0.000101015696925762	0.00180664014366439
CNDP2	0,670028	1,591104	0.000103813442395125	0.00180664014366439
IQGA1	-0,44023	0,737018	0.000106041921475954	0.00180664014366439
ICAM3	-0,66105	0,632418	0.000132639291077847	0.00213193577818003
PGRP1	-1,17297	0,443508	0.000134404646885263	0.00213193577818003
ITAM	-0,43435	0,740025	0.000147775852170548	0.00226589639994841
CH60	0,80701	1,749582	0.000153039935232394	0.00227091516796455
IDHC	0,688683	1,611811	0.000159011922838915	0.00228579639080941
H2B1L	-1,45805	0,363985	0.000177362947612182	0.00242198748157522
E7EVW7	-1,08605	0,471048	0.000179016466029473	0.00242198748157522
E7EX90	-0,45302	0,730514	0.000187195933853377	0.00246028941635866
TCPA	0,771991	1,707625	0.000194208730831616	0.00248155600507065
PUR9	0,62911	1,54661	0.000231747911052572	0.00281114045264921
1433T	0,851819	1,804775	0.000232224646088413	0.00281114045264921
PDXK	-0,44309	0,735556	0.00024633222590944	0.00290545698440601
TRFL	-0,57731	0,670213	0.000282736031960166	0.00325146436754191
MYO1F	-0,33665	0,79188	0.00033024789007912	0.00370522023015598
IQGA2	-0,53553	0,689906	0.000362351539453419	0.00396861209877554
H0YA96	0,420674	1,338553	0.000400946376005919	0.00421506576380321
D6RCD0	-0,5873	0,665588	0.000406269114811886	0.00421506576380321
SAP	1,467342	2,765121	0.00041234338993727	0.00421506576380321
B1AK87	-0,66103	0,632428	0.00043000882379761	0.0043000882379761
BASP1	-1,10536	0,464785	0.000443381498282354	0.0043394784938273
E9PCY7	0,580424	1,495289	0.000470769534719365	0.00451154137439392
PP2AA	-0,3742	0,771534	0.000515378970802659	0.00483825156263721
PROF1	0,682531	1,604953	0.000534319371059996	0.00491573821375197
ITB2	-0,59483	0,662122	0.000573330283397931	0.00517121432084409
Q5T8U5	1,327968	2,510488	0.000588021389089101	0.00520172767271128
PLBL1	-0,77954	0,582552	0.000658431825886464	0.0057146913190146
TCPE	0,717137	1,643916	0.0006876190620926	0.00585749571412215
NCF2	-0,50873	0,702841	0.000739829937319951	0.00612464630005166
PSA6	0,444026	1,360395	0.000745609114788897	0.00612464630005166
LSP1	-1,08112	0,47266	0.000802075814852432	0.00622277756910641
AK1A1	0,510269	1,424316	0.00080386694793935	0.00622277756910641
A0A0U1RQV4	-0,46374	0,725104	0.000806402983445828	0.00622277756910641
CAMP	-1,85438	0,276552	0.000830784004439854	0.00622277756910641
DYN2	-0,5835	0,667342	0.000835774977878851	0.00622277756910641
B4DUR8	0,568247	1,482721	0.000851377873630914	0.00622277756910641
NGAL	-0,69087	0,61948	0.000852249971421096	0.00622277756910641
NBEL2	-0,46929	0,722322	0.000868706006571181	0.00624382442223036
NCF1	-0,41781	0,748558	0.00102117188012414	0.00712775125554875
PLCB2	-0,46424	0,724852	0.00102267735405699	0.00712775125554875
SMHD1	0,447769	1,363929	0.00106244120030378	0.00723590642392959

PDIA6	0,535044	1,448986	0.00108417501345176	0.00723590642392959
APOBR	-0,58572	0,666317	0.00108538596358944	0.00723590642392959
LKHA4	-0,59614	0,66152	0.0012469170911397	0.00819402659891802
RHG09	-0,53613	0,689616	0.00131586654075817	0.00845661570629918
OSTF1	0,765814	1,700329	0.0013236441975077	0.00845661570629918
GDE	-0,55112	0,682492	0.00135682533490137	0.00854985827472095
LDHA	0,496856	1,411135	0.00144225897595955	0.00896539363434312
V9HW75	0,503152	1,417307	0.00153627089458197	0.00932531409606089
ACPH	-0,52732	0,693843	0.00154070406804484	0.00932531409606089
NHRF1	-0,54487	0,685455	0.00158632632751182	0.00947675468383686
LRRK2	-0,62189	0,649821	0.00166300304252894	0.00980745384055526
ARC1B	-0,61263	0,654003	0.00183655738382348	0.0106539616506125
PSMD2	0,431407	1,348548	0.00185286289575869	0.0106539616506125
ALDOC	0,439199	1,355851	0.00188887196946509	0.0106667432241742
FA49B	-0,41154	0,751823	0.00190146292257019	0.0106667432241742
PCAT2	-0,66041	0,6327	0.00202505975509415	0.011223222739076
E7ER45	-0,84567	0,556453	0.00206369974382402	0.0113012128828458
ATPA	0,548217	1,462277	0.00216634402958668	0.0117237441601161
AP2A1	-0,65888	0,63337	0.00228834356785301	0.0122399772233998
SYWC	0,826024	1,772793	0.00238668769260476	0.0125723906550528
F8VZ49	0,643464	1,562075	0.00242692843897657	0.0125723906550528
SQOR	0,516453	1,430434	0.00243248427891239	0.0125723906550528
IMB1	0,368019	1,290579	0.00245991896216537	0.0125729191399564
LOX5	-0,32119	0,800411	0.00255663040985352	0.0129236262476112
ANXA1	0,493113	1,407478	0.00281714490473519	0.0140857245236759
RB11B	-0,30694	0,808357	0.00289003386495894	0.0142947911600119
IF2G	-0,5553	0,680516	0.00292736720564788	0.0143254139850854
PGM2	0,90269	1,869548	0.00316150786494707	0.0152685986537494
TCPH	0,654829	1,574429	0.00318649015382597	0.0152685986537494
FKBP5	0,521136	1,435085	0.00329996333270757	0.0156493106499534
E9PGT1	-0,44581	0,734172	0.00340089322499921	0.0159633763622412
IF4A1	0,381841	1,303004	0.00346215301807322	0.0160867715991281
EF2	0,339299	1,265141	0.00361434011995047	0.0166259645517722
ENOA	0,628091	1,545519	0.00376789431104586	0.0171607067631792
X6RJP6	0,759621	1,693046	0.00391943494620368	0.0176758830907225
S10A9	0,437873	1,354606	0.00401762129707748	0.0178687631465956
LYN	0,324235	1,252	0.00403989427662162	0.0178687631465956
KPYM	0,387978	1,308558	0.00432047320043749	0.0187543534432033
A1AG1	-0,63205	0,645257	0.0043216553586512	0.0187543534432033
HNRPU	-0,4565	0,728752	0.00454821868337407	0.0195530896668418
ENPL	0,52446	1,438395	0.00460475424917896	0.0196128421724289
PP1R7	0,461232	1,376717	0.00477989429896639	0.0201720309864637
TPIS	0,728771	1,657227	0.00491442345678666	0.0205512253647442
VATA	0,825714	1,772412	0.00543016926604002	0.0223591709691703
6PGD	0,448662	1,364774	0.00544397206205887	0.0223591709691703
PSB2	0,542944	1,456942	0.00560453423745784	0.0228149181347841
HNRPK	0,354469	1,278515	0.00567750748658463	0.0229060393461918
S10AC	-1,86627	0,274282	0.00572650983654794	0.0229060393461918

UBA1	0,258633	1,196344	0.00588348800276307	0.0232221815612974
ANXA7	-0,36919	0,774217	0.0059065113971126	0.0232221815612974
PTN6	0,361407	1,284679	0.00610617301728884	0.0238037253216345
TPP2	0,451649	1,367603	0.0069092302373459	0.0267079488166312
RAB2A	-0,41898	0,747956	0.00732274734131953	0.0280705314750582
ANXA5	0,489115	1,403584	0.00832344319328144	0.031625743156691
TKT	-0,42897	0,742794	0.00838769709807892	0.031625743156691
PYGL	-0,30717	0,808228	0.00892205875822761	0.0332407353210388
S10AB	0,732371	1,661367	0.00896054604306263	0.0332407353210388
PRDX3	0,373638	1,295616	0.00986043754346163	0.0358207527659528
SMC1A	-0,39564	0,760154	0.00986806576665174	0.0358207527659528
PDC6I	-0,38468	0,765947	0.00988964261146957	0.0358207527659528
HNRPF	0,396261	1,316092	0.0106630842857002	0.0383204591517352
DHB4	0,423907	1,341556	0.0111706785069713	0.0398334272341614
G6PD	-0,57828	0,669762	0.0113857327311806	0.0402879773564853
ARGI1	0,473232	1,388216	0.0119856255490418	0.0420869294088492
PDIA3	0,294918	1,226815	0.0122561300617975	0.0427107562759609
PEBP1	-0,4478	0,733162	0.0131596424787051	0.0455145529338672
RHG01	-0,31568	0,803474	0.0134786799811758	0.0462700954577678
AMPD3	-0,43009	0,742215	0.0136476887423094	0.0465032357145358
G3V1V0	-0,30492	0,809488	0.0138823270115023	0.0468312794390589
PLCG2	-0,2802	0,823474	0.0139475767025023	0.0468312794390589
PDIA4	0,402137	1,321464	0.0141854922833232	0.047284974277744
A0A1W2PQT3	0,38268	1,303761	0.0145416000835087	0.0476403710058169
CC88B	-0,37514	0,771031	0.0146408854329159	0.0476403710058169
NCKPL	-0,32554	0,798002	0.014692876887799	0.0476403710058169
RAB14	-0,33517	0,792693	0.0147063753974478	0.0476403710058169
A0A087WWU8	-0,30601	0,808878	0.0152012816205161	0.0488992275904714
LYSC	-0,73986	0,598796	0.0154381253496418	0.0489917857590492
BAX	-0,37358	0,771867	0.015443062902309	0.0489917857590492
GDIA	-0,24597	0,843246	0.0162788455116894	0.0512895132560078
EF1A3	0,748041	1,67951	0.0164876870375478	0.0515941227025305
F8W1A4	0,489815	1,404265	0.0170830019358929	0.0530958168277754
D3DSM0	-0,4224	0,746181	0.0176002467288374	0.0542992382901332
TCPD	0,421696	1,339501	0.0177062733554782	0.0542992382901332
A0A1X7SBZ2	0,376913	1,29856	0.0182035019749433	0.0554543768773108
HS90B	0,356409	1,280236	0.0193388617789714	0.0585255027521503
1433B	0,531333	1,445264	0.021415707801593	0.064384492625044
GYS1	-0,29303	0,816187	0.0215548084005582	0.064384492625044
KPCD	-0,26722	0,830921	0.021849741093686	0.0648443929231972
COPD	0,268386	1,204459	0.0225580478382558	0.066517320548703
HP1B3	-0,76121	0,590001	0.0231292289454279	0.0677671676108078
PRS7	-0,29007	0,817862	0.0234609196746565	0.0683039433565948
TERA	0,329638	1,256698	0.0238468797755595	0.0689909729355811
Q32Q12	1,158683	2,232535	0.0246344266602903	0.0708239766483346
1433Z	0,739115	1,669151	0.0261851019946283	0.0747369336475743
RPN1	0,260301	1,197729	0.026320398371537	0.0747369336475743
HMGB2	2,233858	4,703902	0.0265162293180823	0.074831076603177

F5H3G7	-0,54585	0,684989	0.0273117121978374	0.0766060220183244
PDIA1	0,311451	1,240955	0.0279543429025395	0.07793331960708
DPYL2	-0,49607	0,709035	0.0302483645191651	0.0838207691494937
TWF2	0,369778	1,292154	0.0313400375203015	0.0863258518523275
ARP3	-0,2584	0,836015	0.0339479548753331	0.0929527335872217
TBB4B	0,241559	1,182269	0.0365854210057078	0.0995816193054769
CEAM1	-0,32914	0,79601	0.0370082102223604	0.100139862954622
EFHD2	0,326886	1,254303	0.0374301986748922	0.100689423335967
CORO7	0,375458	1,297252	0.0386463856474464	0.102776680506632
ATG7	0,269004	1,204975	0.0386529689731463	0.102776680506632
ACAP1	0,259987	1,197468	0.0396592253995392	0.104846228067747
STK4	-0,23495	0,849715	0.0409281770577112	0.107258473409387
VAT1	-0,26823	0,83034	0.0410380246088089	0.107258473409387
CY24B	-0,33303	0,793868	0.045221295181248	0.116786954045538
PARK7	0,419785	1,337728	0.0452815178517818	0.116786954045538
HOY4R1	0,315768	1,244674	0.0454453582046766	0.116786954045538
NAMPT	0,345646	1,27072	0.0462213447726778	0.118121214419065
HNRPR	0,348226	1,272994	0.0466690931706107	0.118606535129729
DYSF	-0,24111	0,846096	0.0498608378750462	0.126021897925941
TCPZ	1,131588	2,190997	1.07636429415636e-05	0.000825212625519873
IDHP	0,647192	1,566117	1.25050576137168e-07	3.63925924496268e-05
PTPRJ	-0,69583	0,617356	1.38005103546894e-06	0.000126964695263143
PSME1	0,657869	1,577751	1.56626612500718e-05	0.000924429606465863
ASC	1,186934	2,276683	1.60770366341889e-05	0.000924429606465863
A0A1B0GW44	1,042842	2,060282	1.9390347038129e-05	0.000991062181948816
PRS6B	1,513507	2,855032	2.08413463738741e-07	3.63925924496268e-05
HSP7C	0,712343	1,638463	2.3411508454761e-05	0.00107692938891901
IF16	1,630866	3,096989	2.37342994236696e-07	3.63925924496268e-05
GRP75	0,821794	1,767603	3.22213670585424e-05	0.00125306316077374
A0A024R6I7	0,727071	1,655275	3.58717710777193e-05	0.00125306316077374
ROA2	0,876061	1,835357	3.79963365320143e-05	0.00125306316077374
PSME2	0,733379	1,662528	3.87952968992947e-05	0.00125306316077374
AACT	1,022134	2,03092	4.13702519642923e-05	0.00125306316077374
X6R433	-0,48327	0,715353	4.35848055921302e-05	0.00125306316077374
ATPB	0,566324	1,480746	5.44809759716029e-05	0.00134044067665885
1433E	0,645479	1,564258	5.48359476280616e-05	0.00134044067665885
A0A087WX10	-0,59843	0,660472	5.53660279489527e-05	0.00134044067665885
PGM1	-0,58656	0,665929	6.78300260326726e-05	0.00151463362696494
NPM	1,044685	2,062915	6.91463177527472e-05	0.00151463362696494
K7ELL7	-0,60353	0,65814	7.74829886528628e-05	0.00158287060688006
DHRS7	-0,59892	0,660248	7.91435303440031e-05	0.00158287060688006
E9PFN5	0,943012	1,922538	8.04560502857921e-07	9.25244578286609e-05
CPNE3	0,475519	1,390418	8.45454397658284e-05	0.00162045426217838

Table 2 IRIS0 Changes Relative to HVs

Protein	logFC	FC	Unadj. p-value	Adj. p-value
PSMD2	0,660391	1,580511	0.000116345707690248	0.00106691489392547
ENPL	0,443811	1,360192	0.000122220692498304	0.00108011853127855
CATC	0,72639	1,654494	0.000122797698451622	0.00108011853127855
Q5JP53	0,487699	1,402207	0.000146646305231129	0.00126409115109233
B1AK87	-0,70843	0,611984	0.000198150719118265	0.00167456784196024
PTN6	0,604985	1,520963	0.000231172445123839	0.00191606392016105
TCPH	0,925161	1,898897	0.000236578439298032	0.00192387372334815
A0A1B0GW44	1,068355	2,09704	0.000271750531978396	0.00216897183856831
HOYA96	0,594518	1,509968	0.000399396751783505	0.00312981818215801
PUR9	0,801876	1,743367	0.000480155489953258	0.00369548243160454
PDIA4	0,408082	1,326921	0.000519699050540216	0.00392965422426023
ACTY	0,420387	1,338287	0.000570533156069189	0.00423658816464903
CC88B	-0,76697	0,587651	0.000579950584023881	0.00423658816464903
TCPE	0,989715	1,985793	0.000599403675211552	0.00425602379919255
EFHD2	0,586868	1,501983	0.000602360676915883	0.00425602379919255
E7EX90	-0,36876	0,774446	0.000661437571777414	0.00459805795864622
SQOR	0,539141	1,453107	0.000684326365199339	0.0046154067849551
1433T	0,870148	1,82785	0.000685350427464331	0.0046154067849551
HNRPU	-0,51676	0,698938	0.000726305070637591	0.00481596131453541
X6RJP6	0,904871	1,872377	0.000786819589647998	0.00501905090597134
G6PD	-0,68672	0,621266	0.000797498016601962	0.00501905090597134
S10AB	1,016654	2,023221	0.00079916267678099	0.00501905090597134
TERA	0,535711	1,449657	0.000803513950143904	0.00501905090597134
A0A1X7SBZ2	0,485498	1,400069	0.000830822418785514	0.00511549232137938
TKT	-0,38489	0,765839	0.00091865531329287	0.0055766259159046
IMB1	0,486387	1,400932	0.000958104776936634	0.00562922596380443
ACLY	0,341628	1,267186	0.000976296258145245	0.00562922596380443
TRFL	-0,59238	0,663246	0.000984822364940285	0.00562922596380443
SAP	1,236249	2,355852	0.000986019275967126	0.00562922596380443
PGM1	-0,45509	0,729465	0.000992624531900549	0.00562922596380443
PTPRJ	-0,70725	0,612487	0.00110855478885721	0.00620502745451244
VP26A	-0,50634	0,704007	0.0011776224534319	0.00650711894139935
ARC1B	-0,98354	0,505737	0.00120514794970049	0.00654961515623134
PSME2	0,607028	1,523118	0.00121728367500379	0.00654961515623134
BAX	-0,45979	0,727091	0.00123090215233118	0.00654961515623134
RL10A	0,513965	1,427969	0.00126412900294962	0.00663819717149858
DCTN2	0,5178	1,43177	0.00127835351562502	0.00663819717149858
TXNL1	-0,71969	0,607227	0.00153680796005364	0.00788528846170379
UBA1	0,395565	1,315458	0.00161852538424627	0.00806560795349321
G3V1V0	-0,4319	0,741284	0.00162528574722858	0.00806560795349321
H2B1L	-1,23831	0,423869	0.00163038308149414	0.00806560795349321
ARGI1	0,700759	1,62536	0.00164680626428632	0.00806560795349321
ASC	0,979177	1,971341	0.00167817827772316	0.00812690828874924
A0A024R6I7	0,565896	1,480306	0.00183502123350683	0.00878771279601602
LSP1	-0,97537	0,50861	0.00186736665088227	0.00880690585368455

CD97	0,571018	1,485571	0.0018798963771206	0.00880690585368455
TBA1B	0,355763	1,279662	0.00196900522581059	0.00912517475617597
ITB2	-0,49014	0,711957	0.00210520074302023	0.00961902517252741
PCAT2	-0,52115	0,696817	0.00212020276424618	0.00961902517252741
NGAL	-0,70208	0,614684	0.00226862447243302	0.0101851786210274
6PGD	0,453219	1,369091	0.00251397302040076	0.0111703337298219
IQGA2	-0,37288	0,772241	0.00256850026753153	0.0112961593398581
FA49B	-0,37688	0,770102	0.00259993052430315	0.0113188894542895
TLN1	0,300128	1,231253	0.00272116045178097	0.011728201547176
CAMP	-1,87521	0,272588	0.0027858880558373	0.0118882945749097
AT2A3	-0,30902	0,807191	0.00302539585890893	0.012783780541076
COF1	0,419985	1,337914	0.00314822989600248	0.0131736610211366
ITAM	-0,50876	0,702826	0.00320035249067457	0.0132629992642379
EF1D	0,361139	1,28444	0.00344418096452917	0.0140534981872368
NCF2	-0,48476	0,714617	0.00345631277922762	0.0140534981872368
RHG09	-0,57694	0,670386	0.00357605139113695	0.014359618754338
NCF1	-0,36928	0,774171	0.00359823393380163	0.014359618754338
CORO7	0,50943	1,423488	0.00417506167278231	0.0164244055114944
IF4A1	0,302792	1,233529	0.0041918436340241	0.0164244055114944
PARK7	0,676832	1,598626	0.00487743291281391	0.0189385007695747
R4GNH3	0,429472	1,34674	0.00546944163025369	0.0210475834164227
EF2	0,356667	1,280464	0.00567780648400798	0.0214762689444064
PDXK	-0,41408	0,750495	0.00568049805026063	0.0214762689444064
E9PGT1	-0,45958	0,727199	0.00600860992985266	0.0225192250414478
CAP1	0,402461	1,321761	0.00668945080125841	0.0248547697874343
PLEC	-0,46792	0,723005	0.00690117576533125	0.0252268185350566
I3L397	0,365326	1,288173	0.00690664637386701	0.0252268185350566
AP2A1	-0,60943	0,655458	0.00739331171584455	0.0267774567187311
ANXA5	0,477541	1,392368	0.00818114830411981	0.0293839576589636
A0A087X0X3	0,472385	1,387401	0.00908418480476911	0.0323577161227726
MYO1F	-0,31759	0,802407	0.00946613440163748	0.0334418354680799
HS90B	0,252159	1,190988	0.0102909764797815	0.0360602509169579
TCPD	0,518679	1,432643	0.0109910627446885	0.0382028067980705
ANXA7	-0,40118	0,757239	0.0111962011825041	0.0386045016772743
TBB4B	0,283445	1,217097	0.0114383453916592	0.039126403680993
A0A1W2PQT3	0,521759	1,435705	0.0120180522213198	0.0405743915248417
MARE1	-0,24956	0,841155	0.0120499353020412	0.0405743915248417
SNAA	0,409513	1,328237	0.0126131510927659	0.0420091833861224
H3BRU6	0,323303	1,251192	0.0126709833879256	0.0420091833861224
V9HW75	0,477558	1,392385	0.0128312687276193	0.0422158536000299
TCPQ	0,393995	1,314027	0.0137972567623698	0.0450501338225864
COPD	0,295589	1,227386	0.0142167838862584	0.0460709312404315
VINC	0,279072	1,213414	0.0149662923293424	0.0477414854214077
PLCG2	-0,32835	0,796446	0.0150090729051026	0.0477414854214077
LKHA4	-0,35461	0,78208	0.0150645986480544	0.0477414854214077
SMHD1	0,477351	1,392185	0.0153663377086545	0.0480678582708079
H15	-1,95978	0,257068	0.0153906367549223	0.0480678582708079
HOY4R1	0,334725	1,261137	0.0156959123250689	0.0486686202309691

NHRF1	-0,50337	0,705456	0.0159671485821578	0.0488358606890217
RAB14	-0,26807	0,830429	0.015976464865782	0.0488358606890217
GLRX1	0,614221	1,530731	0.0166675177046284	0.0505894375401046
ARPC2	-2,00024	0,249958	0.017118357648047	0.0514981210924769
D3DSMO	-0,33994	0,790076	0.0172058687640758	0.0514981210924769
CAPG	0,517223	1,431197	0.0174766435563644	0.0519478163640902
DYN2	-0,3983	0,758752	0.0176531642115122	0.0521131080490531
GRP78	0,246766	1,186545	0.018511505445295	0.0542752302511709
1433B	0,599219	1,514896	0.0188674935568813	0.0549452008311882
TALDO	-0,42298	0,745882	0.0204505364038953	0.0591555784569051
RALB	-0,58976	0,664454	0.0206711635869114	0.0593951433730587
Q5T8U5	0,887644	1,850152	0.021829666434817	0.0623085181020273
TBA4A	0,587315	1,502448	0.022648301408136	0.0642198546507016
LYSC	-0,67614	0,625838	0.024538999044476	0.0691261999226742
DOPD	0,259078	1,196714	0.0249906131967012	0.0697179234519085
MARCS	-0,86148	0,550389	0.0250725710789926	0.0697179234519085
VAT1	-0,35642	0,781103	0.0255900140993671	0.0703972568854149
FLOT2	0,290017	1,222655	0.0256435483318101	0.0703972568854149
CEAM1	-0,31493	0,803891	0.0259748565009465	0.0708554629867592
A0A087WWU8	-0,43864	0,737831	0.0265783288995212	0.0720456588408405
PLCB2	-0,37402	0,771631	0.0269162479104498	0.0725056428087743
ACTS	0,326451	1,253925	0.0286157590340087	0.0766049201469425
1433Z	0,75731	1,690335	0.0288590437857584	0.0767793078497647
PYGL	-0,23035	0,852426	0.0302778719632743	0.0799632330314118
HNRPF	0,351413	1,27581	0.0304268450513957	0.0799632330314118
MICA1	-0,33876	0,790723	0.0321499901395741	0.0839796712130693
FKBP5	0,368859	1,291331	0.0327029034240009	0.0849093456370145
ANX11	0,282796	1,21655	0.0340013743098891	0.087752049865642
PDIA3	0,297428	1,228951	0.0342868230503953	0.0879620281828594
STAT3	0,262322	1,199407	0.0345552313065123	0.0881260632728213
PSD11	0,264544	1,201256	0.0356481565693832	0.0903785616553185
ANXA6	-0,23733	0,848316	0.0371356828393249	0.093599294173971
EF1A3	0,686856	1,609771	0.0377122992123296	0.094500005584384
COTL1	-0,80869	0,570898	0.0379818141625597	0.0946252133182845
CEAM8	0,466003	1,381277	0.0385767916890341	0.0955551564251361
GDIA	-0,20276	0,868886	0.0407897657876584	0.100459366025605
RETN	-1,15017	0,450571	0.0426448763713011	0.104144126690315
ARP2	-0,38301	0,766836	0.0427691657173683	0.104144126690315
VATA	0,584779	1,499809	0.0440698547806256	0.106708468598032
AP1B1	0,208269	1,155301	0.0457254434321878	0.11009869340376
ARP3	-0,2779	0,82479	0.0463352480801961	0.110717060218733
OLA1	0,220616	1,165231	0.046496027609259	0.110717060218733
PSB2	0,526158	1,440089	0.048082870091608	0.113866576975182
PGM2	0,579538	1,494371	0.049615049914739	0.116852931766407
1433E	0,690316	1,613636	1.0971335281976e-05	0.000181870981020449
PROF1	0,921529	1,894121	1.15450170268189e-06	0.000124397558463974
PRS6B	1,764395	3,397315	1.31121857252399e-10	5.65135204757839e-08
ATPB	0,640969	1,559376	1.37104665640177e-05	0.000218449706145956

SYYC	0,511011	1,425049	1.44167510238772e-05	0.000218449706145956
E9PCY7	0,815912	1,760411	1.46984721072685e-05	0.000218449706145956
K7ENG2	0,833404	1,781885	1.53468806733219e-06	0.000125652850240525
CH60	0,919665	1,891676	1.66831467197298e-05	0.000239681207873452
M0QXS5	0,804783	1,746883	1.7492276135572e-06	0.000125652850240525
PSA6	0,559036	1,473285	2.19523707877178e-06	0.000129060244851584
IDHC	0,837001	1,786333	2.32755978031003e-05	0.000323605892036652
LYN	0,659474	1,579507	2.72614943443185e-06	0.000129060244851584
HSP7C	0,814229	1,758358	2.75698977786029e-06	0.000129060244851584
E9PFN5	1,107667	2,154969	2.99945022033889e-09	6.4638152248303e-07
TCPZ	1,398894	2,636994	3.04477787594619e-06	0.000129060244851584
ROA2	1,081556	2,116317	3.29388095908915e-06	0.000129060244851584
ANXA1	0,624963	1,542172	3.36188891284827e-05	0.000452804412949251
KPYM	0,543617	1,457623	3.60073801916889e-05	0.000469329837403306
AACT	1,031327	2,043904	3.60302341615818e-06	0.000129408591030348
IQGA1	-0,46023	0,726868	3.70236994703304e-05	0.000469329837403306
TCPA	1,010496	2,014603	4.12916650330473e-06	0.000136393568469593
IDHP	0,671412	1,59263	4.33307199653993e-08	6.2251801016957e-06
APOBR	-0,82686	0,563755	4.62411063268871e-05	0.000569426195053952
SYWC	0,913784	1,88398	4.64506339670725e-06	0.000136393568469593
TWF2	0,633026	1,550814	4.88478667525434e-06	0.000136393568469593
K7ELL7	-0,65885	0,633382	5.20756561174038e-06	0.000136393568469593
PDIA6	0,503624	1,417771	5.24645805247064e-05	0.000628117616837457
AMPN	-1,06814	0,476934	5.72221028354263e-06	0.000136393568469593
ATPA	0,68599	1,608806	5.72495141519062e-05	0.000645108966618144
ENOA	0,688282	1,611363	5.80200646746534e-05	0.000645108966618144
PSME1	0,78961	1,728607	5.8374129229948e-05	0.000645108966618144
GRP75	0,837056	1,7864	5.89208520898464e-06	0.000136393568469593
F8VZ49	0,938928	1,917103	6.25743254261439e-06	0.000136393568469593
TPIS	0,973898	1,96414	6.33338674901077e-05	0.000682422422205911
HNRPK	0,556945	1,471151	6.42057985162022e-06	0.000136393568469593
NPM	1,124635	2,180464	6.64562630594304e-06	0.000136393568469593
OSTF1	0,962389	1,948534	7.13342398764532e-06	0.000136440776219556
PIMT	0,752529	1,684743	7.15823023613037e-05	0.000748301543126395
X6R433	-0,56878	0,674186	7.28106230405984e-06	0.000136440776219556
IF16	1,408641	2,654869	7.2920335989115e-05	0.000748301543126395
CNDP2	0,775813	1,712155	8.01748343480922e-05	0.000803612874512273
A0A087WX10	-0,74655	0,596026	8.16528574343697e-06	0.000146634923142556
AK1A1	0,588421	1,5036	8.3034106327228e-05	0.000813356814250801
B4DUR8	0,78779	1,726427	9.22007041904019e-06	0.000158954014024253
GRHPR	-0,65525	0,634965	9.91879611018232e-05	0.000936113626819213
BASP1	-1,42235	0,373104	9.9910039057271e-05	0.000936113626819213

Table 3 IRIS0 Changes Relative to CTRL0

Proteins	logFC	FC	Unadj. p-value	Adj. p-value
LYN	0,335239	1,261587	0.000809425578979284	0.239163674265253
PHB	-0,49439	0,709859	0.00109457059160299	0.239163674265253
C1TC	0,29059	1,223141	0.00359238976175936	0.353487088539033
S10A8	-1,08026	0,472943	0.00533712668234762	0.353487088539033
ARPC2	-1,7044	0,30685	0.00563263255791968	0.353487088539033
PPIA	-0,75507	0,592516	0.00569368060752836	0.353487088539033
RETN	-1,14471	0,45228	0.00598218983560441	0.353487088539033
DCTN2	0,367549	1,290159	0.0064711595155887	0.353487088539033
PSD11	0,261686	1,198879	0.00872445468851161	0.37418375789247
CC88B	-0,39183	0,762163	0.00990564723699649	0.37418375789247
CPNE3	-0,25461	0,838216	0.0104792879626894	0.37418375789247
TCPH	0,270333	1,206086	0.0120031134806147	0.37418375789247
SAMH1	0,449478	1,365546	0.0127163046617654	0.37418375789247
RALB	-0,48923	0,712405	0.0139217538343504	0.37418375789247
R4GNH3	0,323773	1,251599	0.014191699061672	0.37418375789247
ARPC5	-0,75656	0,591906	0.0153012030661435	0.37418375789247
ACPH	0,318637	1,247152	0.0153813331774342	0.37418375789247
CALR	-0,41204	0,751561	0.0160709686572496	0.37418375789247
H15	-1,51013	0,351079	0.0185532743464577	0.37418375789247
PRS8	0,220258	1,164942	0.0185698421688027	0.37418375789247
EFHD2	0,259982	1,197464	0.0200650159208398	0.37418375789247
RS3	-0,33107	0,794949	0.0218272730499999	0.37418375789247
Q4VB86	0,946638	1,927376	0.0228886445734251	0.37418375789247
MARCS	-0,72528	0,604879	0.0236979025108264	0.37418375789247
ACLY	0,174017	1,128195	0.0238054507024113	0.37418375789247
AMPL	0,459622	1,375182	0.0278408028214377	0.37418375789247
Q5JP53	0,246438	1,186275	0.027901486749039	0.37418375789247
EIF3C	-0,35733	0,780607	0.0280710884564312	0.37418375789247
PSMD2	0,228985	1,17201	0.0281899022130397	0.37418375789247
GDIR2	-0,42083	0,746993	0.0288756320110607	0.37418375789247
CAH2	0,762522	1,696454	0.0302030093541759	0.37418375789247
ANX11	0,20579	1,153318	0.030783217837643	0.37418375789247
WDR1	0,265749	1,20226	0.0315502515703416	0.37418375789247
SYK	-0,72733	0,604021	0.0318387071817026	0.37418375789247
SH3L1	0,240065	1,181046	0.0320112639336344	0.37418375789247
PRS4	0,213489	1,159489	0.0326095366768592	0.37418375789247
FLII	0,242085	1,182701	0.0328089552650625	0.37418375789247
SNX2	0,268321	1,204405	0.034315367889986	0.37418375789247
PTN6	0,243578	1,183925	0.0345760317316582	0.37418375789247
H12	-1,26648	0,415674	0.0351625478373702	0.37418375789247
DEOC	0,263185	1,200125	0.0354000022173029	0.37418375789247
A0A087WUQ6	0,309258	1,23907	0.0365813287165204	0.37418375789247
ALBU	0,445316	1,361612	0.036818996772028	0.37418375789247
TWF2	0,263248	1,200178	0.0437906774302982	0.429803949582158
HNRPK	0,202476	1,150672	0.0442589879432428	0.429803949582158

CAN1	0,178351	1,13159	0.0463891548142177	0.440696970735068
BPI	-1,13017	0,456861	0.0495287306538124	0.446620435717946

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Chapter 5: Results & Discussion

Effects of Added ARV Therapy on Neutrophil Functional Profiles, Including Biomarker Candidate Performance

"It is not the expression of [the philosopher's] will to conquer nature. The joys of contemplation are 'immediate enjoyments', joys that belong intrinsically to contemplation. The contribution most his own is that he has the whole in view; that he sees things ... within the horizon of the whole; that he investigates and orders them as parts. Observation requires both proximity and distance." – Heinrich Meier

Abstract

Background TB-IRIS arises in up to 59% of partially ATT-treated TB/HIV-coinfections after initiation of ARV therapy. Various immune mediators – including neutrophils – are implicated in its pathogenesis, which is incompletely understood. Clinical intervention is further complicated by TB-IRIS representing a diagnosis of exclusion, the absence of reliable risk stratification/diagnostic/prognostic/treatment monitoring biomarkers, and non-specific treatment (broadly anti-inflammatory corticosteroids) only partially effective in preventing and treating TB-IRIS. **Methods** Neutrophil proteomic profile changes were followed over two weeks of ARV therapy in partially ATT-treated TB/HIV-coinfected participants (comparing ARV-exposed profiles to those at baseline). Using outputs from this comparison, differential neutrophil proteomic responses to ARV therapy between participants who did or did not go on to develop TB-IRIS (IRIS2, CTRL2) were identified. The same eight neutrophil functional modules discussed in Chapter 4 were interrogated: (1) signalling pathways, (2) PRR and cytokine signalling, (3) the eicosanoid cascade, (4) neutrophil antimicrobial functions, (5) carbon-energy metabolism, (6) protein homeostasis, (7) integrated nitrogen-sulfur-B-vitamin and redox/xenobiotic/glyoxal metabolism, and (8) miscellaneous (including gene expression and cytoskeletal dynamics). Findings were integrated to generate hypotheses regarding TB-IRIS pathogenesis. **Results** Both classes exhibit significant proteomic changes in response to ARV therapy, many of which are shared between classes (i.e. driven by ARV therapy and declining viral load), but some of which are class-unique (i.e. driven by factors preventing/contributing to TB-IRIS pathogenesis). Briefly, ARV exposure induces differential changes in CTRL versus IRIS neutrophil proteomes. Functional profiling suggests that the IRIS group experiences a slower decline in type I IFN signalling (unexpected), and an increase in inflammatory cytokine (e.g. IL-6, TNF α , and IFN γ) signalling (expected). This group may also become more sensitive to epinephrine, but less sensitive to glucocorticoids and vitamin A, and exhibits increased redox, mitochondrial, and ER stress, with simultaneous suppression of autophagy. Further significantly differentially-expressed (SDE) proteins identified during a head-to-head comparison of the CTRL2 and IRIS2 proteomes support and enhance findings of the full analysis (e.g. MMP8 levels are higher in the IRIS group); IRIS-unique proteins identified during the head-to-head comparison further suggest slower decline of ISG expression, among other findings. Various candidate biomarkers distinguish CTRL- and IRIS-group ARV-exposed neutrophils from baseline and from each other. **Conclusions** The IRIS group may enter into ARV exposure with a lesser capacity for adaptive cellular stress responses, and a greater capacity for unrestrained inflammatory responses. Indeed, this group exhibits more indications of neutrophil priming for recruitment and activation, and appears to be more susceptible to inflammatory forms of cell death upon reaching inflammatory sites, which would also impair antigen clearance. A subset of candidates exhibits optimal biomarker characteristics. Both mechanistic hypotheses and biomarker findings require validation in an independent cohort, preferably via orthogonal methods.

1. Proteomic Overview

Neutrophil proteomic differences distinguish (to some degree) between baseline and two weeks' ARV treatment, with more pronounced differences in the IRIS group (fig. 1). Together, the components able to distinguish these classes explain 14.9% (CTRL0 vs. CTRL2) and 19% (IRIS0 vs IRIS2) of variance in the dataset for the CTRL and IRIS groups, respectively.

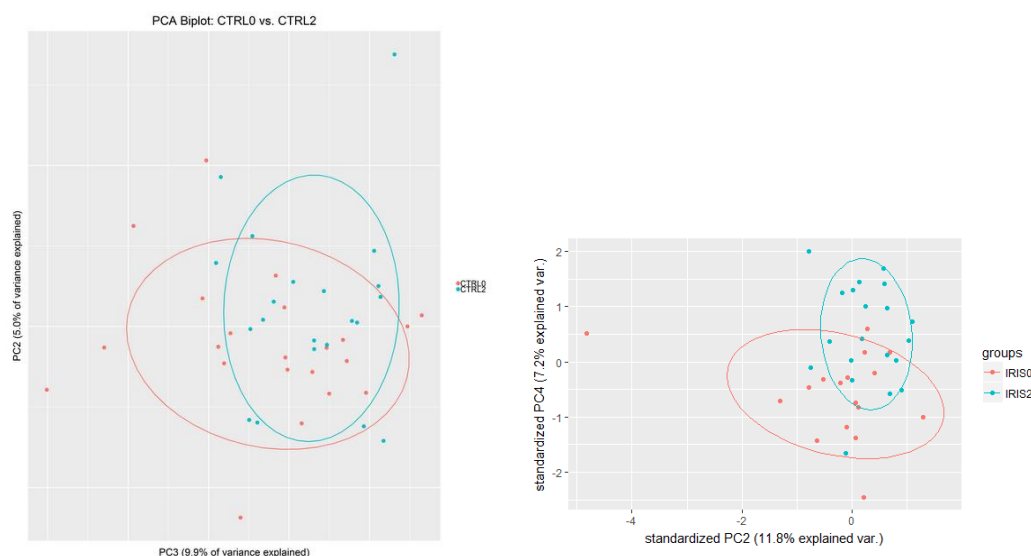


Figure 1 PCA Biplot Demonstrating Between-class Discrimination for both CTRL and IRIS Groups

Indeed, both the analysis determining detectable/undetectable proteins in each class (fig. 2) and differential expression analysis both demonstrate that ARV exposure alters the levels of multiple neutrophil proteins.

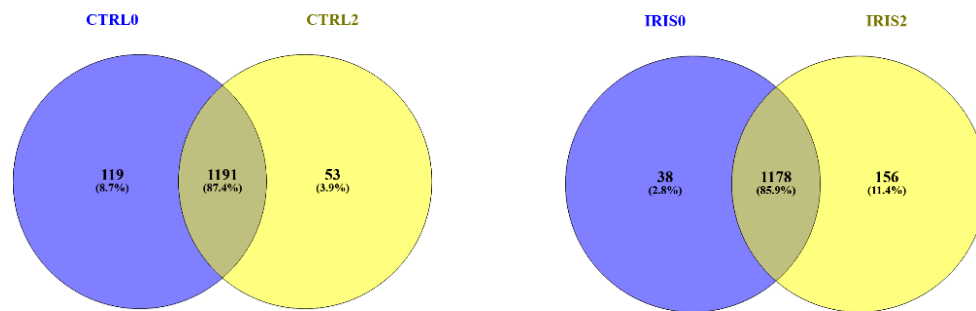


Figure 2 Venn Diagrams Demonstrating Numbers of Detectable/Undetectable Proteins per Class.
In the CTRL2 (relative to the CTRL0) class, 53 and 119 proteins are uniquely detectable and undetectable, respectively.
In the IRIS2 (relative to the IRIS0) class, 156 and 38 proteins are uniquely detectable and undetectable, respectively.

Differential expression analysis (Appendix K) indicates that in the CTRL and IRIS groups, respectively, 50 and 79 proteins become significantly differentially expressed (SDE, $p < 0.05$) during ARV therapy, and that five (LAP3, ALOX5, NAPA, IFI16, STAT1) and 11 (including SAMHD1, NAMPT, FKBP5, HSP90AA1, PSMA6, ALOX5, STAT1, MARCKS, NAPA, IFI35) of these remain SDE after multiple-testing correction (MTC, adjusted $p < 0.05$) (fig.s 3 and 4).

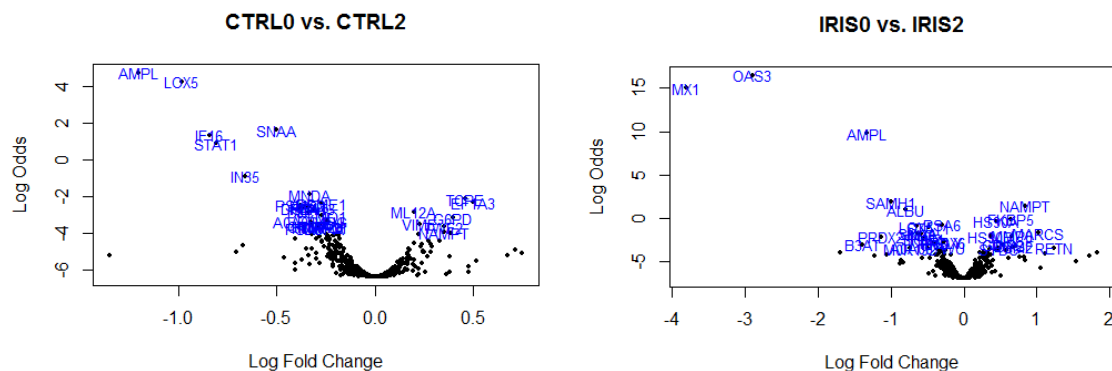


Figure 3 Volcano Plots Demonstrating Distribution of Between-class Proteomic Differences.
Positive logfold-change indicates a relatively higher protein abundance in CTRL2 or IRIS2 (versus baseline) and vice versa.



Figure 4a Representative Grouped Scatter Plots Comparing CTRL Group Inter-Class Protein log2LFQ Values and Spaghetti Plots Demonstrating Trends Over Time in Three CTRL Participants (for whom data from both time-points are available). Protein SNAAL (gene NAPA), down-regulated in response to ARV exposure, is a soluble attachment protein involved in vesicle trafficking and membrane fusion. Protein EF1A3 (gene EF1A3), up-regulated in response to ARV exposure, is a translation elongation factor which promotes loaded tRNA binding to ribosomes.

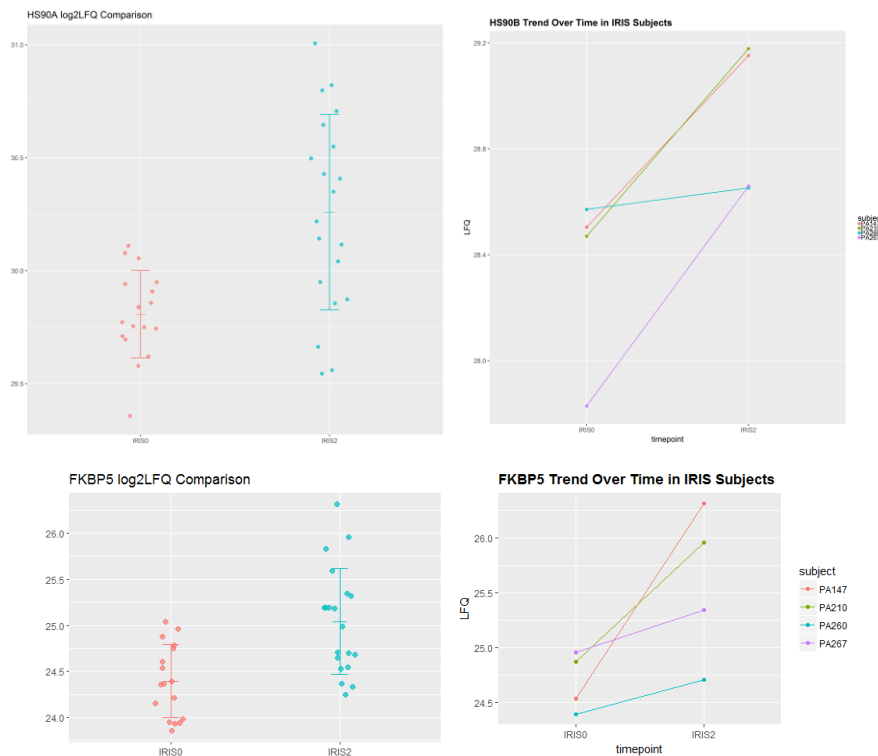


Figure 4b Representative Grouped Scatter Plots Comparing IRIS Group Inter-Class Protein log2LFQ Values and Spaghetti Plots Demonstrating Trends Over Time in Four IRIS Participants (for whom data from both time-points are available). Protein HSP90A (gene HSP90AA1), up-regulated in response to ARV exposure, is an ATP-dependent chaperone which is involved in myeloid TNFa secretion and stabilisation of SMAD3 (for TGFβ signalling). Protein FKBP5 (gene FKBP5), up-regulated in response to ARV exposure, is an immunophilin with roles in AKT1 and immune regulation, and a co-chaperone involved in protein (including steroid receptor) folding/trafficking.

Of all level-altered proteins (i.e. detectable/undetectable *and* SDE) in the CTRL class, many (Table 1a) are 'inference friendly' (as defined in Chapter 3): five rate-limiting enzymes, two protein kinases, two protein phosphatases, and two transcription factors. Of all level-altered proteins in the IRIS class, many (Table 1b) are also 'inference friendly': 14 rate-limiting enzymes, six protein kinases, five protein phosphatases, and one major transcription factor.

Table 1a CTRL Group Response to ARV Therapy: Inference-friendly Level-altered Proteins

	Rate-limiting Enzymes	Protein Kinases	Protein Phosphatases	Transcription Factors
up	NAMPT G6PD	n/a	ANKRD44	STAT6
down	GFPT1 NAGK ALOX5	PKN1 MYLK	DUSP23	STAT3

up uniquely detectable + significantly up-regulated. **down** undetectable + significantly down-regulated

Table 1b IRIS Group Response to ARV Therapy: Inference-friendly Level-altered Proteins

	Rate-limiting Enzymes	Protein Kinases	Protein Phosphatases	Transcription Factors
up	NAMPT COASY HADHB ACAA2 ADK PGM2 MGAM2	TAOK3 MAP2K3 CSNK2A2 STK26 NME1-NME2	ANKRD44 PPP2R2A PTPN1 ACP1 PTPRC	n/a
down	GFPT1 NAGK ALOX5 STT3A AGA ACLY ALDH1A1	AKT1	n/a	STAT1

up uniquely detectable + significantly up-regulated. **down** undetectable + significantly down-regulated

Level-altered proteins represent various functional pathways (Table 2). Members and functioning of various pathways (e.g. carbon-energy metabolism, immune functioning, and protein homeostasis) are considered in greater detail below (Section 3) as part of the eight neutrophil-relevant functional modules identified by deep manual analysis. Cytoskeletal dynamics (including vesicle trafficking) as well as chromatin architecture, RNA Processing, and translation are unfortunately too complex to fit within the scope of this thesis, but are briefly considered.

Table 2a Top 5 Pathways Representing Proteins Up- and Down-regulated During Two Weeks' ARV (CTRL Group)

	KEGG	FDR	Reactome	FDR
represented by ↑ proteins	carbon metabolism	0.0032	immune system	0.0414
	pentose phosphate pathway	0.0065	innate immune system	0.0414
	metabolic pathways	0.0425	neutrophil degranulation	0.0493
	N/A	N/A	N/A	N/A
	N/A	N/A	N/A	N/A
represented by ↓ proteins	proteasome	0.0074	immune system	3.84e-15
	legionellosis	0.0101	innate immune system	1.26e-10
	toxoplasmosis	0.0203	metabolism of proteins	4.44e-09
	metabolic pathways	0.0242	cytokine signalling in immune system	3.15e-08
	herpes simplex infection	0.0301	neutrophil degranulation	5.95e-08

KEGG Kyoto Encyclopedia of Genes and Genomes FDR false discovery rate

Table 2b Top 5 Pathways Representing Proteins Up- and Down-regulated During Two Weeks' ARV (IRIS Group)

	KEGG	FDR	Reactome	FDR
represented by ↑ proteins	endocytosis	0.00081	immune system	2.58e-08
	tuberculosis	0.0445	innate immune system	9.64e-08
	phagosome	0.0445	neutrophil degranulation	2.60e-07
	ubiquitin-mediated proteolysis	0.0445	adaptive immune system	0.00021
	valine, leucine, and isoleucine degradation	0.0445	metabolism	0.00025
represented by ↓ proteins	metabolic pathways	5.65e-05	immune system	3.82e-11
	influenza A	0.00046	neutrophil degranulation	1.51e-07
	lysosome	0.0079	cytokine signalling in immune system	1.01e-06
	measles	0.0084	innate immune system	9.28e-06
	African trypanosomiasis	0.0128	interferon alpha/beta signalling	3.66e-05

KEGG Kyoto Encyclopedia of Genes and Genomes FDR false discovery rate

Although above pathway findings are quite high-level (generic), they already reveal certain not-unexpected divergence in the response to ARV exposure between the CTRL and IRIS groups. Both exhibit apparent bidirectional modulation of immunometabolic function and down-regulation of antiviral responses. However, while the CTRL group experiences mainly carbon-energy metabolism adaptations and *down-regulates* members of the "legionellosis" pathway (encompassing complement-mediated phagocytosis, TLR-MyD88-NFkB signalling, and caspase-mediated functions - e.g. IL-1/18 cytokine maturation and proteotoxic/mitochondrial stress-initiated cell death), the IRIS group mainly *up-regulates* members of endocytosis, the "tuberculosis" pathway (encompassing complement-mediated phagocytosis, TLR-MyD88-NFkB/MAPK signalling, IFN-JAK/STAT signalling, and inhibition of caspase-mediated apoptosis), and protein homeostasis-maintenance responses, while apparently down-regulating certain aspects of metabolism.

2. How Do CTRL2 (Relative to CTRL0) and IRIS2 (Relative to IRIS0) Neutrophils Differ?

A common core of proteins is altered during ARV treatment in both the CTRL and IRIS groups, likely reflecting ARV- (rather than TB-IRIS pathogenesis-) driven changes (Table 3). Far fewer common changes occur over two weeks' ARV therapy than during the transition between health and partially ATT-treated co-infection (Chapter 4, Section 2). Many CTRL- and IRIS-specific (protective/pathogenesis-driven) changes are also noted (*vide infra*).

Table 3. Common Core of ARV-Dysregulated Proteins

Unique or *Up-regulated	Absent or *Down-regulated
AHNAK	ALOX15
ANKRD44	*ALOX5
BRK1	ASNA1
CD82	*CLIC1
CUL4B	CMPK2
DCK	DDX60
DOCK11	DTX3L
*EF1A3/EEF1A1P5	GFPT1
HLA-DRA	*GSTK1
ITGAX	*IFI35
*NAMPT	*LAP3
OGDH	LGALS9
PFKM	*NAGK
PLPBP	*NAPA
PRDX4	PABPN1
QSOX1	PARP10
RAD21	*PSMA6
RIPOR2	RSAD2
RPE	SERPINB9
SNAP23	*STAT1
SNX1	STX3
TXNIP	TTC38
UBAC1	
UBE2M	

3. Altered Neutrophil Immunometabolic Functional Pathways

As noted in Chapter 3, all tabulated information regarding protein function compartments is provided by GeneCards (unless otherwise specified). Fold-changes and p-values for all proteins are provided in Appendix K.

3.1. Signal Transduction

Recall that, at baseline, both classes exhibited likely increased sensitivity to (but decreased production of) pro-inflammatory angiotensin II, pro-inflammatory bradykinin, and anti-inflammatory adrenaline, as well as increased homeostatic/anabolic (e.g. PKA-glucose/lipid) but decreased stress response/inflammatory (e.g. PI3K-AKT, PLC-IP3/Ca²⁺-DAG/PKC, and Ras-MAPK) signalling, including repression of stress/inflammatory signalling by Lyn. These findings suggested a decreased eicosanoid cascade, respiratory burst, and decreased integrin signalling. Both classes – perhaps due to gluconeogenic TGFβ – also exhibited likely-decreased anti-inflammatory AMPK activity, which would de-repress mTORC1 to favour anabolism and repress autophagy. Both classes also exhibited a likely-increased HIF-1α-mediated hypoxic response (including altered iron homeostasis). Finally, both classes appeared more and less susceptible, respectively, to externally- and internally-initiated apoptosis.

Furthermore, the IRIS class exhibited increased insulin sensitivity, especially inhibited MAPK14(-CK-NFkB/STAT1/CREB) signalling (which may de-repress TNF-modulated CASP8), possible de-repressed pro-apoptotic JNK-AP-1-BAX-CytC-CASP9 signalling, altered glucocorticoid signalling, likely-decreased sensitivity to anti-inflammatory progesterone, increased IFNAR signalling (type I IFN oppose mTORC1, and the IRIS class was also missing a protein required for mTORC1 activation), more prominent TLR-4 signalling, and decreased sensitivity to TLR-9 ligands.

During ARV exposure, however, the CTRL class apparently becomes less responsive to adrenaline, as well as to androgens and estrogens (and loses expression of an enzyme which oxidises these), while the IRIS group may become more sensitive to adrenaline and gains expression of the androgen/estrogen-oxidising enzyme (as well as exhibiting a possible decrease in enhancement of androgen signalling) (Table 4). Via intracellular steroid receptors, oestrogen would favour PI3K-AKT- and ERK-mediated cell growth as well as neutrophil infiltration and impaired clearance, while androgens would favour neutrophil maturation, infiltration, and impaired microbicidal/clearance (in association with an N2-like profile with high IL-10 and TGF β secretion). This suggests that adrenaline may be less available while androgens/estrogens are more available in the IRIS class, perhaps enhancing neutrophil recruitment while impairing function and clearance.

Table 4 Dysregulated Proteins Relevant to GPCR- and Enzyme-linked Receptor-mediated Signal Transduction

Class	Protein	Function	Status	Hypothesised Outcome
shared	ANKRD44	putative PPPC6 regulatory subunit PPPC6 is part of a signalling pathway regulating cell cycling in response to IL-2 dephosphorylates (deactivates) MAP3K7, suppressing its activation of the IL-1 signalling pathway dephosphorylates (activates) DDX58/RIG-I	unique	↑ response to IL-2 ↓ MAP3K7-IL-1 signalling ↑ RIG-I signalling
	STAT6	involved in IL-3- and IL-4-mediated signalling key in executing IL-4-induced responses (e.g. expression of anti-apoptotic BCL2L1/BCL-XL, Th2 differentiation, and Ig class-switching)	unique	↑ Th2 cytokine signalling (during CTRL0 this was completely absent)
	WDFY1	PI3P-binding protein localised to early endosome enhances TLR-3/4-mediated signalling by connecting them with TICAM1 promotes TLR-3/4 ligand-induced activation of IRF3 and NFkB and production of IFN β and inflammatory cytokines	unique	↑ TLR-3/4 signalling, including IRF3-mediated type I IFN expression (i.e. slower decline in type I IFN responses compared to IRIS2 group?)
CTRL2-specific	TLR2	PRR that recognises bacterial lipoproteins (e.g. LPS, Mtb LprA, LprG, LpqH, PstS1), PDG, Mtb ndaK, Mtb ESAT-6, MALP-2, soluble tuberculosis factor (STF), PSM, etc. required for normal Mtb uptake (inhibited by Mtb LppM) dimerises with other TLR for differential effects; some of the above recognition requires co-receptors (e.g. TLR-1, TLR-6, CD14, CD36 with TLR-1/6 and LY96: responds to bacterial lipoproteins and other cell wall constituents, promoting apoptosis with TLR-1 and CD14: responds to triacylated lipopeptides with TLR-6, CD14, and CD36: responds to diacylated lipopeptides via MyD88-TRAF6-NFkB induces inflammatory cytokine secretion PstS1 (via TLR-2 and TLR-4) activates p38 MAPK and ERK1/2 PDG (via TLR-2) activates MAPK ESAT-6 inhibits MyD88-mediated downstream signalling	absent	↓ TLR-2-mediated signalling (e.g. for response to LPS, PDG, Mtb lipoproteins/lipopeptides, ESAT-6, etc.) ↓ PDG-induced MAPK activation ↓ Mtb PstS1-induced p38MAPK and ERK1/2 activation (but also transduced via TLR-4) ↓ Mtb phagocytosis ↓ MyD88-TRAF6-NFkB-induced inflammatory cytokine production Recall that CD14 is also absent, so especially inhibited response to acylated lipopeptides
	TMED7-TICAM2	naturally-occurring read-through transcript fusion product inhibits MyD88-independent TLR-4 signalling	absent	de-repressed TLR-4 signalling
	IGF2R	insulin-like growth factor 2 receptor also recognises mannose-6-phosphate transports enzymes to lysosomes activates TGF β degrades insulin-like growth factor 2	absent	↓ sensitivity to insulin-like growth factor 2, mannose-6-phosphate ↓ TGF β activation ↓ lysosomal enzyme loading
	GNAI3	G-protein alpha subunit activates adenylate cyclase associated pathway: beta-adrenergic signalling	absent	↓ cAMP production (possibly in response to epinephrine GPCR)
	GNG5	G-protein gamma subunit required for GTPase activity associated pathway: beta-adrenergic signalling	absent	↓ reassociation of alpha subunit with GPCR complex
	STAT3	activates in response to cytokines and growth factors e.g. IFNs, IL-5, IL-6, IL-31, EGF, HGF, FGF, SCF, BMP2 induces IL-6-mediated acute-phase reactant expression regulates development of T _H 0 into T _H 17 or T _{reg} inhibits autophagy via inhibiting EIF2AK2/PKR	down	↓ IL-6/IFN/Th2/growth factor-induced gene expression de-repression of T _H 17 (over T _{reg}) induction de-repressed autophagy
	PIK3AP1	adaptor for PI3K links immunoreceptors (e.g. TLR) to PI3K-AKT signalling	absent	↓ immunoreceptor-initiated PI3K-AKT signalling
	DGKZ	lowers DAG levels, restraining PKC activity	absent	↑ DAG levels and PKC activity
	RASGRP2	Ca ²⁺ -binding protein with GEF domain activated by DAG and Ca ²⁺ to activate Ras and Rap1 for neutrophil adhesion during inside-out integrin activation	absent	↓ Ras/Rap1 activation during inside-out integrin activation (thus ↓ adhesion)
	RASGRP4	GEF activated by DAG and cations to activate Ras, including in myeloid cells	absent	↓ Ras activation
	KRT1	may regulate PKC and Src kinase activity via binding integrin	absent	altered PKC-Src kinase-integrin signalling

		B1 and RACK1 (receptor of activated protein C kinase 1) associated with cytoskeletal remodelling complexed with C1QBP (which sequesters C1Q to inhibit complement activation)): high-affinity receptor for kininogen-1, which is required for coagulation and kallikrein-kinin system assembly, and contains/releases peptide bradykinin (which also has antimicrobial activity)		?↓ pro-coagulant/bradykinin activity (if C1QBP is present) ?↑ free complement-restraining C1QBP (if this protein is present)
	PKN1	kinase member of PKC superfamily (targets include PPP1R14A, MARCKS, RPS6, etc.) phosphorylated (activated) by PDK1, likely to transduce insulin signals activated by caspase 3 during apoptosis also activated in response to ADRA1B activation, leading to MAPK14 activation key co-activator of androgen receptor-induced transcription	down	↓ insulin and epinephrine (incl. MAPK14) signalling ↓ caspase 3-mediated apoptosis ↓ androgen-induced transcription recall that CASP8 is absent under the CTRL2 condition, so it is not available anyway to activate CASP3/7/10 in response to TNF-type ligands (incl. Fas) MARCKS is only up in IRIS2 (not CTRL2)
	HIST1H4A	H4 histone responds to PKN1 to stimulate transcription of androgen receptor-regulated genes	down	further evidence of ↓ androgen-induced PKN1-mediated transcription
	DOK1	acts downstream of receptor Tyr kinases (e.g. FcγR) as a scaffold for complex assembly inhibits insulin signalling interferes with integrin signalling by competing with talin for ITGB3 binding sites	absent	de-repressed insulin and integrin signalling
	DUSP23	protein Tyr and Ser/Thr phosphatase targets MAPK3 but not MAP10 enhances JNK and MAPK14 activation	absent	↓ dephosphorylation of MAPK3 (a.k.a. ERK1) ↓ enhancement of MAPK14 and JNK activation ERK1 is one of two components with important role in MAPK/ERK cascade activated in response to growth factors such as KIT and SCF, and modulating functions such as growth, adhesion, endosomal dynamics, and survival.
	PDCD4	down-regulates MAP4K1 expression and downstream pro-apoptotic JNK signalling inhibits EIF4A1-EIF4G interaction and thus protein translation	absent	de-repressed MAP4K1 expression and JNK signalling de-repressed protein translation
	LAMTOR2	adaptor enhancing MAPK cascade efficiency and MAPK2 activation part of Ragulator complex, activated by amino acids (via lysosomal V-ATPase) to act as a Rag-activated GEF, thereby recruiting mTORC1 to the lysosomal membrane for activation (facilitating cell growth when amino acid levels are high) mutation is associated with primary immunodeficiency (possibly through disruption of endosomal biogenesis)	absent	↓ enhancement of MAPK cascade and MAPK2 activation ↓ facilitation of mTORC1 activation Stress activates MAPK14, which activates MAPK2 to enhance expression of inflammatory cytokines, such as TNF-alpha and IL-6. Activated MAPK2 also phosphorylates e.g. ALOX5.
	RRAGC	guanine nucleotide-binding protein with dimers with RRAGA and B promotes intracellular localisation of the mTOR complex required for amino acid-induced relocalisation of mTORC1 to lysosomes for activation by RHEB (crucial step in activation of TOR signalling cascade by amino acids)	absent	↓ ability to activate mTORC1
	SMARCE1	part of a chromatin remodelling complex which facilitates de-repression of gene transcription required for co-activation of estrogen-responsive promoters associated pathway: AMPK enzyme complex pathway	absent	↓ capacity for estrogen-induced gene expression ?altered AMPK signalling
	STRN	Ca ²⁺ /calmodulin-binding scaffolding/signalling protein associated pathway: estrogen receptor signalling and dopamine pathway	absent	?altered estrogen signalling
	LCP2	adaptor recruited after PSGL-1 ligation by selectin to activate Fgr, Syk, Btk for convergence of signalling on talin-1 recruitment also recruited (associated with growth factor receptor-bound protein 2) by TCR-activated protein Tyr kinase pathway	absent	↓ selectin-binding-initiated inside-out integrin activation
IRIS2-specific	GNAI2	G-protein alpha subunit involved in hormonal (e.g. β-adrenergic-mediated) inhibition of adenylate cyclase associated pathway: TXA2 receptor signalling	up	↓ AC activation and cAMP production (provided epinephrine is present)
	GNAS	"functions downstream of several GPCRs, including β-adrenergic receptors, e.g. to inhibit AC stimulation by the Gα subunit, or to activate Ras via RAPGEF2"	unique	↓ AC activation and cAMP production (provided epinephrine is present)
	PPP2R2A	subunit of phosphatase 2 which opposes kinases during signal transduction to inhibit anabolism associated pathway: β-adrenergic signalling	unique	↓ anabolism (possibly in response to epinephrine)
	IMPA1	provides inositol for ultimate phosphoinositide synthesis	unique	↑ provision of phosphoinositide precursors
	PPPIP5K2	inositol pyrophosphate kinase synthesizes high-energy inositol pyrophosphates for signalling	unique	↑ provision of inositol pyrophosphates for signalling
	BTK	required for normal BCR signalling via activation of PLCG2 and thus PKC also facilitates recruitment of signalling proteins involved in signalling from cytokine receptors and TLR: induces TIRAP degradation, links TLR-8 and -9 activation to NFκB activation, and is required for NA-activated TLR-9-mediated production of type I IFN, but not for the same effect by TLR-7) [1] experimental B-cell lack of BTK increases production of IL-12 (but decreases production of IL-10) on TLR-9 stimulation [2]	unique	?PLC and PKC signalling initiated by TLR/cytokine receptors ?TLR-9-induced type I IFN, IL-10, and NFκB-mediated pro-inflammatory cytokine production
	PTPN1	founding Tyr phosphatase inhibits growth factor and insulin signalling also dephosphorylates (inactivates) JAK2 and TYK2 that transduce signals from the IFNα receptor likely role in CKII (and SRC) signal transduction	unique	↓ anabolic signalling ↓ type I IFN signalling altered CKII signalling
	PTPRC	dephosphorylates signalling cascade members (e.g. LYN) also suppresses JAK kinase activity to regulate cytokine-	up	↓ LYN activity (normally activated by PKC/enzyme-linked immunoreceptors to dampen integrin

		induced signal transduction		signalling via SHP-1/2, but possibly enhance MAPK signalling) ↓ JAK-mediated cytokine-induced signalling
DGKZ		decreases DAG levels and thus PKC activity (also produces phosphatidic acid)	unique	↓ DAG level and PKC activity
KRT1		regulates PKC activity (via binding ITB1 and RACK1)	unique	↓ PKC activation of selected targets
MARCKS		most abundant PKC substrate Ca ²⁺ /calmodulin or phosphorylation by PKC dissociates it from the membrane and from actin thereby regulating motility and phagocytosis	up	↑ Ca ²⁺ /PKC signalling to alter motility and phagocytosis
PEBP1		phosphatidylethanolamine-binding protein which also binds opioids associated pathways: EGFR and cytokine signalling modulates multiple signalling pathways (e.g. MAPK, NFκB, GSK-3) e.g. inhibits RAF (MAPKKK) activation of MEK (MAPKK) also a serine protease inhibitor for thrombin, neuropsin, and chymotrypsin, tissue type plasminogen activator, and elastase	down	↓ restraint of MAPK pathway activation
LAMTOR2		adaptor that promotes MAPK cascade efficiency and MAPK2 activation also part of Ragulator complex activated by amino acids in a lysosomal V-ATPase-dependent manner to recruit mTORC1 to the lysosomal membrane for activation	unique	↑ enhancement of MAPK2 activation ↑ capacity for lysosomal V-ATPase-dependent mTORC1 activation (provided amino acids are plentiful)
TAOK3		kinase which activates the MAPK14 cascade (e.g. in response to DNA damage), likely via activation of MAP2K3 and MAP2K6 inhibits MAPK8/JNK (e.g. activated in response to EGF) signalling	unique	↓ growth factor-induced MAPK8/JNK signalling ↑ stress (e.g. DNA damage)-induced MAP2K3-MAPK14 pathway signalling
MAP2K3		MAPKK activated by environmental stress, cytokines, adrenergic stimuli (via receptor ADRA1B), mitogens, and insulin (for glucose transporter expression) phosphorylates (activates) MAPK14 also implicated in the integrin pathway	unique	↑ MAPK14 activation (likely in response to stress, cytokines, or epinephrine)
CSNK2A2		casein kinase II (CKII) subunit CKII is a constitutively-active Ser/Thr kinase phosphorylated (activated) by MAPK14 regulatory node which integrates multiple signals, however required for p53-mediated apoptosis in response to DNA damage but prevents cleavage of CASP2 and CASP9 by CASP8, thereby inhibiting apoptosis also interacts with NFκB, STAT1, RNA polymerases, etc. phosphorylates various proteins involved in the HIV lifecycle	unique	↑ epinephrine/cytokine-RAS-MEK-MAP2K3-MAPK14-CKII signalling
C1B1		Ca ²⁺ -binding protein interacts with integrin, DNA-PK, FAK, PKD may be involved in coagulation, angiogenesis, and survival required for recruitment of FAK1 to focal adhesions enhances CDC42-dependent migration on fibronectin inhibits stress-activated MAPK signalling	unique	↑ integrin-mediated adhesion and within-tissue migration ↓ stress-activated MAPK signalling (perhaps post-transmigration?)
RHEB		membrane protein with key role in insulin/TOR/S6K signalling must be farnesylated to be active activates mTORC1 associated pathways: PI3K-AKT-mTOR signalling, insulin-mediated lipid metabolism	unique	↑ mTORC1 activation (provided it is farnesylated and amino acids are plentiful) via PI3K-AKT-mTOR pathway
AKT1		one of three Ser/Thr protein kinases all called AKT kinase recruited by PIP3 involved in cell growth, but also motility (esp. AKT1)	absent	↓ motility branch of PI3K-AKT signalling

Regarding specific signalling pathways, CTRL group ARV exposure suppresses PKA activity (despite possibly-increased KIT/SCF-signalling), further suppresses PI3K-AKT-mTORC1 signalling (although no strong evidence indicates directionality of AMPK modulation, it may be activated), and partially preserves DAG/PKC-mediated effects, while (at least TLR-2-induced) p38 (including MAPK14) and ERK1/2 MAPK signalling remain inhibited. Although an increased capacity for JNK MAPK activation is noted, there is likely decreased *actual* activation. Despite increased adrenaline sensitivity (perhaps due to lower circulating adrenaline levels), the IRIS class, too, exhibits likely-decreased cAMP-PKA-mediated anabolism and (possibly due to increased estrogen oxidation) PI3K-AKT1 signalling, though attempts at mTORC1 activation by RHEB (levels only increased in the IRIS class) actually increase (contingent on FPP-mediated farnesylation and amino acid availability), suggesting strengthened autophagy inhibition. Although ARF6-mediated PLD activation is decreased, RHEB-mediated PLD activation may compensate for this. Decreased DAG-PKC-mediated activity (except via cytoskeleton-modulating MARCKS) and JNK responses to growth factors/insulin persist, but (in opposition to baseline) increased PTPRC-mediated deactivation of Lyn-SHP1/2 inhibitory signalling likely de-represses stress/inflammatory pathways. Indeed there appears to be significant de-repression, enhancement, and activation of MAPK signalling relative to baseline (including increased MAPK14 signalling, thereby activating CKII-NFκB/STAT1 and possibly ALOX5). Indeed, additional findings (Table 10) suggest enhancement of PKD- and CKII-mediated target phosphorylation (e.g. P53, NFκBIA). In opposition to baseline findings, CKII would favour internally-initiated (DNA damage-induced) while inhibiting externally-initiated (CASP8 action on CASP2 and CASP9) apoptosis.

Decreased PKA signalling relative to baseline in both classes suggests de-repression of inflammatory pathways. However, the CTRL class simultaneously exhibits decreased bradykinin activity and becomes less responsive to pro-inflammatory angiotensin II (ACE – known to elevate during BCG-induced granulomatous pulmonary inflammation - generates angiotensin II and degrades bradykinin) while the capacity for responding to androgens, anti-inflammatory glucocorticoids, and possibly vitamin A increases (Table 10). In line with this, the CTRL class exhibits altered PKC-SFK-integrin signalling, including decreased selectin binding-initiated inside-out integrin-mediated adhesion (though absence of DOK1 compensatorily de-represses downstream integrin and insulin signalling), enhanced C1qBP-mediated restraint of complement activation, and decreased pro-coagulant activity. Above signalling findings have already suggested a special tendency in the IRIS class towards especially de-repressed stress/inflammatory pathways (including internally-initiated apoptosis). Indeed, during ARV exposure, the IRIS class becomes *less* responsive to glucocorticoids, and exhibits likely de-repressed integrin signalling, enhanced integrin-mediated adhesion, and cell priming for within-tissue migration. Findings regarding signal transduction pathways are integrated and functional predictions are summarised in fig. 5.

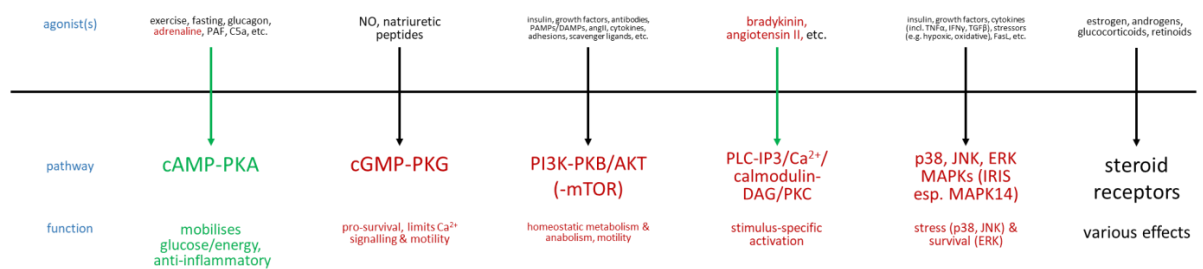


Figure 5a Predicted Baseline Signalling (Both Classes; **active**, **inhibited**, unknown). Inhibition of PI3K-AKT and Ras-Raf-MEK-ERK pathways is likely due to Lyn-mediated (and CD300LF in the CTRL class only) SHP1/2 activation. Retinoids help prevent active TB, and levels are often low during TB. Externally-initiated cell apoptosis is favoured (especially in the IRIS class), while internally-initiated apoptosis is not.

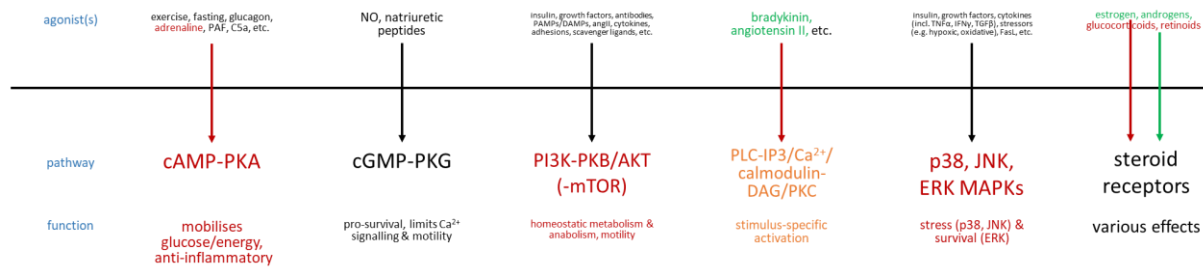


Figure 5b Predicted Signalling During ARV Exposure (CTRL Class; **active**, **inhibited**, unknown). Both Externally- and internally-initiated apoptosis are not favoured.

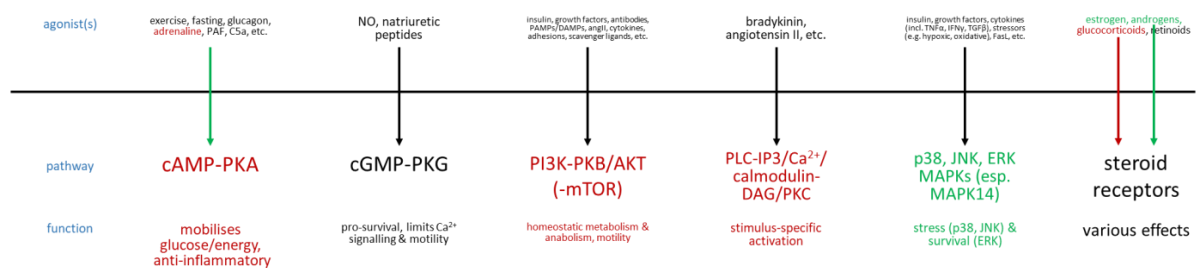


Figure 5c Predicted Signalling During ARV Exposure (IRIS Class). De-repression of MAPK signalling is likely due to a decrease in Lyn-SHP1/2-mediated pathway inhibition. Internally-initiated apoptosis likely becomes favoured, while externally-initiated apoptosis is not.

Specific ARV therapy-induced changes which occur in *opposite directions* in the CTRL versus IRIS groups over time include: DGKZ (which lowers DAG levels to restrain PKC activity), KRT1 (which regulates PKC and Src kinase activity and in co-operation with C1qBP acts as the receptor for pro-coagulation,

pro-inflammatory kininogen), and LAMTOR2 (which enhances the MAPK cascade and as part of the Ragulator complex recruits mTORC1 to the lysosomal membrane for activation when amino acid levels are replete). All decrease to absence in the CTRL group, but increase to uniqueness in the IRIS group.

3.2. Neutrophil Pattern Recognition and Cytokine Receptors

At baseline, both classes exhibited dominant type I IFN signalling signatures, including induction of antiviral ISG such as those which sense intracellular NA and enhance RIG-I, NLRP3, and intranuclear (but not AIM2) inflammasome activation, those which induce further type I IFN and ISG expression via IRF3 (e.g. STAT1 and 2), and those which significantly alter lipid/cholesterol metabolism (e.g. RSAD2, which also binds Fe-S and promotes TLR-7/9-induced IFN β production). Both classes also exhibited decreased TNF α but increased TGF β production (perhaps due to type I IFN-mediated IL-10 induction) and effects. The RELA (p65) subunit of NF κ B was absent from both classes, while NF κ B subunit dimerization-promoting MPTN was unique in both classes, suggesting dysfunction of myeloid chemotaxis, integrin activation, transmigration, phagocytosis, and survival. Although the CTRL class exhibited increased PYCARD, decreased STING suggested that neither intranuclear nor cGAS-STING-activated inflammasome assembly were favoured. Opposing MyD88 inflammatory signalling, the CTRL class exhibited increased inhibitory receptor CD300LF (which signals via SHP1/2), decreased PGLYRP1 (an inflammation-amplifying TREM-1 ligand), and unique SAMHD1 (which would prevent chronic type I IFN induction by host DNA at stalled replication forks). A major difference in the IRIS class was increased STAT3 and 6 (possibly due to especially elevated TGF β signalling), suggesting Th2-type signalling and STAT3-mediated enhancement of the IFN response and ARG1 expression, while also suppressing Th1-type myeloid inflammation, possibly induction of IL-17, and autophagy.

Exposure to ARV therapy (which lowers VL) partially resolves the baseline type I IFN-ISG response, including STAT1 (a change perhaps responsible for the potential decrease in STAT-mediated responses to IL-2 and IFN γ), RSAD2 (a change which would de-repress RHEB farnesylation and cholesterol/steroid hormone synthesis – though levels of RHEB increase only in the IRIS class), and likely RIG-I activation (Table 5). In the CTRL class, additional IRF3-activating ISG decline, in ways suggesting continued suppression of the intranuclear inflammasome (as well as suppression of AMPK activation and phagosome acidification), but de-repression of the AIM2 inflammasome (and JNK signalling), although this class *lacks* CASP1 (DDX58/RIG-I positive regulator ZC3HAV1 is also absent). In agreement, signal transduction findings (Table 4) suggest resolution of elevated baseline type I and II interferon (IFN) and TGF β activity in the CTRL group (including possibly induction of T $_{reg}$). Expression of TLR-2 and CD14 (and thus detection of acylated lipopeptides and possibly Mtb uptake) cease in the CTRL class only.

Table 5 Dysregulated Proteins Relevant to Neutrophil Cytokine Signalling

Class	Protein	Function	Status	Hypothesised Outcome
shared	STAT1	IFN-induced transcriptional response to type I IFN signalling	down	↓ ISG expression
	IFI35	IFN-induced, unknown function paralog NMI interacts with Myc and STAT transcription factors to augment STAT-mediated transcription in response to IL-2 and IFN γ enhances coactivator protein recruitment to STAT1 and 5	down	?↓ STAT-mediated response to IL-2 and IFN γ
	DDX60	antiviral RNA helicase promotes RIG-I-like receptor-mediated signalling enhances DDX58/RIG-I and IFIH1/MDA5-dep type I IFN and ISG expression in response to viral infection	absent	↓ type I IFN- and RIG-I-mediated signalling
	RSAD2	IFN-induced impedes viral budding by disturbing lipid rafts, at least in part via inhibiting enzyme FPPS (required for biosynthesis of sterol and non-sterol isoprenoid lipids)	absent	↑ FPPS activity and isoprenoid-lipid biosynthesis
CTRL2-specific	IFIT1	ssRNA-binding sensors which inhibit viral mRNA expression	absent	↓ need to inhibit viral gene expression
	IFIT3M	disrupts intracellular cholesterol homeostasis to prevent endosome-mediated viral entry newly-budded virions with a cholesterol-depleted membrane may be inactive known activity against HIV-1 critical role in structure and function of V-ATPase (e.g. required for phagosome acidification)	absent	↓ need to inhibit viral entry, activity ↓ V-ATPase-mediated phagosome acidification
	IFI16	cytoplasmic viral dsDNA superstructure-binding transcriptional regulator induces IFN β production after recruiting TMEM173/STING required (at least during HHV-1 infection) to initiate an IRF3 signalling cascade, and may – in association with PYCARD – form	absent	↓ need to inhibit viral gene expression ↓ IRF3-mediated type I IFN production ↓ intranuclear inflammasome activity ↓ restraint of AIM2 inflammasome ↓ capacity for AMPK activation

		on intranuclear CASP1-activating inflammasome also selectively anti-inflammatory, via inhibition of AIM2 inflammasome activation also involved in energy-level-dependent AMPK activation and thus autophagy regulation		
	ISG15	UBE2L6 conjugates ubiquitin-like protein ISG15 to targets (ISGylation) e.g. ubiquitinating enzymes (e.g. UBE2L6 itself), other ISGs (e.g. IFIT1, MX1, IRF3, FLNB), and cytoplasmic viral nucleic acid sensor *DDX58/RIG-I; ISGylation inhibits DDX58/RIG-I, prevents IRF3 degradation, inhibits FLNB-mediated IFN α -induced JNK signalling, prevention of host TSG101 interaction with HIV Gag	absent	↓ IRF3 signalling ↑ JNK signalling ↑ ability of TSG101 to interact with HIV Gag
	OAS2	dsRNA-activated enzymes which activate RNASEL for host and viral RNA degradation	absent	↓ need for host/viral RNA degradation
IRIS2-specific	TSG101	interacts with HIV Gag to support retroviral budding	absent	↓ TSG101-mediated support of HIV budding note that PDCD6IP can also replace TSG101 in its role supporting HIV budding, but here PDCD6IP is also down-regulated
	STEAP4	TNF α -induced Golgi-resident metalloredutase reduces Fe ³⁺ or Cu ²⁺ using NAD as an acceptor helps integrate metabolic and inflammatory responses associated with insulin resistance and obesity involved in inflammatory arthritis	unique	↑ TNF α signalling inferred
	THEMIS2	myeloid inflammatory response regulatory point which promotes LPS-induced TNF production	absent	↓ LPS-induced TNF production
	IL-16	pleiotropic cytokine produced by CASP3 from preproprotein chemoattractant for monocytes, eosinophils, Th signals via CD4 to modulate T-cell activation, priming Th for IL-2 and IL-15 responsiveness (e.g. inducing IL-2R expression) inhibits HIV replication	down	↓ recruitment of monocytes, eosinophils, Th ↓ enhancement of Th sensitivity to IL-2 and IL-15 inferred: presence of CASP3
	WDFY1	PI3P-binding protein interacts with TICAM1 to enhance TLR-3- and 4-induced IRF3 and NF κ B activation for expression of IFN β and pro-inflammatory cytokines	absent	↓ enhancement of TLR-3/4-induced type I IFN and pro-inflammatory cytokine expression
	IFIT3M	IFN-induced disrupts intracellular cholesterol homeostasis to prevent endosome-mediated viral entry newly-budded virions with a cholesterol-depleted membrane may be inactive known activity against HIV-1 critical role in structure and function of V-ATPase (e.g. required for phagosome acidification)	unique	↓ viral entry, activity ↑ V-ATPase-mediated phagosome acidification
	GBP4	IFN-induced antimicrobial protein actually inhibits IRF7-mediated viral-induced type I IFN production [3] associated pathways: cytokine and TLR signalling	unique	↓ type I IFN induction by virus
	GBP5	IFN-induced antimicrobial protein activates NLRP3 inflammasome assembly in response to microbial and soluble agents associated pathways: cytokine and TLR signalling	up	↑ NLRP3 inflammasome assembly
	MX1	type I and II IFN-induced opposes viral replication enhances ER stress-mediated cell death after influenza infection may regulate TRPC calcium channel activity	down	↓ antiviral activity ↓ ER stress-mediated death in response to viral infection ?altered TRPC Ca ²⁺ channel activity
	OAS1	IFN-induced 2-5A synthesiser which is antiviral via RNASEL-dependent and -independent mechanisms	absent	↓ antiviral activity
	OAS3	IFN-induced, dsRNA-activated activates RNASEL for host and viral RNA degradation	down	
	MTPN	promotes NF κ B subunit dimerization	absent	↓ NF κ B dimerization and pro-inflammatory cytokine expression?

In the IRIS class, a protein enhancing TLR-3/4-induced cytokine expression (including type I IFN) becomes absent and additional distinct ISG decline (including one supporting viral-induced ER-initiated cell death), though others in fact *increase* (e.g. GBP4 opposes virus-triggered IRF7-induced type I IFN, while PSPC1 and GBP5 favour cGAS-STING activation and NLRP3 inflammasome assembly respectively, though CASP1, absent at baseline, likely remains so). Thus, *both* classes appear less able to produce inflammasome-matured IL-1 β and IL-18 (unexpected; a major shared change also suggests decreased IL-1 but increased IL-2 and RIG-I signalling). Signal transduction findings (Table 4) suggest enhancement of NA-TLR-9 signalling (including BTK-mediated TLR/cytokine receptor-PLC-PKC signalling, or perhaps TLR-9 induction of type I IFN, NF κ B-induced cytokines, including IL-10) and type II IFN (IFN γ) signalling in the IRIS class. Returning to cytokine-relevant findings (Table 5), elevation of a TNF α -induced protein may additionally suggest increasing TNF α signalling (though NF κ B dimerization may decrease), and intracellular IL-16 levels decrease (IL-16 recruits monocytes, eosinophils, and Th, as well as enhancing sensitivity of the latter to IL-2 and IL-15). Finally, TSG101/PDCD6IP-assisted HIV budding appears to become inhibited.

Both signal transduction and direct cytokine-related findings (Tables 4 and 5) suggest that the CTRL class experiences a more pronounced decline in type I IFN-ISG signalling in response to ARV therapy than does the IRIS class (in which elevated IFIT3M suggests an increased capacity for phagosome

acidification). The CTRL and IRIS groups, respectively, exhibit decreased and increased (e.g. TLR3/4-induced) type I IFN and inflammatory cytokine (IL-1 α , TNF α , IL-6) production (including decreased STAT3-mediated responses to IL-6, and possibly decreased co-induction by IL-6 and TGF β of T_H17, in the CTRL group). It is inferable that STAT1 and 2 (unique at baseline) remain present in the IRIS group, and indeed a head-to-head comparison reveals that ARV exposure results in higher STAT1 levels in the IRIS relative to the CTRL class, suggesting ongoing type I IFN signalling in the former. Similarly, it is inferable that STAT3 and 6 remain present, and a head-to-head comparison reveals that ARV exposure results in higher STAT3 levels in the IRIS relative to the CTRL class, suggesting a response to IL-6, type I IFN, and/or IL-10 (the IL-18R has also been shown to activate STAT3). While STAT6-mediated T_H2-type cytokine signalling likely continues in the IRIS group, T_H2-type cytokine signalling may be increased in the CTRL group. Integrated findings are depicted as models predicting functions of complement, pattern recognition (including inflammasome sensor), and cytokine receptors (fig. 6).

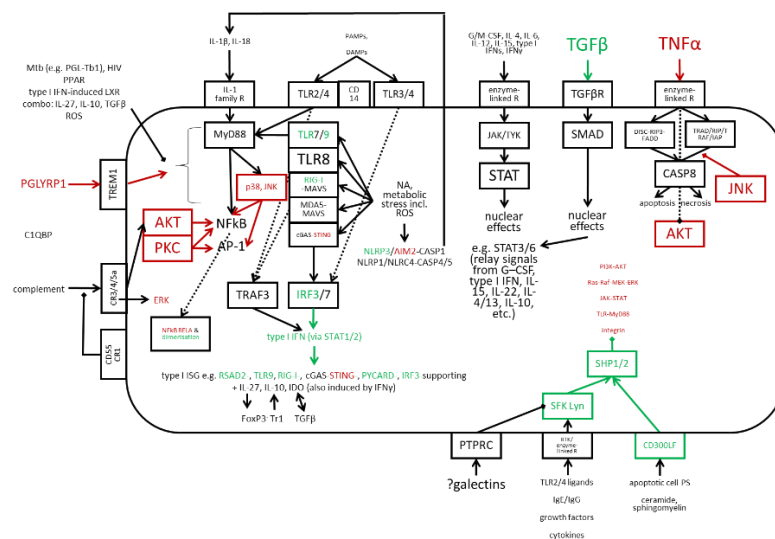


Figure 6a CTRL (baseline)

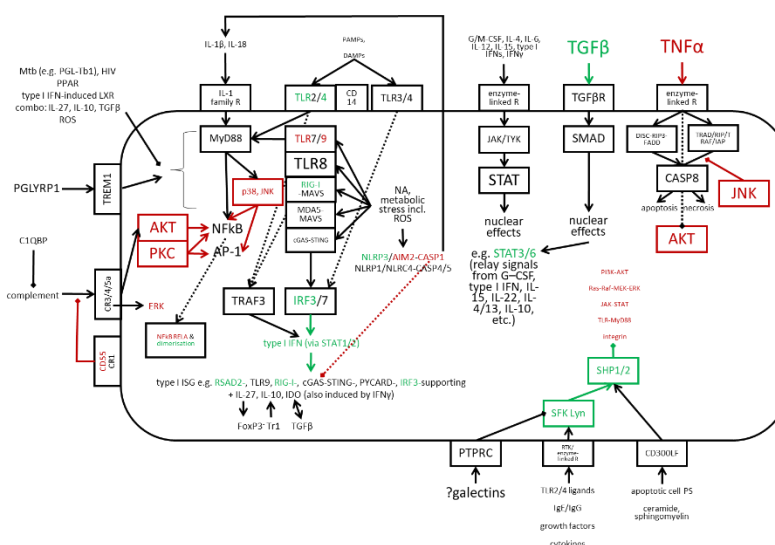


Figure 6b IRIS (baseline)

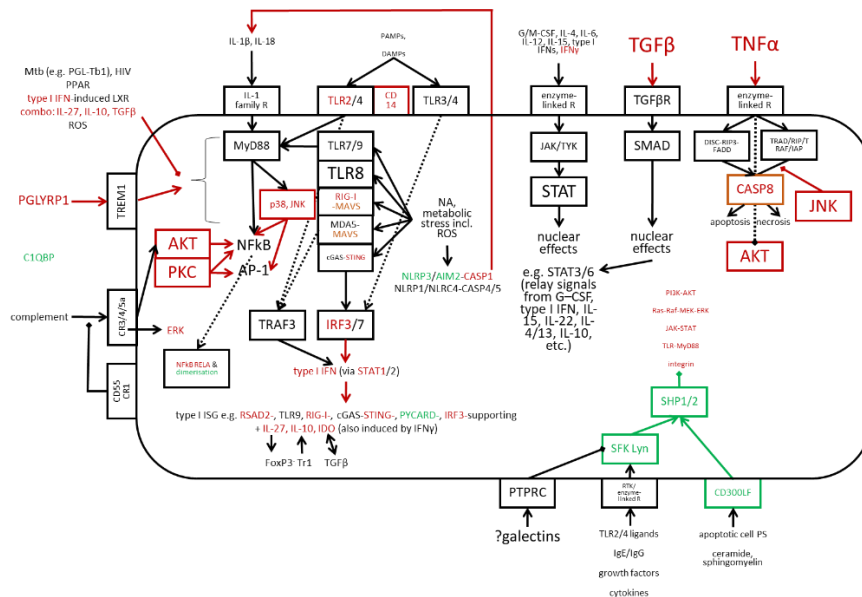


Figure 6c CTRL (ARV-exposed)

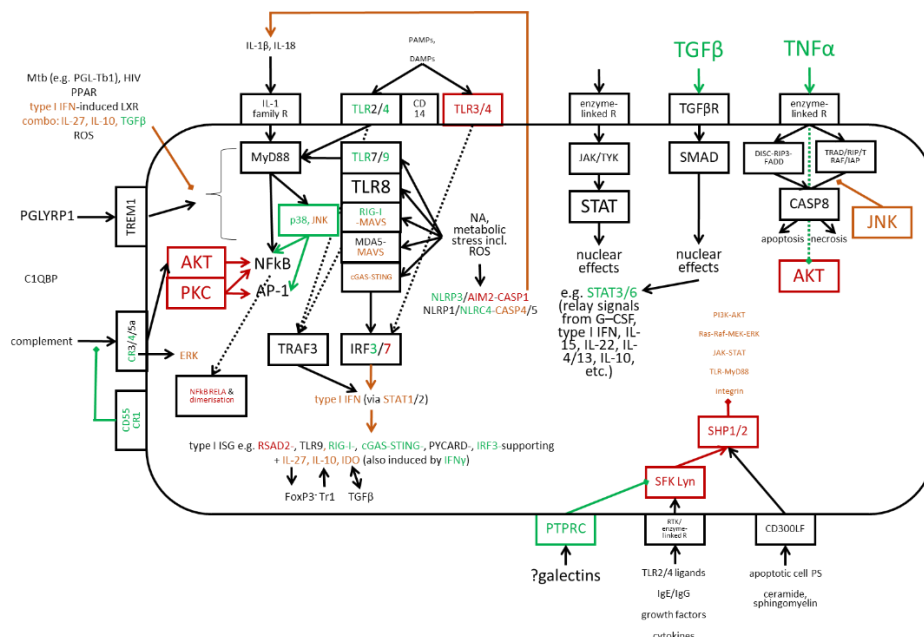


Figure 6d IRIS (ARV-exposed)

3.3. Eicosanoid Cascade

At baseline, both classes exhibited decreased arachidonate provision and LTB₄ production/sensitivity (perhaps due to impaired TNFα signalling; both are usually required for neutrophil influx). The CTRL class also exhibited a decrease in the first step of leukotriene (and thus also lipoxin) synthesis, possibly with funnelling of residual arachidonate towards HETE/HPETE/prostanoid synthesis (though type I IFN oppose PGE₂ synthesis). The IRIS class perhaps also experienced some degree of decreased first-step leukotriene biosynthesis, possibly with concomitantly elevated HETE/HPETE and prostanoid synthesis (indeed literature reports elevated HETE production).

After ARV exposure, both classes exhibit a decreased first step of leukotriene biosynthesis, as well as decreased ALOX15-mediated HETE/HPETE synthesis (Table 6), suggesting that residual arachidonate may be redirected towards prostanoid synthesis. There are indications that arachidonate provision and pro-inflammatory PAF signalling may somewhat recover in the CTRL class, while COX2-mediated prostanoid expression is inhibited in the IRIS class (suppression of PGE2 synthesis prevents its amelioration of immunopathogenesis in response to Mtb).

Table 6 Dysregulated Proteins Relevant to the Neutrophil Eicosanoid Cascade

Class	Protein	Function	Status	Hypothesised Outcome
shared	ALOX5	rate-limiting step of leukotriene synthesis	down	↓ leukotriene and HPETE/HETE synthesis
	ALOX15	involved in response to IL-13 converts PUFA to eicosanoids, hepoxilins, lipoxins, etc. e.g. arachidonate to 12/15-HPETE and HETEs may assist in wound healing through generating LXA4	absent	↓ IL-13 enhancement of efferocytosis and wound healing
CTRL2-specific	ANXA2	Ca ²⁺ -dependent phospholipid-binding protein inhibits PLA ₂ , anticoagulant (via plasmin), required for efficient DNA synthesis and lipid raft formation, prevents apoptosis, involved in uptake of apoptotic cells [4]	absent	↑ arachidonate provision
	PAFAH1B1	non-catalytic subunit of intracellular PAF acetylhydrolase which inactivates chemotactic, pro-inflammatory, PLA ₂ -activating, hypotensive PAF	absent	↓ PAF inactivation ↑ chemotaxis, inflammation, arachidonate provision
IRIS2-specific	ANXA3	Ca ²⁺ - and phospholipid-binding protein inhibits PLA ₂	up	↓ arachidonate provision
	CELF2	inhibits COX2 (PTGS1) mRNA translation thus down-regulating prostaglandin (incl. PGE2) expression	unique	↓ COX2-mediated PG expression

3.4. Neutrophil Inflammatory/Antimicrobial Response Inhibition/Activation

At baseline, both classes exhibited a de-repressed capacity for insulin-induced FA biosynthesis (including due to absent PTPN1 and RETN in the IRIS class) and for inflammatory signalling/activation in response to appropriate stimuli (including due to down-regulated inhibitory receptors), provided that SHP1/2-mediated inhibition is lifted. However, both classes also exhibited changes suggesting decreased Th1/cytotoxic/Th17-type responses concomitant with support of T_H2/T_{reg}-type responses (provided galactoside ligands for TNFa/IL-8-inducible LGALS9 are present, and also mediated by up-regulated ARG1, which may also decrease NO production). Only the CTRL class additionally exhibited CD300LF-mediated inhibition of TLR-MyD88/TRIF-NFκB signalling (perhaps due to higher levels of/sensitivity to vitamin D or another inducer). Despite STAT3 and 6 signalling, the IRIS class did not exhibit up-regulated CD300LF. Also at baseline, both classes appeared undisposed to chemotaxis, adhesion, extravasation, or migration through the ECM, and less capable of iC3b-dependent opsonisation and the respiratory burst. Perhaps due to potentially-elevated TGFβ signalling, the neutrophil phenotype may be more tissue-healing than inflammatory at this time. Although both lysosomal hydrolase (e.g. cathepsin) and anti-protease (e.g. SERPIN) expression were increased, findings of decreased lysozyme and cathelicidin levels may reflect either non-induction (e.g. by IFNγ, TNFa, vitamin D) or exocytosis. The CTRL class exhibited additional findings supporting increased activation (provided LGALS3 galactoside ligands or IgE are present), decreased motility/extravasation, and decreased respiratory burst capacity (although S100A9/calprotectin suggested activation, this was unlikely given inhibited PI3K-AKT and MAPK pathways and unavailability of arachidonate for NOX activation; decreased S100A12 may de-repress MMP-9 activity). The CTRL class remained able to transiently roll on resting endothelia via SELL, while the IRIS class exhibited a more profound decrease in integrin signalling ability, but increased compensatory terminal arrest and transmigration effector levels. In the IRIS class, ligands for up-regulated non-integrin adhesive receptors were absent or down-regulated, along with other findings suggesting decreased restraint of lectin-based complement activation, decreased availability of recruitment signals, and ineffective: degranulation, phagocytosis, respiratory burst, hydrolytic enzyme activity (increased protease activity with the exception of CTSS and lysosomal ceramidase – perhaps due to degranulation), bactericidal, and TNFa activation. Additionally, only the CTRL class exhibited findings suggestive of increased intracellular iron sequestration.

Exposure to ARV therapy, as predicted by baseline findings, indeed appears to favour more unrestrained neutrophil activation in both classes, including enhanced neutrophil-mediated NK cell, Th1, and CD8⁺ T-cell activation (in agreement with above suggestions of increased IL-2 signalling), although CTRL and IRIS neutrophils may focus more on CD8⁺ T-cells and NK cells, respectively (Table 7).

This is consistent with existing literature (Chapter 1) suggesting that: (a) at baseline IRIS-group NK cell frequencies (and expression of their activating and inhibitory receptors) are higher, that there is larger proportion of NK cells sensitised to IL-18, and that NK cells are already activated/degranulating, and (b) at the time of IRIS manifestation Similarly, NK counts and IL-18 sensitisation increase (although apparently now cells are less activated and degranulation levels are on par with those of the CTRL group). At first glance, CTRL class findings indeed suggest decreased homeostatic restraint (of e.g. cytotoxicity, autoreactivity, NETosis), de-repressed TLR signalling (though TLR-2 and CD14 are absent, suggesting decreased Mtb uptake and decreased responses to inflammatory LPS, PDG, oxLDL, and Mtb lipoproteins), increased activation (including CD300LF becoming absent), adhesion, lysosomal glycogen catabolism, antigen presentation to/activation of CD8⁺ T-cells, and opsonic efferocytosis (provided receptor ligands are present). However, activation does appear somewhat more restrained in the CTRL class, including increased expression of regulatory receptors (e.g. restraining lectin-based complement fixation, the respiratory burst, and NETosis, as well as favouring self/viral tolerance) perhaps *in response to* inflammation, an increased capacity for response to glucocorticoids, the noted decreased sensitivity to angiotensin II, and likely-decreased expression of inflammatory cytokines such as IL-1 α , IL-6, and TNF α (as well as decreased apoptosis mediated by TNF/Fas-CASP8-CASP10-CASP3/7, due to absence of CASP8), though IL-10 production may also decrease (findings also suggest decreased enhancement of IL-4/IL-13 signalling). Indeed, evidence indicates decreased: chemotaxis, transient rolling on resting endothelia, ITGB2 activation, arrest, transmigration, and lysosomal cathepsin Z/protective anti-protease activity (unless these latter findings are due to degranulation).

Table 7 Dysregulated Proteins Relevant to Neutrophil Inhibition/Activation

Class	Protein	Function	Status	Hypothesised Outcome
shared	HLA-DRA	non-polymorphic alpha chain of HLA-DR one of three highly-polymorphic human MHC-II isotypes: HLA-DR, -DQ, and -DP MHC-II antigens derive from endocytosis and autophagy and initially associate with CD74 in the ER after entry into endolysosomal system, proteases – e.g. CTSS and CTSL – degrade CD74 remaining ‘CLIP’ removed by a β CLIP complex (facilitated by HLA-DM, regulated by HLA-DO)	unique	↑ antigen presentation to CD4 ⁺ T-cells
	CD82	T-cell co-stimulatory molecule, expression strongly correlates with that of p53	unique	↑ T-cell co-stimulation ?↑ neutrophil proneness to apoptosis
	ITGAX	combines with ITGB2 to form iC3b receptor 4 for phagocytosis of complement-opsonised particles this combination also facilitates neutrophil adhesion to activated endothelia and fibrinogen	unique	↑ capacity for adhesion and for phagocytosis of opsonised particles
	LGALS9	galactoside-specific helps activate macrophages (incl. IL-1 β secretion) via ERK1/2 phosphorylation induces myeloid IL-6, IL-8, IL-12, and CCL2 production induces DC maturation and migration also inhibits mast cell degranulation and induces their apoptosis inhibits NK cell function promotes stromal suppression of T-cell proliferation induces – via TIM3 ligation – Th1 death induces – via CD44 and SMAD3 – FOXP3 expression and Treg expansion and function induces post-viral CD8 ⁺ T-cell apoptosis via PDIA1 (also up-regulated) ligation alters Th2 membrane redox state to enhance migration	absent	↓ myeloid activation capacity specifically in response to galactoside ligands (incl. IL-1 β , IL-6, IL-8, and IL-12 production); such ligands may not be present in this context ↑ NK, Th1, and CD8 ⁺ T activation ↓ Th2 migration
	SERPINB9	acute-phase neutrophil granule protease inhibitor also inhibits granzyme B	absent	↑ neutrophil degranulation with inhibition of lymphocyte cytotoxicity OR ↓ production with restraint of neutrophil hydrolase activity and of lymphocyte cytotoxicity
CTRL2-specific	LILRA3	monocyte/B-cell immunoregulatory immunoreceptor high-affinity binding to monocytes abolishes LPS-induced TNF α production soluble receptor for MHC-I antigens	unique	↑ neutrophil regulation ↓ TNF α production ↑ MHC-I antigen-scavenging
	AGTRAP	inhibits angiotensin II receptor-mediated signalling	unique	↓ response to angiotensin II
	TPP2	neutral N-terminal tripeptidase part of proteolytic cascade downstream of ubiquitin-26S proteasome-mediated protein degradation essential for certain MHC-I antigen presentation activities	up	↑ MHC-I antigen presentation to CD8 ⁺ T-cells
	GAA	lysosomal alpha-glucosidase required glycogen breakdown to glucose	unique	↑ lysosomal glycogen catabolism

	SELL	L-selectin	absent	↓ transient rolling on resting endothelia
	MYLK	Ca ²⁺ /calmodulin-dependent myosin light chain kinase involved in inflammatory response (apoptosis, vascular permeability, diapedesis, motility and morphology) PTK2B/PYK2 phosphorylation mediates ITGB2 activation and is essential to trigger neutrophil transmigration during AII	absent	↓ ITGB2 activation ↓ inflammatory responses dependent on motility
	MEMO1	likely relays extracellular chemotactic signals to the cytoskeleton mediator of ERBB2 and GPCR signalling	absent	↓ transmission of chemotactic signals to cytoskeleton
	CD300LF	enhances uptake and clearance of phosphatidylserine-expressing apoptotic cells and negatively regulates MyD88/TRIF-mediated TLR signalling-mediated activation of NFκB through activation of PTPN6/SHP-1 and PTPN11/SHP-2 (protein phosphatases which negatively regulate signalling cascades; up under both conditions) may also be an IL-4 co-receptor enhancing IL-4 and IL-13-induced signalling [5] in dendritic cells, active vitamin D, which programs them to become tolerogenic, induce increased CD300LF expression, among other genes [6] treatment with CD300LF ligands blocks TLR2,3,4, and 9-mediated expression of IL-8 and MMP-9 [7]	absent	↑ TLR signalling ↓ IL-4 signalling ↓ resolution/skewing
	SIGLEC-5	ligation by major ligand, sialic acid (ubiquitous in the ECM and on host cell surfaces, including cis-binding), causes ITIM phosphorylation, SHP-1/2 recruitment, and inhibition of signalling cascades provides baseline level of inflammatory inhibition, including of NETosis ligation of SIGLEC-5 and -9 are known to dampen NETosis [8]	absent	↓ baseline anti-inflammatory activity de-repression of: - neutrophil inflammation - NETosis
	VSIR	immunoregulatory receptor inhibits T-cell response may stimulate MMP14-mediated MMP2 activation	absent	↓ restraint of neutrophil activation
	VSTM1	inhibitory immunoreceptor regulating phagocytes isoform 2: cytokine favouring Th17 differentiation and promoting IL17A secretion by T-cells paralog of LILRB1 (binds MHC-I on APC to transduce inhibiting signals to limit inflammation, cytotoxicity, and autoreactivity)	absent	↓ restrain of neutrophil activation
	TLR-2	as above with TLR-1 and CD14: responds to triacylated lipopeptides with TLR-6, CD14, and CD36: responds to diacylated lipopeptides via MyD88-TRAF6-NFκB induces inflammatory cytokine secretion	absent	↓ Mtb uptake ↓ response to lipoproteins (incl. Mtb-generated) and PDG ↓ inflammatory cytokine production
	CD14	monocyte/macrophage surface antigen with PPR mediates innate response to LPS (i.e. co-receptor for LPS) with LBP, delivers LPS to TLR-4/LY96 complex, signalling via MyD88-TIRAP/TRAFF6-NFκB activation to induce cytokine secretion and an inflammatory response binds electronegative LDL and mediates cytokine release	absent	↓ response to LPS and electronegative LDL ↓ inflammatory cytokine production
	HNRNPM	Kupffer cell receptor for carcinoembryonic antigen activation may culminate in induction of IL-1α, IL-6, IL-10, and TNFα	down	↓ inflammatory cytokine (and IL-10) production in the presence of ligand
	HNRNPU	negative regulator of glucocorticoid-mediated transcription enhances mRNA stability and expression of TNFα prevents accumulation of HIV transcripts in the cytoplasm	down	↑ capacity for response to glucocorticoids ↓ enhancement of TNFα expression ↓ need for anti-HIV activity
	CASP8	on ligation of TNFRSF1A or Fas, a DISC complex activates CASP8, which activates downstream apoptotic caspases (e.g. 3, 4, 6, 7, 9, and 10)	absent	↓ responsiveness to TNF and Fas ↓ TNF/Fas-triggered apoptosis
	CTSZ	lysosomal cathepsin role in peptide hormone metabolism	absent	↓ lysosomal cathepsin Z activity (protease capacity) OR degranulation
	SERPINA1	a.k.a. alpha-1-antitrypsin secreted serine protease (elastase, plasmin, thrombin, trypsin, chymotrypsin, plasminogen activator) inhibitor protects LRT from elastase-mediated damage (e.g. emphysema)	down	↓ host-protective anti-protease response during inflammation OR degranulation
	SERPINB1	suicide inhibitor of neutrophil elastase, CTSG, kallikrein-3, granzyme H, proteinase-3, etc. for host tissue protection during inflammation	down	
IRIS2-	LILRB2	inhibits FcγRIIA- (but not FPR)-mediated	unique	↑ restraint of neutrophil activation and ROS production

specific SELL & SIGLEC-5 are absent under CTRL2 condition (relative to CTRL0)		neutrophil activation for early attenuation of ROS production major ligand for LILRB2 is soluble or cell-associated MHC-I (including cis-binding) ligation produces ITIM phosphorylation, SHP-1 recruitment, and Syk inhibition highest affinity for non-classical HLA-G (whose expression increases under pathological conditions), and avidity is highest for HLA-A, then -B, then -C ligation by "soluble HLA-G inhibits phagocytosis of opsonised bacterial particles" [8]		(provided MHC-I is available as a ligand)
	SIGLEC5	ligation by major ligand, sialic acid (ubiquitous in the ECM and on host cell surfaces, including cis-binding), causes ITIM phosphorylation, SHP-1/2 recruitment, and inhibition of signalling cascades provides baseline level of inflammatory inhibition, including of NETosis ligation of SIGLEC-5 and -9 are known to dampen NETosis [8]	unique	↑ restraint of neutrophil activation and inflammatory function, incl. NETosis this is an opposite-direction change compared to CTRL2
	HLA-E	non-classical MHC-Ib which participates in self-tolerance with β2-microglobulin binds VL9 peptides originating from classical MHC-Ia complex forms inhibitory ligand for NK cell KLRD1-C1 during cell stress, HLA-E-β2M instead binds peptides derived from stress-induced chaperones and non-classical MHC-Ib HLA-G complex is then an activating ligand for NK cell KLRD1-C2 also presents pathogen peptides to unconventional CD8+ T-cells viral infection often down-regulates MHC-I molecules while HLA-E presents viral peptides to NK cells, inducing tolerance	unique likely due to decreased viral load?	↑ self/viral-tolerance altered NK cell inhibition/activation (activation if cell stress provides chaperone-derived peptides)
	CD55	complement decay accelerating factor surface glycoprotein accelerates decay of bound complement proteins – e.g. C3b, C4b - to prevent host damage also interferes with the ability of C3b and C4b to convert C2 and factor B to active C2a and Bb, preventing formation of C3bBb and C4b2a (amplification convertases, i.e. C3 and C5 convertase) also exhibits antigens which are part of the Cromer blood group system, is exploited as a receptor for certain coxsackie- and echoviruses, and is implicated in the lectin-activated complement pathway	unique	↓ complement fixation and production of amplification convertases for lectin-activated complement pathway
	LGALS3	galactose-specific lectin which binds IgE may contribute (with alpha-3/beta-1 integrin and CSPG4) to endothelial migration during acute inflammation is involved in "neutrophil activation and adhesion, monocyte recruitment, opsonization of apoptotic neutrophils, and activation of mast cells"	unique	↑ neutrophil activation, adhesion, opsonic neutrophil efferocytosis (provided ligands are present)
	SELL	L-selectin, mediates transient and rapid rolling on resting endothelium mediates Ca ²⁺ -dependent adhesion to neighbouring cell glycoproteins e.g. lymphocytes to endothelia IL-8 induces neutrophil ROS production during neutrophil rolling, inducing L-selectin shedding [9]	unique	↑ transient endothelial rolling/tethering
	MEMO1	relays extracellular chemotactic signals to cytoskeleton mediator of ERBB2 signalling	unique	↑ recruitment/chemotaxis
	FCGR1A	high-affinity Fcγ receptor	unique	↑ phagocytosis of IgG-opsonised particles ↑ neutrophil activation
	PLAUR	GPI-anchored receptor for serine protease urokinase plasminogen activator (U-PA), which cleaves plasminogen plasminogen proteolytic cleavage generates active plasmin (fibrinolytic) and angiostatin (anti-angiogenic) plasmin also cleaves fibronectin, laminin, thrombospondin, and von Willebrand's factor, and activates collagenases, complement zymogens (C1, C5), and U-PA itself (which also negatively feeds back by cleaving the receptor) soluble form of the receptor also exists	unique	↑ ability to produce active plasmin (fibrinolytic for clot dissolution, but also breaks down ECM components and activates complement) and angiostatin
	PRG3	proteoglycan 3 cytotoxic/cytostimulatory effects resembling those of MBP/PRG2 stimulates neutrophil ROS and IL-8 production (and basophil histamine and LTC4 production)	absent	↓ neutrophil activation (e.g. ROS and IL-8 production, likely LTC4 production, and cytotoxicity)
	MMP8	degrades type I-III collagens to degrade	up	↑ ECM degradation

		ECM		
	MMP9	degrades type IV and V collagens and fibronectin may be involved in IL-8-induced mobilisation of hematopoietic progenitors from marrow	up	
	CTSC	lysosomal cysteine proteinase that activates many leukocyte serine proteinases (e.g. elastase, CTSG, granzymes A and B), neuraminidase, and factor XIII	down	↓ activation of hydrolases OR ↑ degranulation
	CTSS	lysosomal cysteine proteinase (which can function as an elastase) that may process peptides for MHC-I presentation (also responsible for "removal of the invariant chain from MHC-II), and is "involved in collagen chain trimerization"	unique	↑ antigen and MHC processing
	ASAH1	hydrolyses ceramides to produce sphingosine and free FA, and thus sphingosine-1-phosphate proteolytic processing of the ASAH1 preproprotein generates subunits which dimerize to form the mature enzyme mutations in this gene associate with lysosomal storage disorder	unique	↑ lysosomal catabolism of ceramides to sphingosine-1-P and free fatty acids
	ARSB	lysosomal hydrolyses sulfate groups of N-Acetyl-D-galactosamine, chondroitin sulfate, and dermatan sulfate storage disorder results from malfunction involved in regulation of cell adhesion, migration, and invasion	absent	↓ lysosomal de-sulfation of galactosamine and chondroitin/dermatan sulfate
	GUSB	lysosomal glucuronidase that degrades GAGs incl. heparan sulfate, dermatan sulfate, keratan sulfate, and chondroitin sulfate malfunction produces mucopolysaccharidosis	down	↓ lysosomal degradation of heparin/dermatan/keratin/chondroitin sulfate
	AGA	proteolytically processed to maturity involved in catabolism of N-linked oligosaccharides of glycoproteins cleaves asparagine from N-acetylglucosamines as one of the final steps in lysosomal breakdown of glycoproteins malfunctions cause lysosomal storage disorder	absent	↓ lysosomal removal of N-linked oligosaccharides from glycoproteins during lysosomal glycoprotein degradation
	ATP6V0A1	subunit of lysosomal V-ATPase required for its assembly and activity	absent	↓ lysosomal acidification (?due to AMPK non-activation)
	AP2M1	subunit of AP-2 complex (one of two clathrin-associated adaptor complexes) required for activity of V-ATPase responsible for acidification of endosomes and lysosomes may play an important role in regulating intracellular trafficking and function of CTLA4 associated with MHC-I antigen processing and EGF/EGFR signalling	unique	↑ endosomal/lysosomal acidification attempts ?altered CTLA4 trafficking
	AP2S1	small chain of AP-2 also associated with MHC-I antigen processing and EGF/EGFR signalling	unique	suggests enhanced AP-2 function
	S100A8	exists mainly as calprotectin (S100A8/A9 complex) with various functions (intracellular: facilitating arachidonic acid trafficking and metabolism, activation of neutrophil NADPH oxidase via facilitating its assembly at the membrane and providing arachidonate – an essential cofactor) (extracellular: proinflammatory, antimicrobial, oxidant-scavenging, apoptosis-inducing; e.g. leukocyte recruitment, favouring chemokine/cytokine production, regulation of adhesion and migration; acts as alarmin/DAMP and binds TLR-4 and AGER thereby activating MAPK and NFκB signalling pathways to amplify proinflammatory cascade can induce cell death via autophagy and apoptosis (via cross-talk between mitochondria and lysosomes via ROS)	up	altered Ca ²⁺ homeostasis ↑ NADPH-mediated respiratory burst (provided arachidonate is available) possible extracellular functions
	S100A11	may function in motility, invasion, and tubulin polymerization facilitates differentiation and cornification of keratinocytes related pathway: innate immune system	down (may be due to degranulation)	altered motility/invasion
	DDT	D-dopachrome decarboxylase homologue of macrophage inhibitory factor (MIF), with which it synergises to increase neutrophil recruitment [10] at least in cervical cancer, inactivity of DDT and MIF restores E-cadherin expression while suppressing expression of vimentin and phosphorylation of AKT and GSK3-β [11]	down	↓ neutrophil recruitment ?↑ E-cadherin expression capacity ?↓ AKT phosphorylation and vimentin expression capacity ?↓ *GSK3-β phosphorylation capacity *serine-threonine kinase belonging to glycogen synthase kinase subfamily; negative regulator of glucose homeostasis and involved in energy metabolism, inflammation, ER-stress, mitochondrial dysfunction, and apoptotic pathways; defects associated with Parkinson disease and Alzheimer disease

MCEMP1	likely involved in mast cell degranulation	unique	↑ degranulation
PTMA	confers resistance to certain opportunistic infections	unique	↑ resistance to infection
FTH1	ferritin heavy chain subunit	unique	↑ intracellular iron storage/sequestration

Unlike the CTRL class, IRIS class findings suggest that in response to ARV therapy neutrophils increase: recruitment/chemotaxis, transient rolling on resting endothelia, fibrinolytic plasmin (complement-activating, proteolytically facilitates movement through ECM) and angiotensin activation, MMP8 and 9 production (also favouring ECM degradation), phagocytosis of IgG-opsonised particles, antigen and MHC processing, and production of antimicrobial proteins. However, findings also suggest decreased: respiratory burst activity (though S100A8 activates it, provided arachidonate is available), IL-8 production, LTC4 production, cytotoxicity, hydrolase activation, and degranulation. These neutrophils can lysosomally catabolise ceramides to sphingosine (and thus sphingosine-1-P) and VL chain free FA (unlike the IRIS class at baseline, in which the relevant ceramidase ASAH1 is absent) and are competent to degrade ECM, but lysosomal acidification (via ATP6V0A1) and lysosomal catabolism of ECM/pathogen glycosaminoglycans and N-linked glycoproteins is impaired. Nevertheless, compensatory endosomal/lysosomal acidification attempts continue. Increased intracellular iron sequestration is likely in response to inflammation.

Thus, there is apparently relative neutrophil inactivity in the CTRL class (including C1qBP-mediated prevention of classical complement activation, decreased recruitment and transmigration, and decreased lysosomal hydrolase activity), with increased neutrophil activation in the IRIS class (including CD55-mediated restraint – absent at baseline – of complement cascade amplification, increased migration through the ECM, complement- and IgG-mediated phagocytosis, and phagosomal acidification). Thus, both classes exhibit down-regulation of (differential) lysosomal hydrolase activity, except for carbohydrate-degrading enzymes. As mentioned earlier, ARV exposure also induces in both classes a further decrease in early leukotriene (but now also HETE/HPETE) synthesis, and the IRIS class also down-regulates LTC4 and COX2-mediated prostanoid synthesis. Recall that literature suggests both the LOX and COX arms of the eicosanoid cascade are active at the time of TB-IRIS (higher levels of ALOX5-derived linoleic acid non-leukotriene HODE/HPODE products and COX-1/PTGS1) (Chapter 1). The unique presence of the urokinase receptor PLAUR (which typically associates with CR3) in IRIS-class neutrophils likely reflects increased myeloid activation; it is possible that 9-HODE-induces secretion of PAI-1/SERPINE1 to inhibit tissue plasminogen activator and urokinase (and thus fibrinolysis) (Chapter 1).

Specific ARV therapy-induced changes which occur in *opposite directions* in the CTRL versus IRIS groups over time include: SIGLEC-5 (inhibits inflammation via SHP-1/2 in presence of ubiquitous ligand sialic acid), SELL (mediates transient rolling on resting endothelium), and MEMO1 (relays extracellular chemotactic signals to cytoskeleton). All decrease to absence in the CTRL group, but increase to uniqueness in the IRIS group. Integrated findings are summarised in Table 8.

Table 8 Predicted Neutrophil Activity Change from Baseline through ARV Exposure

	CTRL	IRIS
capacity for inflammatory signalling (provided SHP1/2 restraint lifted)	↑ becoming -	↑ becoming -
regulatory receptor expression	↑ becoming ↑	-
activation	- becoming ↑	-
recruitment	-	↓ becoming ↑
chemotaxis	↓ becoming ↓	↓ becoming ↑
adhesion	↓ (preserved SELL rolling, ↓ selectin-activated integrin) becoming ↓ (SELL rolling & integrin)	↓ (non-integrin & profound integrin) becoming ↑ (transient rolling)
extravasation	↓ becoming ↓	↓ becoming -
migration through ECM	-	- becoming ↑
degranulation	↓ becoming -	↓ becoming ↓
phagocytosis	↓ (esp. complement-opsonised) becoming ↑ (efferocytosis)	↓ (esp. complement-opsonised) becoming ↑ (esp. complement- & IgG-opsonised)
respiratory burst	↓ becoming -	↓ becoming ↓
microbicidal substances	-	↓ becoming -
cytotoxicity	-	- becoming ↓
Fe sequestration	↑ becoming -	- becoming ↑
complement inhibition	↑ becoming ↑ (C1QBP inhibits classical pathway)	↓ becoming ↑ (CD55 inhibits cascade amplification)
Th1/cytotoxic/Th17 induction	↓ becoming ↑ (NK, Th1, & esp. CD8 ⁺ T-cells) but ↓ IL-1α, IL-6, TNFα, IL-10, TGFβ (ARV)	↓ becoming ↑ (esp. NK, but also Th1 & CD8 ⁺ T-cells) with ↑ TNFα, IFNγ (ARV)
Th2/Treg induction	↑ becoming - but ↑ IL-4/13 (ARV)	↑ becoming - but ↑ TGFβ (ARV)
eicosanoid cascade	↓ arachidonate provision, LT (incl. LTB4) synthesis, LTB4R becoming ↓ LT & ALOX15-mediated HETE/HPETE	↓ arachidonate provision, LT (incl. LTB4) synthesis becoming ↓ LT (incl. LTC4), ALOX15-mediated HETE/HPETE,

	synthesis (preservation of PAF)	& COX-2-mediated prostanoid synthesis (↓ protective PGE2) (possible PAF inactivation)
coagulation	-	- becoming ↑ (PLAUR, plasmin)

3.5. Carbon-Energy Metabolism (Including Mitochondrial Function)

At baseline, glycogen synthesis and catabolism were decreased in both classes, likely reflecting the effects of PKA signalling (or opposing activities of anabolic insulin versus glucose-mobilising adrenaline). As mentioned above, both classes exhibited likely-increased sensitivity to adrenaline, increased baseline homeostatic/anabolic (e.g. PKA-glucose/lipid) signalling, a de-repressed capacity for insulin-induced FA synthesis, and increased insulin sensitivity in the IRIS class, all of which would support enhanced glucose uptake and utilisation. However, at baseline, glucose entry into early glycolysis and the PPP actually appeared to be decreased (perhaps due to insufficient glucose availability, and likely decreasing NADPH production), while post-G3P/DHAP glycolysis (generating pyruvate and ATP) as well as the downstream PPP (generating ribulose-5-P and compensatory NADPH) were enhanced. Glycolytic rates are commonly elevated during inflammation. Although both classes de-repressed HIF1a activity, only the CTRL class engaged in conversion of pyruvate to lactate + NAD (while also increasing NAD synthesis from ribulose-5-P-derived PRPP and Trp), and only the IRIS class decreased conversion of ribose-5-P (inter-convertible with ribulose-5-P) to G3P (despite increased G3P use for serine biosynthesis; Section 3.8.).

Also at baseline, both classes exhibited increased NAD-dependent purine (guanine) synthesis and altered purine (adenine) metabolism, with increased mitochondrial pyrimidine salvage and pyrimidine over purine catabolism. Regarding the TCA cycle, both classes exhibited increased conversion of malate to pyruvate, and a likely build-up of α-KG (instead of conversion – with glutamate consumption – to succinyl-CoA, also derived from FAO), consequently altering complex V activity and FA/arginine metabolism. The CTRL class perhaps engaged in increased branched-chain amino acid catabolism, while the IRIS class likely exhibited increased aspartate and decreased conversion of adenylosuccinate (from purine nucleotide metabolism) to fumarate (despite the TCA likely requiring fumarate or succinate replenishment). Since de-repressed mTORC1 would enhance glycolysis, protein/lipid/pyrimidine synthesis, and mitochondrial biogenesis, but also suppresses autophagy/mitophagy, this could have exacerbated any potential mitochondrial stress (including decreased ATP output, increased mtROS production, and altered transport dynamics of pyruvate, malate, aspartate, glutamate, and adenine nucleotides). Both classes exhibited respiratory chain compromise at naturally-dysfunctional neutrophil complex IV, including compensatory increases in complex V activity. The CTRL class exhibited increased complex I activity (perhaps due to increased NADH provision), while the IRIS class exhibited dysfunctional complex III activity (required to maintain mitochondrial potential) as well as compensatory increases in the other respiratory complexes. High TGFβ levels would further exacerbate mtROS production due to dysfunctional complex III activity, further favouring permeability transition and cell death. Indeed, both classes exhibited differential additional indications of mitochondrial stress (e.g. only the CTRL class may engage in NRF1-mediated induction of protective mitochondrial biogenesis, though NRF1 – in response to ER cholesterol overload – can also de-repress LXR activity), but also of increased opposition to intrinsic pathway apoptosis. At least in the IRIS class, both TGFβ and ROS (as well as EFHD2) perhaps suppressed NFκB to prevent CASP1 expression, suggesting that (instead of NLRP3) NLRP1/NLRC4-induced CASP4/5 activation is responsible for IL-1β/IL-18 maturation and possibly a predisposition to pyroptosis (indeed, a head-to-head comparison - after ARV exposure - demonstrates that NLRC4 levels are elevated in the IRIS versus CTRL class, and low-level CASP4 is indeed detectable in a subset of IRIS-class samples). Given that G3P exists at the intersection between dysregulated glycolysis, the PPP, nucleotide breakdown product utilisation, oxidative phosphorylation, NAD regeneration, glycerolipid synthesis, and serine biosynthesis (Fig. 7), its metabolism was likely significantly altered.

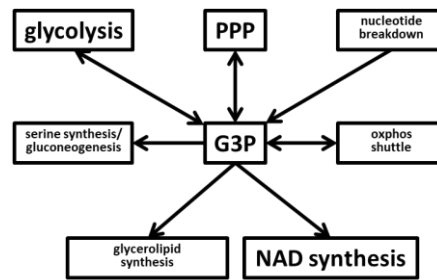


Figure 7 The Central Role of Glyceraldehyde-3-Phosphate in Metabolism

In both classes, ARV exposure corrects many baseline metabolic abnormalities (Table 9): over-anabolism induced by insulin/growth factors, impaired glucose flux through early glycolysis (including likely-increased cellular monosaccharide uptake and a preference for G1P conversion to G6P in the IRIS class), the build-up of α -KG (which may have been feeding into glycolysis/the PPP as salvaged sugars, but can also be used to supplement glutamate in the unlikely event that it becomes limiting), pyrimidine catabolism for purine synthesis, FPPS and thus isoprenoid and cholesterol/steroid hormone synthesis as well as prenylation, and possible PLP deficiency (perhaps due to adaptation to INH). The CTRL class may also exhibit increased glucose flux through the early PPP, with concomitant generation of the NADPH that was likely lacking at baseline. Both classes exhibit increased conversion of ribulose to xylulose; CTRL class findings suggest ribose is funnelled into xylulose production, perhaps favouring G3P instead of NAD/nucleotide precursor synthesis (e.g. decreased adenosine and AMP generation), unlike at baseline. IRIS class findings suggest an increase in DNA precursor pool size and increased generation of ribose from nucleotides (perhaps to provide G3P to glycolysis or serine biosynthesis), but decreased provision of thymidine-based intermediates for pyrimidine synthesis (possibly exacerbating mtDNA depletion by ARVs or oxidative stress) or carbon-energy metabolism, and possibly a preference for purine or ATP synthesis instead. The CTRL and IRIS classes, respectively, appear to undergo decreased and increased conversion of glycolytic pyruvate to acetyl-CoA (with concomitant increase and decrease in glycine levels), though of course this remains to be confirmed by metabolomics studies. The intra-mitochondrial portion of the malate-aspartate shuttle (for transfer of NADH electrons to the respiratory chain) appears to be relatively up-regulated in the IRIS group (which would consume glutamate, also producing aspartate and α -KG). Although the IRIS group apparently produces more ammonia, requiring increased detoxification, both conversion to glutamine (via consumption of glutamate) and early-stage urea cycle reactions are decreased, although later-stage urea cycle reactions (producing arginine and fumarate) are increased.

Table 9 Dysregulated Proteins Relevant to Neutrophil Carbon-Energy Metabolism

Class	Protein	Function	Status	Hypothesised Outcome
shared	PFKM	catalyzes committing and ATP-consuming step of glycolysis (conversion of F-6-P to F-1,6-BP)	unique	↑ initial glycolytic steps
	RPE	within PPP, reversibly epimerises ribulose-5-P to xylulose-5-P	unique	↑ conversion of ribulose-5-P to xylulose-5-P, possibly to support increased production of F-6-P for entry into glycolysis (or because glycolysis is providing G-3-P to feed into PPP)
	OGDH	α KG dehydrogenase subunit within TCA cycle converts α KG to succinyl-CoA + CO_2 requires thiamine pyrophosphate co-factor α KG dehydrogenase deficiency causes lactic acidosis	unique	↑ conversion of α KG to succinyl-CoA (provided thiamine pyrophosphate is available)
	NAMPT	rate-limiting enzyme for NAD biosynthesis	unique	↑ NAD biosynthesis (from PPP ribulose-5-P → ribose-5-P → PRPP)
	DCK	required for phosphorylation of various deoxyribonucleotides and their nucleoside analogs (e.g. as part of pyrimidine biosynthesis)	unique	↑ pyrimidine biosynthesis
	PLPBP	buffers intracellular levels of PLP by binding it	unique	↑ availability of PLP
	NAGK	N-acetylhexosamine kinase converts GlcNAc to GlcNAc-6-P major enzyme recovering amino sugars involved in ingested Neu5Gc degradation	down	↓ conversion of amino acid carbon skeletons to usable sugars
	CMPK2	nucleotide salvage pathway enzyme may participate in mitochondrial pyrimidine (e.g. dUTP/CTP) synthesis possibly involved in mtDNA depletion due to treatment with pyrimidine analogs	absent	↓ nucleotide salvage for pyrimidine synthesis, possibly due to NRTI-type ARV-mediated mtDNA depletion
	RAD21	chromosomal phosphoprotein cleaved by CASP-3 or -7 during early apoptosis C-terminal product may initiate cytoplasmic apoptotic events	unique	↑ neutrophil proneness to apoptosis
CTRL2-	G6PD	funnels G-6-P into the PPP	up	↑ glucose flux through early parts of PPP, with

specific		major mechanism for regenerating NADPH		concomitant NADPH generation
	RPIA	reversibly inter-converts ribose-5-P and ribulose-5-P within the PPP	absent	↓ ribose-5-P and ribulose-5-P interconversion (recall above increased conversion of ribulose-5-P to xylulose-5-P)
	AK2	regulates adenine nucleotide composition via reversible transfer of phosphate groups among adenine nucleotides – e.g. between ATP and AMP – as part of energy homeostasis and adenine nucleotide metabolism, and is required for AMP de novo biosynthesis	down	↓ phosphate group inter-change between ATP and AMP ↓ AMP biosynthesis
	ADA2	one of two human adenosine deaminases which lowers high adenosine levels; secretion from myeloid cells regulates extracellular adenosine, and through enzyme binding to cell surfaces – via proteoglycans – influences cell proliferation and differentiation	absent	↓ lowering of high adenosine levels
	NUDT2	nucleotide pyrophosphatase which hydrolyzes Ap4A to produce AMP and ATP, thereby regulating intracellular levels of dinucleotide Ap4A and playing a key role in homeostasis maintenance	absent	↓ AMP and ATP generation
	ENPP4	converts extracellular Ap3A into AMP and ADP, and Ap4A into AMP and ATP; ADP receptor ligation has pro-coagulant activity at thrombotic sites	absent	↓ extracellular AMP and ADP or ATP generation ↓ pro-coagulant activity
	DLD	component of glycine cleavage system and of three different α-ketoacid dehydrogenase complexes (pyruvate-, α-KG-, and branched-chain amino acid-dehydrogenases) α-KG-dehydrogenase operates mainly in the mitochondrion, but in the nucleus is required for histone Lys succinylation (provides succinyl-CoA to histone-modifying KAT2A) pyruvate dehydrogenase converts pyruvate into acetyl-CoA on its own may also be a serine protease	absent	↑ glycine ↓ conversion of pyruvate to acetyl-CoA some ↓ conversion of α-KG to succinyl-CoA?
	NDUFS1	complex I largest subunit (part of Fe-S group) transfers electrons from NADH to coenzyme Q	absent	↓ electron transfer from NADH to respiratory chain via complex I
	NDUFS3	Fe-S component of complex I	absent	
	SDHB	Fe-S subunit of respiratory chain complex II (oxidises succinate) transfers electrons from FADH2 to CoQ (ubiquinone)	unique	↑ transfer of electrons from FADH2 to respiratory chain via complex II
	CYC1	complex III subunit (part of Fe-S group) transfers electrons from coenzyme Q to cytochrome C	absent	interruption of respiratory chain at complex III ?impaired proton-motive force generation, ↑ mtROS generation
	ATP5PO	architectural component of mitochondrial ATPase for ATP synthesis	absent	↓ ATP generation
	SFXN1	may help transport an Fe-utilising component into/out of mitochondria	unique	?↑ Fe flux through mitochondria
	PPA2	inorganic pyrophosphatases (hydrolyse inorganic pyrophosphates) PPA2 is mitochondrial and essential for maintenance of mitochondrial membrane potential, organisation, and function	absent	↓ requirement for alternate energy source to ATP
	VDAC3	voltage-dependent mitochondrial outer membrane anion channel conducts ATP and small hydrophilic metabolites (e.g. adenine nucleotides) may form part of mPTP	unique	?↑ import of ADP into mitochondria ?↑ capacity for permeability transition
	DDI2	protease that releases ubiquitinated NRF1 from the ER membrane	unique	?↑ capacity for NRF1-mediated mitochondrial stress-response gene expression OR for NRF1 degradation
	RPS3	ribosomal protein with highly-specific functions when mitochondrially-located, down-regulates ROS levels and mtDNA damage suppresses DNA repair in the presence of hydrogen peroxide, while protecting p53 from ubiquitination enhances NFκB-mediated transcription: binds RELA to enhance DNA binding and transcription contributes to activation of CASP8 to induce apoptosis	down	?↓ need for amelioration of mtROS production and mtDNA damage ↓ enhancement of NFκB-mediated transcription ↓ enhancement of apoptosis (CASP8 is absent under the CTRL2 condition anyway)
	ACADM	β-oxidation step 1 enzyme specific for medium-chain (4-16-carbon) FAs	absent	↓ initiation of mitochondrial FA β-oxidation, with ↓ FADH2 production
	HSD17B10	enzyme which performs the same function as β-oxidation step 3 enzyme, HADHA essential for mitochondrial structural and functional integrity of mitochondria also catalyzes beta-oxidation at carbon 17 of androgens and estrogens (including 3-α-hydroxysteroid dehydrogenase activity on androsterone) exhibits 20-β-OH and 21-OH dehydrogenase activities with C21 steroids interacts with intracellular amyloid-beta	absent	↓ step 3 execution and production of NADH ↓ mitochondrial homeostasis ?altered steroid hormone metabolism
	HSD17B4	peroxisomal FA β-oxidation enzyme; catalyzes formation of 3-ketoacyl-CoA intermediates	down	↓ step of peroxisomal FA oxidation that produces 3-ketoacyl-CoA
	RALY	required for cholesterol biosynthetic gene expression	absent	↓ cholesterol biosynthesis
	FNTA	essential subunit of the farnesyltransferase and geranylgeranyltransferase complexes which transfer farnesyl or geranylgeranyl moieties from diphosphates to target proteins; may also function in RAC1 prenylation and activation	absent	↓ farnesylation and geranylgeranylation of proteins
	GPAT3	mediates an essential step in glycerolipid synthesis: conversion of G-3-P (through addition of acyl-CoA) to lysophosphatidic (and thus phosphatidic) acid for triacylglycerol synthesis likely PPARα-regulated	unique	↑ (?PPARα-induced) triacylglycerol (and probably glycerolipid) synthesis, provided G-3-P and acyl-CoA are available
	ERLIN2	with GP78, mediates sterol-accelerated ERAD of HMGCR also regulates cholesterol homeostasis [12]: when cholesterol levels are high, interacts with INSIG1 inhibit activation of SREBP1c (key regulator of de novo lipogenesis)	unique	↓ cholesterol and SREBP1c-mediated lipid biosynthesis
IRIS2-specific	SLC2A3	facilitative glucose transporter can also take up various other monosaccharides (not fructose)	up	↑ monosaccharide (e.g. glucose) uptake (usually followed by HK-mediated conversion to G-6-P for

				cellular retention)
	PGM1	converts G-6-P to G-1-P	down	↓ conversion of G-6-P to G-1-P
	PGM2	catalyzes first step (conversion of ribose-1-P to ribose-5-P) of sub-pathway synthesising G-3-P from degradation of nucleoside breakdown product ribose-1-P (may also convert G-1-P to G-6-P)	up	↑ ribose-5-P production from nucleotides for feeding into glycolysis as G-3-P ↑ conversion of G-1-P to G-6-P (i.e. overall favouring of G-6-P production and conservation)
	TYMP	reversibly removes phosphate from thymidine products are used for pyrimidine salvage or as carbon-energy sources angiogenic associated disorder: mtDNA depletion syndrome 1	down	↓ provision of thymidine-based intermediates for pyrimidine synthesis or carbon-energy metabolism
	SAMHD1	antiviral restriction factor – restrict early HIV replication in myeloid cells dNTPase: lowers deoxyribonucleoside triphosphate levels under those required for retroviral reverse transcription thus also regulating DNA precursor pool size also promotes DNA degradation at stalled replication forks to prevent induction of type I IFN and chronic inflammation	down	↑ DNA precursor pool size
	NME1-NME2	fusion protein resulting from naturally-occurring read-through transcript unknown function NME1 has key role in synthesis of non-ATP nucleoside triphosphates (e.g. NTP) while also acting as a Ser/Thr protein kinase and geranyl and farnesyl pyrophosphate kinase	up	↑ DNA precursor pool size
	NUDT2	nucleotide pyrophosphatase hydrolyses Ap4A (di-adenosine tetraphosphate, possible alarmin) to produce ATP and AMP	unique	↑ provision of ATP and AMP OPPOSITE-DIRECTION-CHANGE vs CTRL2
	BPNT1	converts PAPS to AMP	unique	↑ provision of AMP was absent under both CTRL0 and IRIS0 conditions (relative to HV)
	ADK	highly-conserved adenosine kinase transfers phosphate from ATP to adenosine, producing ADP and AMP regulates intracellular acenine nucleotide and extracellular adenosine levels major route of adenosine metabolism during steady state	unique	↑ conversion of ATP to ADP and adenosine to AMP
	RETN	insulin effect-suppressing (i.e. anti-adipogenic) hormone decreased in response to gamma-chain family cytokine signalling	up	↓ adipogenesis, likely in response to down-regulating IFN signalling
	HSD17B10	some enzymatic function as HADHA (which catalyses penultimate step of mitochondrial fatty acid β-oxidation) required for mitochondrial integrity also oxidises androgens and estrogens at carbon 17 exhibits 20-beta-OH and 21-OH dehydrogenase activities with C21 steroids interacts with intracellular amyloid-beta	unique	↑ latter steps of FA oxidation (with concomitant production of NADH) ↓ androgen/estrogen/C21 steroid activity
	HADHB	beta subunit of mitochondrial trifunctional protein that catalyses final three steps of mitochondrial long-chain fatty acid β-oxidation this subunit catalyses the final step, producing acetyl-CoA	unique	↑ final step of FA oxidation cycle, releasing acetyl-CoA from the FA chain
	ACAA2	catalyses final step of mitochondrial fatty acid β-oxidation associated with PPARα regulation also abolishes BNIP3-mediated apoptosis and mitochondrial damage	unique	
	ACLY	main enzyme producing cytosolic acetyl-CoA (and oxaloacetate) from citrate and CoA acetyl-CoA is then used in lipogenesis and cholesterol biosynthesis key regulator reprogramming metabolism to high rates of aerobic glycolysis and de novo lipid synthesis	down	↓ lipid (incl. sterol) biosynthesis
	APOBR	macrophage receptor that binds apolipoprotein B48 of dietary TG-rich lipoproteins may provide essential lipids, lipid-soluble vitamins, and other nutrients to reticuloendothelial cells when overwhelmed with elevated plasma TG can contribute to foam cell formation also binds APOB in hypertriglyceridemic VLDL binds and internalises TLR mediates rapid high-affinity uptake of chylomicrons and trypsinised VLDL devoid of APOE	up	↑ uptake of circulating triglycerides and lipid-soluble vitamins
	APOA1	major protein component of circulating HDL mature form transports cholesterol from tissues to liver co-factor for LCAT, which enzymatically forms the majority of plasma cholesterol esters	absent	↓ cholesterol biosynthesis and export
	FNTA	essential subunit of farnesyltransferase and geranylgeranyltransferase complexes which transfer farnesyl or geranylgeranyl moieties from diphosphates to target proteins	unique	↑ prenylation (PTM) of target proteins, which will alter their intracellular trafficking, including impacts on inflammation, AMPK-mTOR signalling, and viral budding previously unique only to CTRL0 but not IRIS0 condition (versus HVs)
	ZMPSTE24	cleaves C-terminal residues from farnesylated proteins	unique	↑ cleavage of hydrophobic C-terminal from farnesylated proteins, likely releasing them from membrane associations
	NDFUS1	complex I subunit which transfers electrons from NADH to the respiratory chain	unique	↑ transfer of electrons from NADH to dysfunctional respiratory chain by complex I, ↑ mtROS production
	MT-CO3	complex IV functional core subunit (mitochondrially-encoded)	absent	interruption of respiratory chain at final complex

	COX6B1	complex IV regulatory/assembly subunit which transfers electrons from reduced cytochrome C to oxygen associated pathway: AMPK	absent	(perhaps due to mtDNA depletion) ↓ generation of proton-motive force
	PPA2	mitochondrial inorganic pyrophosphatase essential for maintenance of mitochondrial membrane potential, architecture, and function hydrolysis of inorganic pyrophosphates can provide an ATP-alternate energy source	unique	↑ use of ATP-alternate pyrophosphates for energy ↑ attempt to maintain mitochondrial integrity
	DDI2	facilitates release of ubiquitinated NRF1 from the endoplasmic reticulum membrane	absent	↓ NRF1-mediated induction of mitochondrial gene expression OR ↓ UPS-mediated degradation of NRF1
	VDAC2	part of voltage-dependent MoM anion channels that provide major route for diffusion of small hydrophilic molecules closed state is cation-selective opens at lower membrane potentials also thought to be involved in the mitochondrial apoptotic pathway via regulation of BCL2-antagonist/killer 1 activity	unique	↑ capacity for MoM permeabilisation
	SLC25A24	cytosolic Ca ²⁺ -dependent mitochondrial solute exchanger (Mg-ATP/ADP for phosphate) catalyzes net uptake of adenine nucleotides across mitochondrial inner membrane when cytosolic Ca ²⁺ rises may protect against oxidative stress-induced cell death (promoting formation of calcium-phosphate precipitates in the mitochondrial matrix probably buffers mitochondrial Ca ²⁺ levels)	unique	↑ mitochondrial uptake of adenine dinucleotides (in response to cytoplasmic Ca ²⁺ signalling) in a potentially-futile attempt to increase ATP synthesis
	HEBP2	enhances mitochondrial inner and outer membrane permeabilisation, especially during oxidative stress involved in mitochondrial membrane depolarisation prior to necrosis	unique	↑ capacity for mitochondrial membrane permeability and depolarisation (probably in response to mtROS) prior to cell death
	SH3GLB1	interacts with pro-apoptotic Bcl-2 member Bax involved in activation of caspase-dependent apoptosis by promoting BAX/BAK1 activation may help maintain normal MoM dynamics may regulate apoptotic signalling pathways also involved in caspase-independent apoptosis during starvation involved in regulation of autophagy	unique	↑ likelihood of mitochondrially-initiated caspase-dependent or -independent cell death altered autophagy
	CASP10	recruited to TNF/Fas-type receptors for activation by CASP8 then cleaves and activates CASP3 and CASP7 as part of apoptotic signalling cascade also activates CASP8 (and 4, 6, and 9) may participate in granzyme B-induced apoptosis as well	unique	↑ capacity for granzyme-, TNF/Fas-, or mitochondrially-initiated caspase-dependent cell death
	STK26	Golgi-resident Ser/Thr kinase activated by Golgi protein GM130 with STK24 inhibits Golgi reorientation in polarized cell migration on Rho activation also cleaved by CASP3, possibly as part of the apoptotic cascade pathway	unique	↑ TNF/Fas-CASP8-CASP10-CASP3-STK26 signalling
	PDCD6IP	functions within ESCRT pathway during intraluminal endosomal vesicle formation, and in enveloped virus budding overexpression blocks apoptosis, likely by Ca ²⁺ -dep binding of pro-apoptotic PDCD6 also binds endophilins that regulate membrane shape during endocytosis also involved in membrane repair and vesicle trafficking	down	↓ opposition of apoptosis altered endocytosis, vesicle trafficking, viral budding

Regarding oxidative phosphorylation, the IRIS class apparently experiences an increase in electron transfer from NADH to the respiratory chain by complex I, while the CTRL class exhibits complex I dysfunction (with possible increased import of respiratory chain Fe components into the mitochondrion), increased electron transfer from FADH₂ to the respiratory chain by complex II, and further dysfunction at complex III (which can transfer electrons from FADH₂ and the G3P shuttle directly to oxygen, and whose dysfunction would significantly impact cellular function). Only the IRIS class exhibits respiratory chain interruption at complex IV (already relatively inactive in neutrophils). The CTRL and IRIS classes, respectively, exhibit decreased and increased complex V (ATP synthase) function, but since neutrophil mitochondrial membrane potential is not as closely coupled to ADP phosphorylation as in other cells, this may in fact reflect co-operation - in the IRIS class - of F₁-ATPase with the mitochondrial adenine dinucleotide transporter to maintain mitochondrial potential despite respiratory chain dysfunction. Respiratory chain/oxidative phosphorylation dysfunction should result in an impaired proton-motive force/membrane potential, as well as increased mtROS production – which can damage mt/nuclear DNA - and reliance on anaerobic energy generation (i.e. lactate production).

Indeed, while the CTRL class undergoes increased mitochondrial protein degradation, and suppression of mtROS production, mtDNA damage, and mitochondrially-induced plus CASP8-mediated apoptosis, the IRIS class exhibits various convincing signs of increasing mitochondrial stress (including enhanced folding quality-control especially for mitochondrial proteins imported under stress conditions, increased mitochondrial ADP uptake in an attempt to increase ATP synthesis, ATP-alternate energy production, an increased capacity for mitochondrial membrane permeabilisation and depolarisation). The IRIS class may also experience increased attempts at DNA repair (e.g. via PARP1-dependent PARP9-DTX3L-

mediated ubiquitination promoting recruitment of repair proteins to sites of DNA damage), and possibly necroptosis when this fails due to absence of DTXL3 in both classes. Indeed, the baseline resistance to apoptosis resolves in both classes. However, the CTRL class exhibits decreased TNF/Fas-CASP8-CASP10-CASP3/7-mediated apoptosis (CASP8 is absent), while CASP10 expression becomes up-regulated in the IRIS class, which would up-regulate the capacity for granzyme-, TNF/Fas-, or mitochondrially-initiated caspase-dependent or -independent cell death (especially via BAX and/or the CASP8-CASP10-CASP3/7 pathway). The IRIS class may also become more susceptible to NLRC4-CASP4/5-mediated pyroptosis, lipid peroxidation-mediated ferroptosis, or uncorrected DNA damage-mediated necroptosis. Recall that even at baseline the IRIS group appeared more prone to necrosis.

Specific ARV therapy-induced changes which occur in *opposite directions* in the CTRL versus IRIS groups over time include: NUDT2 (hydrolyses possible alarmin Ap4A to produce ATP and AMP), HSD17B10 (catalyses the penultimate step of mitochondrial FA β -oxidation, required for mitochondrial integrity, oxidises androgens and estrogens, and dehydrogenates steroid hormones), FNTA (essential subunit of complexes transferring farnesyl or geranylgeranyl from diphosphates to target proteins), NDUFS1 (largest subunit of respiratory complex I; part of Fe-S group that transfers electrons from NADH to coenzyme Q), and PPA2 (mitochondrial enzyme which hydrolyses inorganic pyrophosphates; essential for maintenance of mitochondrial membrane potential, organisation, and function). All decrease to absence in the CTRL group, but increase to uniqueness in the IRIS group. Furthermore, DDI2 (a protease that releases ubiquitinated NRF1 from the ER membrane) is unique in the CTRL group, but absent in the IRIS group.

3.6. Lipid & Isoprenoid/Sterol Metabolism

At baseline, both classes exhibited decreased cellular long-chain FA and mitochondrial FA-CoA uptake despite a build-up of FA/glycerol/mevalonate/IPP (preventing protein prenylation as well as steroid hormone and Q10 synthesis), and simultaneously increased (compensatory?) FAO-derived FADH2 provision to the respiratory chain. The CTRL class, especially, exhibited NRF1-mediated mitochondrial biogenesis and enhancement of both lipid synthesis and FAO capacity (including increased NADH and FADH2 provision to the respiratory chain), as well as an increase in the peroxisomal FAO steps producing 3-ketoacyl-CoA. NRF1 would also inhibit FA synthesis while favouring cholesterol esterification and efflux. The CTRL class also exhibited attempts at increased steroid hormone synthesis and decreased degradation. Fewer additional baseline findings (increased: acetyl-CoA formation, glycerolipid synthesis from G3P, and HDL export; decreased steroidogenesis) were noted in the IRIS class, perhaps reflecting more complete inhibition of the AMPK-PGC1 α -NRF pathway by potentially-higher levels of TGF β .

Exposure to ARV therapy normalises elevated baseline CTRL class rates of FAO (Table 9, *vide supra*) while further enhancing lipid synthesis, perhaps focused more on PPAR α -mediated glycerolipid than SREBP-mediated sterol synthesis (and thus protein prenylation, despite de-repression of FPPS activity due to declining RSAD2). Potential down-regulation of HMGcR activity further suggests amelioration of the IPP accumulation likely occurring at baseline under both conditions. In the IRIS class, on the other hand, triglyceride/fat-soluble vitamin uptake and lipid droplet breakdown/FAO increases, while lipid and sterol biosynthesis (including cholesterol export) decreases (though de-repression of FPPS activity due to declining RSAD2, and an increase in protein prenylation, may suggest some conservation of isoprenoid synthesis). Increased prenylation likely alters membrane trafficking of target proteins, and may impact inflammation, AMPK-mTOR signalling, and viral budding.

3.7. Endoplasmic Reticulum/Protein Homeostasis

Apart from protein folding, glycosylation, and trafficking, the ER also plays a significant role in lipid synthesis and xenobiotic detoxification. At baseline, both classes exhibited altered protein (including insulin/growth factor and respiratory complex) expression and ER architecture/Ca²⁺/glycosylation dynamics, including decreased folding QC, increased chaperone activity (especially in the IRIS class), and differentially-enhanced ubiquitin-proteasome system (UPS) flux. The CTRL class exhibited possible compensatory attempts to enhance autophagy/mitophagy, while the IRIS class exhibited increased

repair/degradation of damaged proteins, but decreased translocation of misfolded/fusion ER proteins for UPS-mediated degradation, and inhibited auto/mitophagy.

Both classes respond to ARV therapy (Table 10) by decreasing certain aspects of ER-mediated protein-processing and glycosylation (suggesting decreased protein synthesis/co-translational translocation into the ER), and decreasing protein insertion into the ER membrane (perhaps suggesting a reversal of ER lumen expansion as lipid biosynthesis and protein-folding requirements de-escalate). However, both classes also further elevate chaperone expression (significantly more so in the IRIS than the CTRL class, including especially for proteins entering the mitochondrion under stress conditions) and ubiquitination (plus neddylation) of selected targets for UPS-mediated degradation. The CTRL and IRIS classes, respectively, engage in decreased and increased: processing, folding QC, and glycosylation of nascent proteins entering the ER; SUMOylation of selected targets; and UPS-mediated degradation of misfolded/fusion proteins (though many 26S proteasomal components are up-regulated, down-regulation of some may be due to regulation by adrenaline, either to oppose HIF-1 α degradation, or to de-repress MAVS-mediated innate responses). Nevertheless, the CTRL class (via uniqueness of one of the most abundant ER chaperones) exhibits likely-increased secretory protein export from the ER (and targeting of abnormally-folded proteins to ERAD), while the IRIS class exhibits increased rates of glycoprotein and glycolipid transport (possibly including retrograde transport of aberrant cargo) between the ER and Golgi. In the CTRL class, the absence of UFD1 suggests decreased RIG-I degradation (an increased capacity to respond to PAMPs with type I IFN generation), down-regulation of PSME1 and 2 clearly suggest down-regulation of IFN γ stimulation (and immunoproteasome activity). In the IRIS class, there is some suggestion of increased RIG-I degradation (unexpected).

Table 10 Dysregulated Proteins Relevant to Protein Homeostasis

Class	Protein	Function	Status	Hypothesised Outcome
shared	CUL4B	core component of E3 ubiquitin ligase complexes which ubiquitinate targets (e.g. histones) for UPS-mediated degradation co-operates with MLST8 and 26S proteasome to regulate mTOR pathway	unique	↑ ubiquitination of selected targets ?altered mTOR signalling
	UBAC1	non-catalytic subunit of E3 ubiquitin ligase complex associated with MHC-I antigen processing	unique	↑ ubiquitination of selected targets
	UBE2M	E2 ubiquitin ligase transfers ubiquitin-like NEDD8 from the UBA3-NAE1 E1 ligase to targets (e.g. CDC53, CUL1-4) associated with MHC-I antigen processing and cytokine signalling	unique	↑ neddylation of selected targets
	DTX3L	E3 ubiquitin ligase co-operates with PARP9 for IFN-mediated enhancement of STAT1-dependent ISG expression	absent	↓ IFN-mediated antiviral signalling
	ASNA1	ATPase component of arsenite exporter core component of complex which inserts new proteins into the ER membrane may be involved in insulin signalling associated pathway: UPR	absent	?↓ stimulation by arsenicals ↓ insertion of new proteins into the ER membrane (possibly due to ↓ insulin signalling or ↑ UPR activation)
	GFPT1	catalyses hexosamine pathway rate-limiting step (formation of glucosamine-6-P) to control flux of glucose into pathway thus regulates availability of precursors for protein N- and O-linked glycosylation	absent	↓ protein glycosylation precursor availability
	LAP3	N-terminal peptidase for cellular protein processing and turnover associated pathway: glutathione metabolism	down	↓ cellular protein turnover
	PSMA6	20S core alpha subunit T1A peptidase associated pathway: beta-adrenergic signalling	down	↓ UPS-mediated protein degradation
CTRL2-specific	TCPE	CCT5 of TRiC chaperonin complex for folding unfolded proteins	up	↑ protein-folding quality-control capacity
	HSPH1	promotes ATP-dependent prevention of denature protein aggregation by other chaperones (e.g. HSPA1A and B) esp. during severe stress that decreases ATP levels	unique	↑ protein-folding/deaggregation quality-control capacity
	DNAJC13	HSP co-chaperone for HSPs (e.g. Hsc70) role in clathrin-mediated endocytosis also interacts with SNX1 for involvement in post-endocytic transport implicated in recycling of transferrin in early endosome to later endosome transport implicated in degradation of EGF and EGFR	down	↓ co-chaperone activity for Hsc70-type HSPs
	BCAP31	chaperone that is among the most ER-abundant proteins helps export secreted proteins from the ER also targets abnormally-folded proteins to ERAD "may be involved in caspase-8-mediated apoptosis"	unique	↑ protein-folding/degradation quality-control capacity and export of secretory proteins from the ER ?↑ capacity for apoptosis
	DNAJB11	soluble ER lumen glycoprotein co-chaperones immunoglobulin-binding heat-shock protein (required for proper folding and assembly of proteins in the ER) also a co-chaperone for HSPA5 directly binds both unfolded proteins that are ERAD substrates	absent	↓ co-chaperone activity for Ig-binding HSP and HSPA5

	and nascent unfolded peptide chains		
UBE2I	E2 ubiquitin ligase with E3 ligases (e.g. RANBP2, CBX4, ZNF451) attaches SUMO1-5 to targets e.g. FOXL2, KAT5, p53	absent	↓ SUMOylation of selected targets
SUGT1	may play a role in ubiquitination and proteasomal degradation of target proteins	absent	↓ UPS-mediated degradation
COPS2	essential part of COP9 signalosome regulates various signalling pathways, incl. ubiquitination mediates de-neddylation of SCF-type E3 ligase subunits leading to decreased ubiquitination of target proteins also helps phosphorylate p53, JUN, NFKBIA, ITPK1, IRF8 possibly via association with casein kinase II and PKD phosphorylation of p53 and JUN respectively enhance and inhibit UPS-mediated degradation	absent	↓ de-neddylation of E3 ligases, thereby ↑ ubiquitination of selected targets ↓ enhancement of PKD- and CKII-mediated phosphorylation of certain targets, incl. p53, NFKBIA
ANKRD13A	binds Lys63-linked ubiquitin to promote internalisation of activated ubiquitinated EGFR	absent	↓ ubiquitin-mediated activated EGFR internalisation
UFD1	part of complex (with VCP and NPLOC4) required for export of misfolded/fusion proteins from ER to the cytoplasm for UPS-mediated degradation complex also inhibits type I IFN generation by promoting DDX58/RIG-I degradation	absent	↓ export of misfolded/fusion proteins from ER to cytoplasm for UPS-mediated degradation ↓ RIG-I activity
ADRM1	Ub receptor subunit of the 26S proteasome engages and activates deubiquitinases UCHL5 (19S-associated) and PSMD14 (19S subunit)	absent	↓ capture of ubiquitinated proteins for 26S proteasomal degradation ↓ activation of UCHL5
UCHL5	19S-associated de-ubiquitinating enzyme activated by transient interaction – via ADRM1 – of the INO80 complex with the 26S proteasome	absent	↓ deubiquitination
PSMA2	core alpha subunits of the 20S proteasome	absent	↓ 20S/26S proteasomal activity
PSMD9	non-ATPase subunits of the 19S regulator acts as a chaperone during 26S proteasome assembly	absent	↓ 26S proteasomal activity
PSME1, PSME2	IFNγ-induced immunoproteasome 11S component	down-regulated	↓ IFNγ-induced immunoproteasomal activity
SRPRB	signal recognition particle (SRP) receptor (SR) subunit which anchors it to ER membrane SR mediates co-translational targeting of secretory and membrane proteins to ER associated pathways: UPR, viral mRNA translation	absent	↓ co-translational targeting of secretory and membrane proteins to ER
SPCS3	component of microsomal signal peptidase complex which removes signal and other N-terminal peptides from nascent proteins being translocated into ER lumen associated pathway: peptide hormone metabolism	absent	↓ processing of nascent proteins entering ER
DDOST	essential subunit of ER lumen OST complex which transfers high-mannose oligosaccharides to asparagine residues in nascent polypeptides (N-linked glycosylation) also implicated in processing of AGEs	down	↓ N-linked glycosylation of nascent proteins entering ER
OGA	adds and removes O-GlcNAc to/from protein Ser/Thr residues as part of O-glycosylation	absent	↓ O-linked glycosylation flux
CALR	multifunctional calcium-storage protein of ER important lectin member of ER CALR/CANX cycle inhibits response to glucocorticoids, androgens, and retinoic acid responds to vitamin D receptor binds antibodies in some autoimmune diseases	down	↓ ER CALR/CANX quality-control cycle (retention by lectins) de-repressed ability to respond to glucocorticoids, androgens, vitamin A
PDIA4	ER PDI helps protein folding via disulfide bridge formation enhances IgG inter-chain disulfide bond formation	down	↓ ER disulfide bridge formation/protein-folding enhancement
PDIA6	helps protein folding via disulfide bridge formation helps prevent protein aggregation negatively regulates UPR through binding sensors ERN1 and possibly EIF2AK3	down	↓ disulfide bridge formation/protein-folding enhancement de-repression of ability to activate UPR
TMX4	participates in protein folding and thiol-disulfide interchange which reversibly oxidises reactive protein thiols to facilitate disulfide bond formation	unique	↑ TMX-mediated enhancement of disulfide bond formation and protein folding
ERLIN2	ER lipid raft protein mediates ERAD of: - activated IP3 receptors e.g. ITPR1 (regulating IP3 signalling) - HMGCR (as noted above)	unique	↓ capacity for ERAD
CLPP	mitochondrial peptidase hydrolyses proteins into small peptides part of housekeeping Clp complex (similar to a proteasome) which degrades proteins/peptides	unique	↑ mitochondrial protein degradation
CNDP2	non-specific dipeptidase hydrolyses various dipeptides including L-carnosine (alanine + histidine) elevated level activates p38 MAPK pro-apoptotic pathways lower level activates ERK MAPK pathway favouring proliferation	down	↓ dipeptide hydrolysis ↓ MAPK/ERK signalling
NELFB	subunit of negative elongation factor NELF acts with DSIF, SUPT4H1 and 5 to pause RNAPII transcription complex also involved in HIV latency	absent	↓ restraint of transcription
CISD2	ER protein that binds Fe-S clusters regulator of autophagy: antagonises BECN1-mediated autophagy, but contributes to BIK-initiated autophagy participates in interaction of BCL2 with BECN1 required for BCL2-mediated depression of Ca2+ stores during autophagy	absent	↓ ER Fe-S cluster-binding ↓ restraint of BECN1-mediated autophagy ↓ capacity for BIK-mediated autophagy ↓ BCL2-mediated Ca2+ store depression during autophagy
MANF	ER, Golgi, and secreted protein retained in ER by HSPA5 under normal conditions expression up-regulated in response to ER stress and hypoxia secreted form binds a lipid sulfatide on target cells, promoting endocytosis for alleviation of ER stress and hypoxic cell toxicity decreased expression increases susceptibility to ER stress-induced death	absent	inferred: ↓ ER stress, hypoxia ↑ susceptibility to ER stress-induced death (when ER stress is present)

IRIS2-specific	HSP90AA1, AB1	aids proper folding of target (e.g. signal transduction) proteins in an ATP-dependent manner targets alter depending on which co-chaperones are associated also modulates transcription at 3 levels	up	↑ protein-folding quality-control capacity
	HSP90B1	specifically located in the ER, "functions in processing and transport of secreted proteins", is – along with CNPY3 – required for appropriate TLR folding, and has a role in ERAD	up	↑ ER protein-folding quality-control capacity ↑ transport of secretory proteins ?↑ ERAD capacity
	HSPA4	Hsp70 family member associated with glucocorticoid receptor signalling (as is one of the other HSPs in this list)	up	↑ protein-folding quality-control capacity
	HSPD1	mitochondrial chaperonin essential for folding and assembly of proteins imported into mitochondrion may also promote proper refolding and assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix may function as a signalling molecule in innate immunity	up	↑ protein-folding quality-control capacity for proteins entering mitochondrion under stress conditions
	HSPE1-MOB4	naturally-occurring read-through transcript fusion protein that shares sequence identity with each individual protein MOB4 may play a role in membrane trafficking e.g. budding HSPE1 heat-shock chaperonin functions with co-chaperonin HSPD1 implicated in mitochondrial protein import and macromolecular assembly with Hsp60 facilitates correct folding of imported proteins, prevents misfolding, and promotes refolding of proteins generated under stress conditions in the mitochondrial matrix	unique	↑ protein-folding quality-control capacity for proteins entering mitochondrion under stress conditions
	CDC37	molecular chaperone which functions in signal transduction complexes with Hsp90 and various kinases incl. Src, Raf-1, eIF2 alpha kinases associated with EGFR and TNFR1 pathways promotes interaction of kinases with Hsp90 complex to stabilise them and promote their activity	up	↑ protein-folding quality-control capacity, esp. for signalling kinases
	FKBP5	immunophilin and prolyl isomerase protein-folding co-chaperone that also regulates basic cellular processes and immunity via modulation of AKT phosphorylation and NFkB activation	up	↑ protein-folding quality-control capacity
	UBA2	E1 ligase with SAE2 activates SUMO1-4 for protein SUMOylation	unique	↑ SUMOylation of selected targets
	SUGT1	may play a role in ubiquitination and proteasomal degradation of target proteins	unique	↑ UPS-mediated degradation
	CUL2	core part of many cullin-RING-based ECS E3 ubiquitin ligase complexes which ubiquitinate target proteins e.g. HIF-1	unique	↑ ubiquitination (incl. of HIF-1) recall that under both conditions, unique UBE2M neddylates CUL1-4, likely ↑ neddylation of CUL2
	COPS2	essential part of COP9 signalosome regulates various signalling pathways, incl. ubiquitination mediates de-neddylation of SCF-type E3 ligase subunits leading to decreased ubiquitination of target proteins also helps phosphorylate p53, JUN, NFKBIA, ITPK1, IRF8 possibly via association with casein kinase II and PKD phosphorylation of p53 and JUN respectively enhance and inhibit UPS-mediated degradation	unique	↑ de-neddylation of E3 ligases, thereby ↓ ubiquitination of selected targets ?↑ enhancement of PKD- and CKII-mediated phosphorylation of certain targets, incl. p53, NFKBIA
	CAND1	essential regulator of cullin-RING E3 ubiquitin ligase complexes inhibits cullin neddylation and prevents complex formation knockdown blocks adipogenesis	down	↑ neddylation ↑ ubiquitination ?↓ adipogenesis
	RNF213	Zn ²⁺ -binding ATPase, E3 ubiquitin ligase mediates ubiquitin-dependent FLNA degradation inhibits angiogenesis, promotes vessel regression	down	↓ ubiquitination and UPS-mediated degradation (incl. of FLNA) de-repressed angiogenesis
	PRPF19	ubiquitin ligase core component of several complexes involved in pre-mRNA splicing and DNA repair may play a role in the biogenesis of lipid droplets	unique	↑ ubiquitination ?↑ RNA processing, DNA repair ?lipid droplet biogenesis
	OTULINL	deubiquitinase	unique	↑ de-ubiquitination
	UFD1	part of complex (with VCP and NPLOC4) required for export of misfolded/fusion proteins from ER to the cytoplasm for UPS-mediated degradation complex also inhibits type I IFN generation by promoting DDX58/RIG-I degradation	unique	↑ export of misfolded/fusion proteins from ER to cytoplasm for UPS-mediated degradation ?↓ RIG-I activity
	ADRM1	Ub receptor subunit of the 26S proteasome engages and activates deubiquitinases UCHL5 (19S-associated) and PSMD14 (19S subunit)	unique	↑ capture of ubiquitinated proteins for 26S proteasomal degradation ↑ activation of UCHL5
	PSMA2	20S core alpha subunit	unique	↑ 20/26S UPS-mediated degradation
	PSMA7	core alpha subunit of 20S interacts with HBV protein increases HIF-1a degradation under hypoxic conditions related pathway: beta-adrenergic signalling mediates nuclear translocation of androgen receptor and thus enhances androgen-mediated transactivation inhibits MAVS-mediated innate responses through MAVS degradation	absent	↓ 20/26S UPS-mediated degradation ↓ HIF-1a degradation (?hypoxia/inflammation) ↓ enhancement of response to androgens de-repression of MAVS-mediated innate responses
	PSMB3	core beta subunit of 20S also associated with beta-adrenergic signalling	absent	?↓ beta-adrenergic signalling leading to specific ↓ 20/26S UPS-mediated degradation
	PSMD9	non-ATPase subunit of 19S regulator acts as a chaperone during 26S proteasome assembly	unique	↑ 26S UPS-mediated degradation
	SSR1	glycosylated ER membrane receptor associated with protein translocation across ER membrane signal sequence receptor binds Ca ²⁺ to ER membrane to regulate retention of ER proteins may function as membrane-bound chaperone for folding of	unique	↑ translocation and ?retention/folding of nascent proteins into ER

		translocated proteins		
	SPCS3	component of microsomal signal peptidase complex which removes signal peptides and other N-terminal peptides from nascent proteins entering the ER lumen	unique	↑ processing of nascent proteins entering the ER
	DDOST	essential component of ER OST for N-linked glycosylation also implicated in processing of AGEs required for assembly of both SST3A and B-containing OSTs	up	↑ N-glycosylation of nascent proteins in ER (maybe in response to lack of STT3A)
	STT3A	catalytic subunit of OST complex which catalyzes first step of co-translational N-glycosylation (glycan transfer from a lipid carrier to asparagine)	absent	↓ N-glycosylation of nascent proteins in ER
	PRKCSH	regulatory subunit of glucosylase II known PKC substrate thought to stabilise the catalytic subunit, as well as "enhancing N-glycan processing rates"	up	↑ CALR/CANX quality-control folding cycle and N-glycosylation enhancement
	CANX	Ca ²⁺ -binding ER-associated chaperone for nascent N-glycosylated protein folding as part of CANX/CALR cycle associated with MHC-I antigen processing may play a role in receptor-mediated endocytosis	up	↑ CALR/CANX quality-control folding cycle
	PPIA	PPIases cis-trans isomerise proline imidic peptide bonds in oligopeptides and accelerate protein folding binds and may help mediate cyclosporin A-mediated immunosuppression can also interact with several HIV proteins and is necessary for formation of infectious HIV virions	up	↑ protein-folding rates ?↑ HIV virion formation
	LMAN1	mannose-binding lectin 1 a.k.a. ERGIC cycles between ER and Golgi cargo receptor for glycoprotein and glycolipid transport	up	↑ transport of glycoproteins and glycolipids between ER and Golgi

At baseline, CAMKK2 and an AMPK subunit were absent in both groups (recall that AMPK is pro-autophagic). Since ARV therapy apparently does not further dysregulate their levels, AMPK activity likely remains repressed. Indeed, there is indirect evidence of this in the CTRL class, although other findings in this class (Table 4, *vide supra*) also suggest activated AMPK, de-repressed autophagy, translation, and altered flux through the UPS. In the IRIS class, however, there is indirect evidence of attempts to activate (likely absent) CAMKK2/AMPK. Although there are indications of mTORC1 inhibition (e.g. via TXNIP) and thus de-repressed autophagy in both classes (especially CTRLs), the IRIS class also exhibits up-regulated (now likely farnesylated) RHEB, which should help activate mTORC1 (provided amino acids are available and lysosomal V-ATPase is active) via the PI3K-AKT-mTOR pathway, thereby further repressing autophagy. Furthermore, up-regulated HSPA5 and PDIA6 in the IRIS class likely also repress the unfolded-protein response (UPR; though there is some suggestion of increased ERAD capacity). Although the CTRL class remains capable of the UPR (including ERAD) and autophagy, it apparently does not experience misfolded protein stress. Specifically in support of mitochondrial stress are CTRL class indications of decreased ER Fe-S cluster binding, and of increased mitochondrial-site protein degradation.

Specific ARV therapy-induced changes which occur in *opposite directions* in the CTRL versus IRIS groups over time include: SUGT1 (likely plays a role in ubiquitination and proteasomal degradation of target proteins), COPS2 (an essential part of COP9 signalosome involved in various signalling pathways including ubiquitination), UFD1 (complexed with VCP and NPLOC4 is required to export misfolded/fusion proteins from ER to the cytoplasm for UPS-mediated degradation; also promotes DDX58/RIG-I degradation), ADRM1 (ubiquitin receptor subunit of the 26S proteasome; also activates 19S-associated deubiquitinases UCHL5 and PSMD14), PSMA2 (20S proteasome core alpha subunit), PSMD9 (non-ATPase subunit of 19S regulator; chaperones 26S proteasome assembly), SPCS3 (part of complex removing signal peptides and other N-terminal peptides from nascent proteins entering the ER lumen), and DDOST (essential component of ER OST for N-linked glycosylation). All decrease to absence in the CTRL group, but increase to uniqueness in the IRIS group.

3.8. Integrated Amino Acid, Vitamin, H₂S/Sulfate, Glutathione, Redox, Glyoxylate, Xenobiotic, and Fe-S Protein Metabolism (not tabulated)

At baseline, various amino acid metabolic pathways were likely altered: decreased PLP-dependent glutamate → glutamine and lysine + (built-up) α-KG → saccharopine + glutamate, increased G3P + glutamate → serine and late-stage urea cycle arginine → ornithine + urea. The CTRL class also exhibited likely increased tryptophan + PRPP → NAD, and decreased entry of cytosolic serine into mitochondria. The IRIS class also exhibited likely increased asparagine → aspartate + NH₄, neither combining with glutamate to form glutamine nor being fully detoxified by the urea cycle (due to decreased mid-cycle arginosuccinate → arginine + fumarate). Regarding the methionine-S-adenosylmethionine-cysteine-GSH continuum,

only the CTRL class exhibited likely increased ATP/NAD-dependent methionine → SAME, and likely-present oxidative stress would have prevented resulting homocysteine remethylation to methionine, instead driving trans-sulfuration to cysteine and thus H₂S/GSH/GPx/Trx reductase, but the latter could only occur provided PLP and vitamin B5 were available. Indeed, at baseline, both classes exhibited evidence suggesting decreased PLP synthesis. Only the CTRL class exhibited decreased recycling of D-pantetheine back to vitamin B5 (required for the antioxidant response and for CoA synthesis), both classes exhibited decreased early-stage CoA synthesis from vitamin B5, and only the CTRL class exhibited compensatory increased late-stage CoA synthesis (CoA is used to activate FA for entry into metabolic pathways). Acetyl-CoA can feed into the TCA cycle, or is used for lipid synthesis. At baseline, both classes exhibited decreased mitochondrial FA-CoA uptake, increased FA/glycerol levels, and increased FAO (provision of NADH and FADH₂ from FAO increased in the CTRL class, especially). The IRIS class exhibited increased acetyl-CoA production, as well as glycerolipid synthesis from G3P.

As mentioned, in both classes ARV exposure somewhat corrects the likely baseline build-up of α-KG as well as pyrimidine catabolism for purine synthesis, and increases NAD biosynthesis despite findings simultaneously suggesting increased G3P generation from ribose/ribulose → xylulose (perhaps for serine biosynthesis). As per Table 11, in the IRIS class further-dysregulated nucleotide precursor metabolism (decreased provision of thymidine intermediates for pyrimidine synthesis, as well as a continuing preference for purine synthesis) may exacerbate potential ARV/oxidative stress-induced mtDNA depletion (to which neutrophils may be particularly susceptible), with elevated mtROS likely resulting from mitochondrial dysfunction. While the CTRL class apparently decreases conversion of glycolytic pyruvate to acetyl-CoA with a concomitant rise in glycine, the opposite occurs in the IRIS class, which also up-regulates the intra-mitochondrial portion of the malate-aspartate shuttle (for provision of NADH electrons to the respiratory chain), thereby consuming glutamate and producing aspartate and α-KG. Increased IRIS class ammonia detoxification via an intracellular urea cycle would also consume glutamate and generate glutamine, arginine, and fumarate.

Table 11 Dysregulated Proteins Relevant to Protein Homeostasis

Class	Protein	Function	Status	Hypothesised Outcome
shared	PRDX4	senses hydrogen peroxide-mediated signalling events converts hydrogen peroxide and alkyl hydroperoxides to water and alcohol regulates NfκB activation	unique	↑ antioxidant response to ROS
	TXNIP	binds antioxidant thioredoxin, causing ROS accumulation and ER stress also inhibits DDIT4 degradation, contributing to mTORC1 inhibition	unique	↑ ROS accumulation, ER stress ↓ mTORC1 activity
	QSOX1	catalyses peptide/protein thiol-sulphydryl group oxidation to disulfides (possibly contributing to disulfide bond formation), in the process forming hydrogen peroxide	unique	↑ ROS production ↑ protein folding
	CLIC1	chloride channel that inserts into membrane apparently only under oxidising conditions	down	↑ insertion into membrane (↓ detection)
	GSTK1	peroxisomal glutathione transferase which conjugates glutathione to various hydrophobic substrates – including xenobiotics – for elimination from the cell	absent	↓ xenobiotic elimination
CTRL2- specific	MPST	paralog of TST (unique in the CTRL0 class) transfers sulphur from 3-mercaptopyruvate to thiol compounds (e.g. to generate thiosulfate for Ca ²⁺ -regulated H ₂ S biosynthesis by 3MST/CAT) or cyanide, as part of cysteine metabolism and cyanide detoxification also has antioxidant functions	unique	↑ cyanide detoxification and/or H ₂ S catabolism
	TSTD1	transfers sulfane from thiosulfate to glutathione to generate GSSH	absent	↓ sulfane transfer (unless TST paralog MPST can compensate)
	SELENBP1	selenium-binding protein (absent in CTRL0 class) may sense intracellular reactive xenobiotics may oxidise organosulfur compounds	unique	altered sulfur metabolism ↑ altered selenium-based antioxidant activity
	VNN1	absent in CTRL0 class recycles D-pantetheine to usable pantothenate (vitamin B5) with release of cysteamine	unique	↑ antioxidant capacity ↑ capacity to synthesise CoA altered cysteine metabolism
	GSR	NADPH- and FAD (vitamin B2)-dependently converts oxidised glutathione disulfide (GSSG) back to reduced glutathione for use in antioxidant defense and sulfur metabolism key enzyme in antioxidant defense (via glutathione maintenance)	up	↑ antioxidant capacity altered sulfur metabolism
	PRDX3	thiol-specific peroxidase converts hydrogen peroxides and organic hydroperoxides to water and alcohols, respectively	down	↑ requirement for this type of antioxidant activity
	XRCC6	DNA repair helicase part of an XRCC5-containing dimer which increases DNA affinity of DNA- dependent protein kinase 100-fold also regulates DNA virus-mediated innate responses by forming part of a complex which is a platform for IRF3 phosphorylation and cGAS-STING activation	down	↓ DNA repair ↑ due to drop in antiviral response
	CDKN2AIP	regulates DNA damage response via several pathways	absent	↓ DNA repair
	AIP	enhances AhR-mediated induction of the xenobiotic response (e.g. CYP450 expression) can inhibit HBV activity	absent	↓ xenobiotic response enhancement
	ABHD14B	hydrolase for p-nitrophenyl butyrate associated with CYP450 metabolism	absent	↓ xenobiotic response
IRIS2- specific	TSTD1	part of H ₂ S catabolism transfers sulfane from thiosulfate to glutathione, producing GSSH	unique	↑ sulfane transfer to glutathione

SELENBP1	see above	absent	altered sulfur/cysteine metabolism
COASY	mediates first step of CoA synthesis	unique	↑ CoA synthesis
ORM2	circulatory carrier for hydrophobic substances including xenobiotics	unique	↑ xenobiotic response
HMOX2	converts heme to biliverdin	unique	↑ biliverdin-mediated AhR activation to induce xenobiotic response ↑ heme catabolism, providing ↑ cellular iron and (from biliverdin catabolism to bilirubin) CO
ATP6AP1	location- and assembly-determining subunit of a non-lysosomal (i.e. granule) V-ATPase which under aerobic conditions activates Fe ²⁺ prolyl hydroxylase (PHD) enzymes (for iron homeostasis) as well as causing HIF1A degradation i.e. involved in iron and hypoxic homeostasis	unique	response to ferric and hypoxic stress
APMAP	arylesterase which hydrolyses aromatic carboxylic acid esters (incl. converting phenyl/naphthyl acetates to phenol and acetate) helps protect lipoproteins from oxidation [13]	up	↑ protection against lipoprotein oxidation
ALDH1A1	follows alcohol dehydrogenase in the major alcohol catabolism pathway also converts retinaldehyde to retinoic acid	absent	↓ acetaldehyde catabolism ↓ provision of retinoic acid
MTHFD1	interconverts tetrahydrofolate 1-carbon derivatives required for methionine, thymidilate, and <i>de novo</i> purine synthesis	down	↓ methionine and NA synthesis
ACP1	Tyr phosphatase associated with thiamine metabolism	unique	altered thymine metabolism
DLD	converts pyruvate to acetyl-CoA consumes glycine	unique	↑ acetyl-CoA synthesis ↓ glycine
GLUL	converts glutamate and ammonia to glutamine	unique	↑ ammonia detoxification, glutamine synthesis ↓ glutamate
ASL	converts arginosuccinate to arginine and fumarate as part of urea cycle	unique	↑ arginine, fumarate synthesis, ammonia detoxification
CA4	provides bicarbonate for urea cycle	unique	↑ ammonia detoxification
ARMT1	methylation-dependent gene expression alteration/DNA repair	unique	↑ need for DNA repair/altered gene expression
PRDX2 and PRDX6	peroxiredoxins PRDX2 senses hydrogen peroxide signalling and may – via regulating hydrogen peroxide concentration – participate in signalling cascades of growth factors and TNFα PRDX6 protects against lipid peroxidation	down	↓ protection against peroxides, perhaps due to ↓NADPH or Se-cysteine
SH3BGR13	modulates activity of antioxidant GLRX (which reduces low-MW disulfides and proteins in the presence of glutathione and NADPH) also involved in S-glutathionylation of signalling mediators and β-amyloid toxicity	down	altered activity of glutaredoxin class of antioxidant enzymes
GPX1	reduces both hydrogen peroxide and organic peroxides in the presence of glutathione also associated with HPETE/HETE biosynthesis and metabolism	down	↓ capacity for antioxidant response
GPX4	specifically prevents membrane and lipoprotein lipid/phospholipid peroxidation, as well as preventing ferroptosis resulting from iron-dependent lipid-ROS accumulation	unique	response to ↑ ferric peroxidative stress

The CTRL class recovers the ability to regenerate vitamin B5 (perhaps due to increased CoA catabolism), while the IRIS class experiences increased CoA synthesis. Relevant to acetyl-CoA levels, ARV exposure decreases FAO and further increases (likely glycerol-)lipid synthesis in the CTRL class, while increasing TG uptake and FAO, but decreasing lipid synthesis, in the IRIS class. The observed down-regulation of ATP6V0A1-mediated lysosome acidification in the IRIS class may reflect elevated homocysteine levels, which could suggest decreased conversion of homocysteine to cysteine. Although IRIS-class cysteine status is uncertain, levels of the other two GSH precursors (glycine and glutamate) apparently become decreased, suggesting that GSH may be especially limiting under this condition (while there is increased GSR-mediated regeneration of reduced GSH in the CTRL class). While at baseline it was the CTRL class which did not use serine to generate 1-carbon-donor charged folates, ARV exposure makes the IRIS class unable to generate 1-carbon-donor charged folates (required for methionine, thymidylate, and *de novo* purine synthesis). Recall that both vitamins B9 and B12 (folate) are required for DNA synthesis. Both classes exhibit some evidence of DNA repair responses, but these may be relatively down-regulated and up-regulated in the CTRL and IRIS classes, respectively.

At baseline, both classes attempt to enhance generation of H₂S, though (based on Chapter 1 literature) it is likely that insufficient GSH is available. Although the initial step (cysteine → pyruvate) is down-modulated in both classes, step 2 (SAME-stimulated homocysteine + cysteine → serine) is increased in the CTRL class, and steps 3 (aspartate-stimulated cysteine + α-KG amino group → 3MCP) and 4 (GSH/thioredoxin/dihydrolipoic acid-dependent 3MCP → persulfides → H₂S, or sulfate → thiosulfate) are increased in both classes. Only the CTRL class also engages in reconversion of thiosulfate to sulfite, which also detoxifies cyanide to thiocyanate (detoxification of endogenous cyanide – produced by microbicidal leukocytes/muscarinic cholinergic signalling – can also consume vitamin B12). Simultaneously, both classes exhibited an attempt to increase H₂S catabolism to thiosulfate (dependent on sufficient quinone availability) and thus glutathione persulfide (dependent on sufficient GSH availability). Though the CTRL class (via TST) can convert thiosulfate back to

H₂S/sulphite-thiocyanate, the IRIS class appears to undergo thiosulfate buildup in the absence of an enzyme to transfer the thiol group to GSH. Altered H₂S levels would be able to explain many alterations suggested by the dataset, including of Ca²⁺, K⁺, HIF-1α/SIRT1/NRF2/GSH/thioredoxin, insulin/glucose/G3P, NFκB/iNOS/NO/RNS/proinflammatory cytokines, respiratory complex II/IV inhibition, oxidised DNA/mt depolarisation, formation of sulfhemoglobin, etc. Exposure to ARV apparently further up-regulates H₂S biosynthesis in the CTRL class (provided cysteine and GSH/thioredoxin are available) but up-regulates H₂S catabolism in the IRIS class (although both classes may engage in cyanide detoxification).

At baseline, both classes exhibited findings suggestive of oxidative stress, exacerbated by NADPH, GSH, and GPx/Trx reductase depletion, perhaps especially so in the IRIS class. Although there is evidence of increased ROS (e.g. H₂O₂) production in both groups during ARV exposure (suggesting damage to biomolecules such as lipids and DNA), different parts of the anti-oxidant response are both increased and decreased in both classes. For example, both classes exhibit TXNIP-mediated repression of thioredoxin activity and decreased levels of peroxiredoxins (which rely on active-site cysteine residues to detoxify peroxides, including lipid peroxides). The IRIS class also exhibits decreased glutathione peroxidase (GPX) 1 (and thus peroxide detoxification) and glutaredoxin regulation by SH3BGR1. However, GPX4 is up-regulated, specifically suggesting protection against lipid peroxidation and ferroptosis. In the CTRL class, most protein-folding QC enzymes (including those assisting in disulphide bridge formation) are down-regulated, with the exception of unique TMX4 (perhaps a response to altered redox conditions, rather than to ER protein-folding capacity requirements).

At baseline, xenobiotic metabolism appeared to be increased in both classes, including an attempt to enhance immunoregulatory AhR signalling (AhR is induced, for example, by NFκB). However, only the IRIS class exhibited decreased conversion of heme to biliverdin, again suggesting altered/impaired Fe metabolism (recall that Fe-S cluster and Fe-S-containing protein biosynthesis was increased in both classes). After ARV exposure, both classes – surprisingly – exhibit decreased GSTK1-mediated conjugation of GSH to e.g. xenobiotics for elimination. The CTRL class exhibits additional evidences of a decreased xenobiotic response, while the IRIS class exhibits decreased acetaldehyde detoxification (as well as decreased activation of vitamin A by the same enzyme), but otherwise evidence of ongoing xenobiotic responses (including to prevent lipoprotein oxidation, and for increased heme breakdown/AhR activation).

Although at baseline both classes exhibited indications of increased glycolysis-derived glyoxal stress (especially in the IRIS class), there are no direct indications in the dataset regarding glyoxal metabolism after ARV exposure (although glycolytic flux continues to be elevated, suggesting ongoing production of reactive carbonyl species). Class-specific predictions regarding integrated metabolic profiles are summarised in Table 12 and depicted in fig. 8 (green: enhanced, red: suppressed, orange: hypothetically suppressed).

Table 12 Predicted Neutrophil Metabolic Changes from Baseline through ARV Exposure

	CTRL	IRIS
insulin sensitivity	- becoming corrected over-anabolism	↑ becoming corrected over-anabolism
PKA signalling (favours FAO)	likely-dominant becoming ↓	likely-dominant becoming ↓
AKT-mTOR signalling (favours lipid synth)	↓ (but inactive AMPK de-represses mTORC1) becoming further ↓ (incl. possible AMPK activation attempts repressing mTORC1)	↓ (but inactive AMPK de-represses mTORC1) becoming further ↓ (but possible ↑ AMPK & mTORC1 activation by farnesylated RHEB)
glycogen flux	↓ becoming -	↓ becoming -
glycolysis (enhanced by mTORC1, HIF1α, etc.)	↓ glucose entry, ↑ post-G3P/DHAP activity becoming ↑ glucose flux through early glycolysis, ↓ glycolytic pyruvate → acetyl-CoA (↑ Gly)	↓ glucose entry, ↑ post-G3P/DHAP activity becoming ↑ glucose flux through early glycolysis (incl. monosaccharide uptake & glucose entry), ↑ glycolytic pyruvate → acetyl-CoA (↓ Gly)
HIF1α induction (also alters Fe homeostasis)	↑ (incl. pyruvate → lactate + NAD) becoming -	↑ becoming -
PPP	↓ glucose entry (↓ NADPH), ↑ downstream activity becoming ↑ glucose flux through early PPP (↑ NADPH)	↓ glucose entry, ↑ downstream activity becoming -
glyoxal stress	↑ becoming ?↑ (high glycolytic flux persists)	esp. ↑ becoming ?↑ (high glycolytic flux persists)
TCA cycle	↑ malate → pyruvate, ↓ α-KG + glutamate → succinyl-coA (α-KG buildup may feed glycolysis/PPP) becoming corrected α-KG buildup	↑ malate → pyruvate, ↑ malate → oxaloacetate suggests ↑ intra-mt portion of malate-aspartate shuttle (transfers NADH electrons to respiratory chain, consumes glutamate, produces aspartate + α-KG), ↓ α-KG + glutamate → succinyl-coA (α-KG buildup may feed glycolysis/PPP), ↓ adenylosuccinate → fumarate becoming corrected α-KG buildup
oxphos	↓ complex I & II, ↑ complex V, ↑ reliance on pyruvate → lactate becoming: further ↓ complex I, ↑ electron transfer from FADH2 via complex II, & ↓ complex III (*possible mt depolarisation), ↓ complex V	↓ complex II & III (*possible mt depolarisation), ↑ complex V becoming: ↑ electron transfer from NADH via complex I, ↓ complex IV, ↑ complex V (*possible attempt to prevent mt depolarisation)

mitochondrial stress & cell death	likely ↑ NRF1-mediated mt biogenesis & antioxidant response, ↑ inhibition of apoptosis (but higher capacity for externally- over internally-initiated apoptosis), de-repressed mTORC1 may ↓ mitophagy (↑ mt stress, mROS, mtDNA damage) becoming ↓ ER Fe-S cluster binding, possible ↑ mt import of respiratory chain Fe components, ↑ mt-site protein degradation, ↑ inhibition of mROS production & mtDNA damage, further ↑ inhibition of apoptosis (both externally-induced *CASP8-mediated & internally-induced mt-mediated apoptosis) *absent CASP8 means ↓ TNF/Fas-CASP8-CASP10-CASP3/7-mediated apoptosis	no evidence of NRF1-mediated signalling, ↑ inhibition of apoptosis (but higher capacity for externally- over internally-initiated apoptosis), likely ↑ proneness to necrosis, de-repressed mTORC1 may ↓ mitophagy (↑ mt stress, mROS, mtDNA damage) becoming ↑ folding QC esp. for mt proteins imported under stress conditions, ↑ mt ADP uptake, ↑ ATP-alternate E-production, ↑ capacity for mt membrane permeabilisation, ↓ resistance to apoptosis (↑ capacity for both externally-induced granzyme/TNF/Fas-CASP8-*CASP10-CASP3/7 & especially internally-induced mt-BAX-mediated apoptosis), possible ↑ NLR4-CASP4/5-mediated pyroptosis &/or lipid peroxidation-mediated ferroptosis &/or DNA damage-induced necroptosis ** CASP10 expression
oxidative stress (ROS) damages lipids, DNA, & other biomolecules	↑ (likely exacerbated by ↓ NADPH, GSH, GPx/Trx reductase, esp. in IRIS) becoming further ↑ with ↑ & ↓ antioxidant responses (e.g. TXNIP-mediated Trx inhibition, ↓ cysteine-peroxiredoxins for lipid peroxide detoxification)	↑ (likely exacerbated by ↓ NADPH, GSH, GPx/Trx reductase, esp. in IRIS) becoming further ↑ with ↑ & ↓ antioxidant responses (e.g. TXNIP-mediated Trx inhibition, ↓ cysteine-peroxiredoxins for lipid peroxide detoxification, ↓ GPx1 for peroxide detoxification, SH3BGRL-mediated glutaredoxin regulation, & ↑ GPx4 to prevent lipid peroxidation & ferroptosis)
nucleotide metabolism mTORC1 would enhance pyrimidine synthesis both vitamins B9 and B12 (folate) are required for DNA synthesis nt can be broken down into PRPP/ribose/ribulose, which can be used to generate nt, NAD, or G3P serine can be derived from diet or synthesised in vivo (incl. from glycine, which is increased in CTRL but decreased in IRIS), serine & its metabolites (ethanolamine, choline) are nitrogen bases in glycerophospholipid synthesis; serine also gives rise to sphingosine & thus ceramides; serine is also gluconeogenic (enters dicarboxylic acid shuttle – which requires aspartate – as pyruvate); serine also participates in charged folate synthesis. Serine is thus NB in phospho- and glyco-lipid metabolism. Aspartate is also required for malate-aspartate shuttle. α-KG can be siphoned off the TCA cycle and utilized for the synthesis of glutamate	↑ mt pyrimidine salvage & catabolism for ↑ purine (guanine) synthesis (NAD-dep), Δ purine (adenine) metabolism, ↑ PRPP + tryptophan → NAD (NAD also derives from anaerobic glycolysis), ↑ G3P + glutamate (possibly from α-KG buildup) → serine (not entering mt & not used to generate charged folates required for methionine & de novo purine synthesis, suggesting instead use for glycerophospholipid &/or sphingosine/ceramide synthesis) becoming: ↑ DNA repair (esp. in IRIS class) but necroptosis if this fails (e.g. due to absent DTXL3), ↓ cannibalising pyrimidines for purine synthesis (incl. ↓ adenosine/AMP synthesis), further ↑ PRPP + tryptophan → NAD, ↑ ribose/ribulose → xylulose → G3P (rather than nt synthesis) → serine	↑ mt pyrimidine salvage & catabolism for ↑ purine (guanine) synthesis (NAD-dep), Δ purine (adenine) metabolism, ↓ ribose-5-P → G3P & ↓ α-KG + lysine → glutamate, but ↑ G3P + glutamate → serine (& glutamate is also consumed by other processes in IRIS class) becoming: ↑ DNA repair (esp. in IRIS class) but necroptosis if this fails (e.g. due to absent DTXL3), ↑ DNA precursor pool size, somewhat corrected pyrimidine cannibalism for purine synthesis, but still ↓ pyrimidine synthesis precursors (may exacerbate ARV-induced mtDNA depletion, to which neutrophils may be esp. susceptible) perhaps prioritising purine/ATP synthesis, ↑ PRPP + tryptophan → NAD, ↑ nt → ribose/ribulose → xylulose → G3P (rather than nt synthesis) → serine (but serine not used to generate charged folates required for methionine & de novo purine synthesis, suggesting instead use for glycerophospholipid &/or sphingosine/ceramide synthesis)
lipid metabolism mTORC1 would enhance lipid synthesis recall that succinyl-CoA is produced not only from α-KG metabolism, but also via FAO	↑ FA synthesis & ↑ FAO (incl. NADH FADH2 to oxphos) becoming further ↑ lipid synthesis (possibly preferential PPARα-glycerolipid) but normalised (↓) FAO	↑ acetyl-CoA/FA/glycerolipid (from G3P) synthesis, ↑ FAO-derived FADH2 to oxphos becoming ↓ lipid synthesis but ↑ TG/fat-soluble vitamin uptake & ↑ FAO
integrated CoA, TCA cycle, amino acid, B-vitamin, & NH₄ metabolism vitB5 (required for antioxidant response) produces CoA, a cofactor (acyl carrier) in many metabolic reactions Involved in linking glycolysis to TCA, & activates FA for metabolism CoA, carnitine, & creatine are related to sulfur AA. CoA & carnitine → muscle ATP production (formation of NADH & FADH2 for oxphos) & l-carnitine import into mt for FAO (low carnitine → carb use, high carnitine → FA use for energy); creatine → muscle ATP regeneration. acetyl-CoA comes from mt pyruvate, phenylalanine/tyr/leu/lys/trp catabolism, & FAO. "transported" to cytosol via citrate shuttle (acetyl-CoA + OA → citrate, transported to cytosol to recombine with CoA using ATP → acetyl-CoA + OA). Also exchanged for carnitine (facilitating acyl-CoA in & , minority, acetyl-CoA out of mt). acetyl-CoA feeds into TCA/lipid synth (FA synth requires NADPH from PPP) during fasting, AA are used for gluconeogenesis to maintain glucose for rapidly-metabolising cells. In repletion, ketogenic AA (producing acetyl-CoA/acetoacetyl) enter into FA/TAG synthesis or are metabolised as fuel (but cannot become glucose), only Leu & Lys are ketogenic only; all other AA (incl. also-ketogenic isoleu, phenylAla, & Trp) can enter gluconeogenesis. AA producing ketoglutarate, succinyl-CoA, fumarate, OA directly supplement TCA cycle, allowing withdrawal of OA, the major gluconeogenesis substrate. AA can also produce OA via pyruvate: pyruvate → oxaloacetate → glucose (via malate) or citrate (in mt)	carbon/lipid findings relevant to acetyl-CoA: ↑ glycolytic pyruvate → lactate (rather than acetyl-CoA), ↓ mt acyl-CoA uptake in exchange for carnitine (favouring carbohydrate rather than FA oxidation), ↑ FA/glycerol levels, ↑ FAO (with ↑ provision of NADH and FADH2 to oxphos), ↑ branched-chain AA catabolism, becoming ↓ glycolytic pyruvate → acetyl-CoA, ↑ (likely glycerolipid synthesis, ↓ FAO. Thus, acetyl-CoA likely initially derives from FAO/ketogenic AA rather than glycolysis, then from none of these (despite consumption by lipid synthesis) – perhaps formation from AA continues. ↓ vitB5 recycling, ↓ early vitB5 → CoA but ↑ late CoA synthesis, ↓ PLP synthesis, ↓ PLP-dep glutamate → glutamine, ↓ lysine + built-up α-KG → glutamate, ↑ G3P + glutamate → serine (↓ entry into mt), ↑ late urea cycle arginine → ornithine + urea, ↑ tryptophan + PRPP → NAD, becoming ↑ vitB5 regeneration (due to increased CoA catabolism), possible PLP normalisation (adaptation to INH). Initial decreased CoA would impair glycolysis-TCA linking (pyruvate converted to lactate rather than acetyl-CoA anyway), and it appears that G3P/serine are diverted to glycerolipid synthesis, while α-KG may feed into glycolysis/PPP (requiring TCA replenishment, likely by succinyl-CoA from FAO), & both arginine and tryptophan are consumed (for NH₄ detoxification and NAD synthesis, respectively). Arginine consumption may impair NO synthesis. However, ARV therapy corrects many of these (including B-vitamin) abnormalities, though acetyl-CoA for ongoing increased lipid synthesis must derive from AA catabolism given that pyruvate production & FAO become decreased.	carbon/lipid findings relevant to acetyl-CoA: unknown glycolytic pyruvate → lactate (HIF1α suggests, but LDHA only dysregulated in CTRL), ↓ mt FA-CoA uptake in exchange for carnitine, ↑ FA/glycerol levels, ↑ glycerolipid synthesis from G3P, ↑ FAO, becoming ↓ glycolytic pyruvate → acetyl-CoA, ↑ TG uptake, ↓ lipid synthesis, further ↑ FAO. Thus, acetyl-CoA likely initially derives from FAO rather than glycolysis (increased OA production may suggest gluconeogenesis) & is consumed by lipid synthesis, then from glycolysis & FAO (with decreased consumption by lipid synthesis). ↓ PLP synthesis, ↓ PLP-dep glutamate → glutamine, ↓ lysine + built-up α-KG → glutamate, ↑ G3P + glutamate → serine, ↑ late-stage urea cycle arginine → ornithine + urea becoming ↑ CoA synthesis, possible PLP normalisation, ↑ intra-mt portion of malate-aspartate shuttle (delivers NADH to oxphos, consumes glutamate, produces aspartate & α-KG), ↑ asparagine → aspartate + NH₄ (neither combining with glutamate to form glutamine nor fully detoxified by urea cycle, given ↓ mid-cycle arginosuccinate → arginine + fumarate). Here glycolysis-TCA linking via acetyl-CoA formation is apparently not impaired. Increased OA production may suggest that this is facilitated by gluconeogenesis (though OA may also be participating in the citrate shuttle, transporting mt acetyl-CoA into the cytosol), but there are also indications that FA rather than carbohydrates are favoured as the energy-producing substrate (though acetyl-CoA & G3P/serine are simultaneously consumed for lipid synthesis). Again, α-KG may feed into glycolysis/PPP (with TCA likely being replenished by succinyl-CoA from FAO), & arginine consumption may impair NO synthesis. Here, too, ARV therapy corrects many of these (including B-vitamin) abnormalities, but further increases glycolytic driving of the TCA as well as rates of FAO, & although there is enhanced production of NH₄, its detoxification appears impaired (including failure to regenerate arginine). Production of aspartate is also enhanced, and may be utilised by the dicarboxylic acid cycle which helps convert serine-derived pyruvate into glucose (gluconeogenesis); indeed, cellular uptake of monosaccharides is also enhanced.

methionine-SAMe-cysteine-GSH continuum (recall charged folate is also required to regenerate methionine)	↑ methionine → SAMe (ATP/NAD-dep) likely (due to ROS) ↓ homocysteine → methionine likely ↑ (due to ROS) trans-sulfuration to cysteine & thus GSH/GPx/Trx reductase (requiring likely ↓ PLP & vitB5) or H₂S (requiring GSH) becoming ?corrected ability of trans-sulfuration to produce GSH (due to recovery from relative PLP & vitB5 deficiency) & ↑ GSR-mediated regeneration of reduced GSH (i.e. ↓ homocysteine buildup & partial maintenance of GSH levels)	literature suggests ↓ baseline GSH, which (if high ROS) would ↑ homocysteine buildup; high ROS & ↓ PLP would thus further ↓ GSH synthesis becoming ?further ↑ homocysteine (↓ trans-sulfuration to cysteine & thus GSH/GPx/Trx reductase – despite PLP recovery - or H₂S) GSH may become especially limiting: all 3 precursors (glycine, glutamate, likely cysteine) are ↓
H₂S metabolism & cyanide detoxification	attempt at ↑ generation (but GSH likely insufficient) ↓ step 1 (cysteine → pyruvate) ↑ step 2 (SAMe-stimulated homocysteine + cysteine → serine) ↑ steps 3 (aspartate-stimulated cysteine + α-KG → 3MCP) & 4 (GSH/thioredoxin/dihydrolipoic acid-dependent 3MCP → persulfides → H₂S, or sulfate → thiosulfate) ↑ reconversion of thiosulfate to sulphite (also detoxifies cyanide to thiosulfate; detoxification of endogenous cyanide – produced by microbicidal leukocytes/muscarinic cholinergic signalling – can also consume vitamin B12). ↑ H₂S catabolism (quinone-dependently) to thiosulfate and thus (GSH-dependently) to glutathione persulfide can convert thiosulfate back to H₂S/sulphite-thiocyanate becoming: further ↑ H₂S synthesis (if cysteine & GSH/Trx available) & possibly cyanide detox	attempt at ↑ generation (but GSH likely insufficient) ↓ step 1 (cysteine → pyruvate) - step 2 ↑ steps 3 (aspartate-stimulated cysteine + α-KG → 3MCP) & 4 (GSH/thioredoxin/dihydrolipoic acid-dependent 3MCP → persulfides → H₂S, or sulfate → thiosulfate) ↑ H₂S catabolism (quinone-dependently) to thiosulfate and thus (GSH-dependently) to glutathione persulfide appears to undergo thiosulfate buildup in the absence of an enzyme to transfer the thiol group to GSH becoming: further ↑ H₂S catabolism & possibly cyanide detox
Fe & xenobiotic metabolism	↑ intracellular Fe sequestration, ↑ Fe-S cluster & Fe-S protein synthesis (possibly due to Fe-S binding by ISG RSAD2), ↑ xenobiotic metabolism (incl. AhR signalling) becoming ↓ conjugation to GSH for elimination (& ↓ in additional xenobiotic responses)	↑ Fe-S cluster & Fe-S protein synthesis (possibly due to Fe-S binding by ISG RSAD2), ↓ heme → biliverdin, ↑ xenobiotic metabolism (incl. AhR signalling) becoming ↓ conjugation to GSH for elimination & ↓ acetaldehyde breakdown/vitamin A activation by ALDH, but otherwise ongoing xenobiotic responses (e.g. to prevent lipoprotein oxidation & for ↑ heme breakdown/AhR activation), ↑ intracellular Fe sequestration
isoprenoid/sterol metabolism	FA/glycerol/mevalonate/IPP buildup, ↓ protein prenylation/cholesterol/steroid hormone/Q10 synthesis (with attempts at ↑ steroid hormone synthesis/↓ steroid hormone degradation) becoming likely ↓ SREBP-induced sterol synthesis, but de-repressed FPPS relieving buildup & facilitating prenylation & thus RHEB AMPK/mTORC1 activation (though RHEB levels become elevated only in IRIS)	FA/glycerol/mevalonate/IPP buildup, ↓ protein prenylation/cholesterol/steroid hormone/Q10 synthesis; ↑ HDL export, ↓ steroidogenesis becoming ↓ sterol synthesis, but de-repressed FPPS relieving buildup & facilitating prenylation & thus RHEB AMPK/mTORC1 activation
amino acid & protein homeostasis mTORC1 would enhance protein synthesis (ER also relevant to lipid/cholesterol synthesis & xenobiotic detoxification)	altered protein expression & ER architecture/Ca²⁺/glycosylation dynamics, incl. ↓ folding QC, ↑ chaperone activity (esp. IRIS), ↑ UPS flux, de-repressed mTORC1 may ↓ autophagy, with possible compensatory ↑ auto/mitophagy attempts becoming: ↓ ER nascent protein processing, folding QC (TMX4 up-regulation is likely a response to altered redox rather than folding requirement conditions), & glycosylation (suggesting ↓ co-translational translocation OR ER dysfunction; additional findings suggest de-repressed protein translation), ↓ protein insertion into ER membrane (?reversal of ER lumen expansion as lipid biosynthesis and protein-folding requirements decline), but further ↑ chaperones (esp. IRIS), ↑ ubiquitination (& neddylation) of selected targets for UPS-mediated degradation, but ↓ SUMOylation of selected targets, ↓ UPS-mediated degradation of misfolded/fusion proteins (incl. ↓ immunoproteasome activity), likely-↑ secretory protein export from ER (& targeting of abnormally-folded proteins to ERAD), retaining capacity for UPR (incl. ERAD) although apparently no misfolded protein stress, likely-continued AMPK inactivity despite activation attempts, though mTORC1 may be inhibited (e.g. via TXNIP) esp. in CTRL (with decreased likelihood of activation attempts), which may de-repress auto/mitophagy	altered protein expression & ER architecture/Ca²⁺/glycosylation dynamics, incl. ↓ folding QC, ↑ chaperone activity (esp. IRIS), ↑ UPS flux, ↑ repair/degradation of damaged proteins, but ↓ translocation of misfolded/fusion ER proteins for UPS-mediated degradation, de-repressed mTORC1 may ↓ autophagy, with indications of ↓ auto/mitophagy becoming: ↓ parts of ER nascent protein processing & glycosylation (but overall ↑ processing, folding QC, and glycosylation of nascent proteins entering the ER), ↓ protein insertion into ER membrane, but further ↑ chaperones (esp. for proteins entering mt under stress conditions), ↑ ubiquitination (& neddylation) of selected targets for UPS-mediated degradation, ↑ SUMOylation of selected targets, ↑ UPS-mediated degradation of misfolded/fusion proteins, ↑ glycoprotein (& glycolipid) transport (possibly incl. retrograde transport of aberrant cargo) between the ER and Golgi, likely likely ↓ UPR capacity (with possible ↑ ERAD branch), likely-continued AMPK inactivity despite activation attempts, though mTORC1 may be inhibited (e.g. via TXNIP, though activation attempts by farnesylated RHEB are likely), which may partially de-repress auto/mitophagy

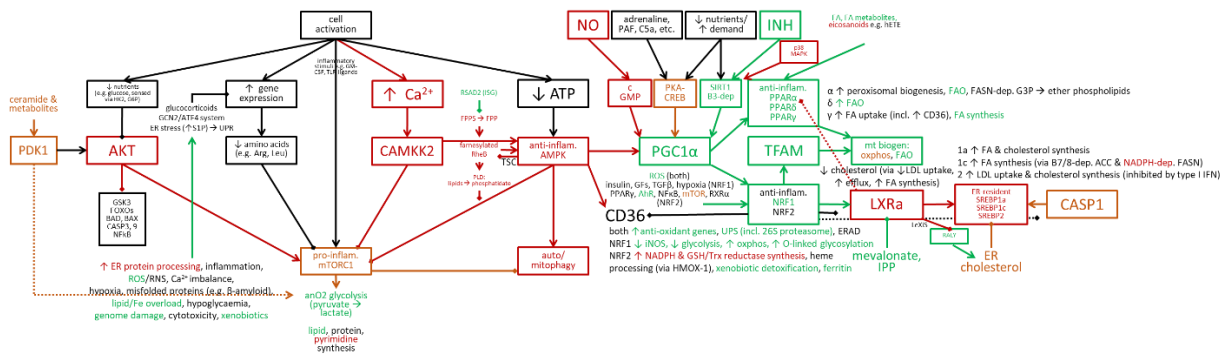


Figure 8a Predicted Neutrophil Metabolic Control Model (CTRL, Baseline)

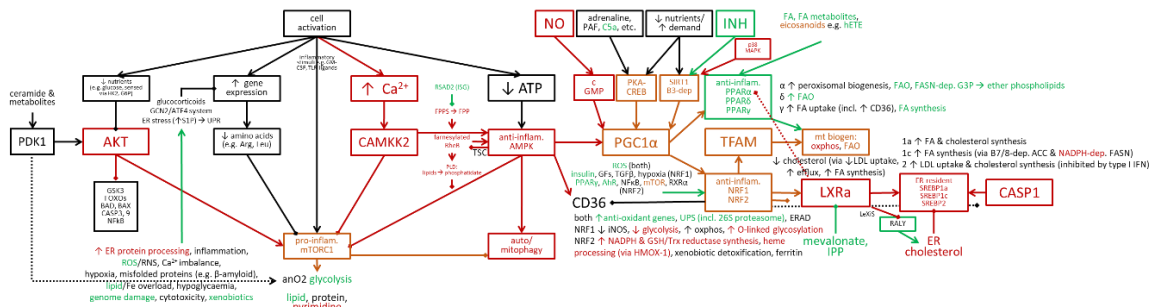


Figure 8b Predicted Neutrophil Metabolic Control Model (IRIS, Baseline)

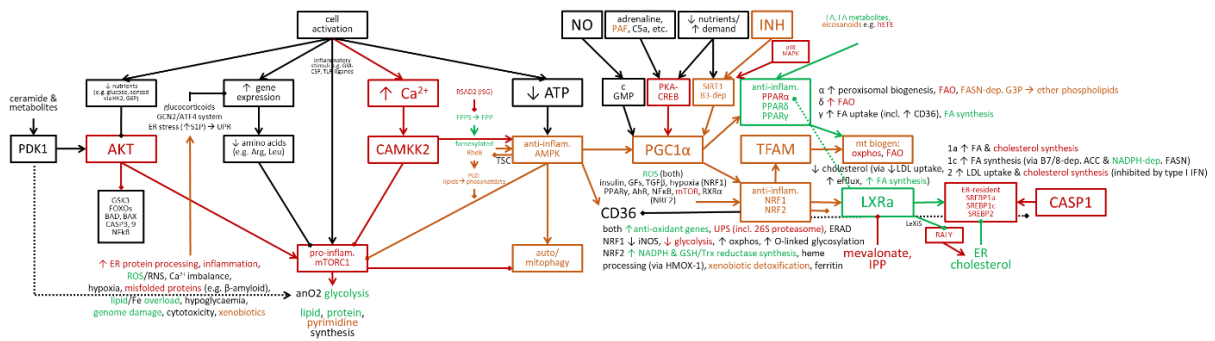


Figure 8c Predicted Neutrophil Metabolic Control Model (CTRL, ARV-exposed)

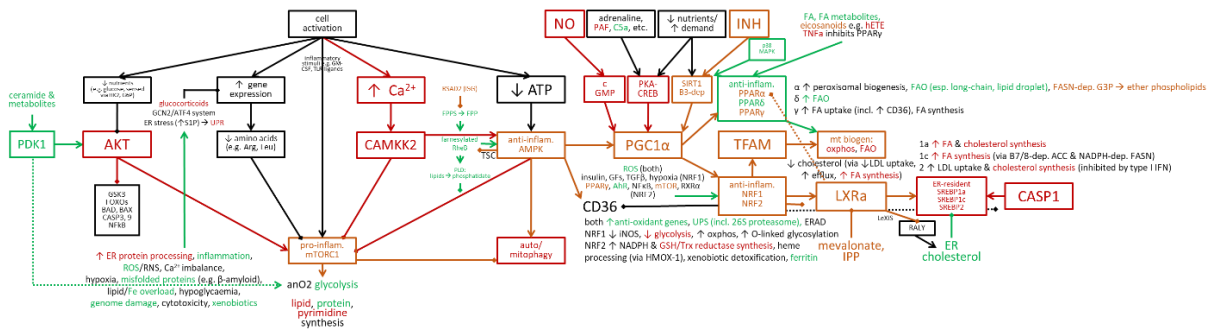


Figure 8d Predicted Neutrophil Metabolic Control Model (IRIS, ARV-exposed)

3.6. Miscellaneous Additional Dysregulated Pathways

Various additional neutrophil proteins whose levels are altered in the CTRL2 and IRIS2 classes belong to pathways modulating cytoskeletal dynamics and vesicle trafficking (including exocytosis/degranulation) (Table 13), as well as chromatin architecture, mRNA/ncRNA processing, and protein translation (Table 14). Indeed, CTRL2 versus IRIS2 changes to these pathways appear quite distinct from each other. However, given their incredible complexity, details of these pathways are beyond the scope of this thesis, but will be addressed in a subsequent research paper in order to maximise data utility.

Table 13 Dysregulated Proteins Relevant to Cytoskeletal Dynamics and Vesicle Trafficking After 2 Weeks' ARV Therapy

Unique to Class		Up-regulated in Class		Absent from Class		Down-regulated in Class	
CTRL2	IRIS2	CTRL2	IRIS2	CTRL2	IRIS2	CTRL2	IRIS2
AHNAK BRK1 CKAP5 DOCK1 EXOC2 LMNB2 MYL1 RIPOR2 SEPT5 SNAP23 SNX1 VPS16	AHNAK ANKFY1 AP1G1 ARF6 ARHGAP15 ARHGAP26 BRK1 CCDC93 CORO1B DOCK1 FAM107B GOLPH3 GRIPAP1 HERC4 HIP1 NUDC NUP155 PACSIN2 PITPNB PSTPIP2 RAB35 RAB5C RAB8A RANGAP1 RAP1GAP2 RIPOR2 RUFY1 SAR1A SIPA1 SNAP23 SNX1 SNX27 STX11 UTRN VPS13C VPS26C VPS29	MYL12A TAGLN2 TWF2 VIM	ARHGFB ARPC2 ARPC5 BIN2 CPNE3 CSE1L DYSF MAPRE1	ADGRG3 AP1S2 AP3M1 ARFGEF1 CORO1B CYTH1 DOP1B DYNC1LI1 EEA1 EXOC8 FAM107B KPNA1 RAB33B STX3 STX11 STXB3 RUFY1 STAG2 TM9SF2 VPS29 VPS35L VTA1	DYNLT1 EXOC2 RDX STX3 TMED4 VPS11	ARF1 COPA ELMO1 FMNL1 KPNB2 NAPA	ACTR1B DCTN1 NAPA

Red text indicates opposite-direction changes. Selected changes in the IRIS2 class further support findings already discussed: unique AP1G1 (associated with MHC-I antigen processing and CTLA4 signalling), unique ARHGAP26 (associated with the integrin pathway), unique FAM107B (stimulates RELA ubiquitination and likely degradation), unique RAB35 and 8A (may help regulate insulin-induced GLUT4 translocation to plasma membrane), unique GOLPH3 (implicated in modulation of mTOR signalling and regulation of mitochondrial lipid biosynthesis), unique GRIPAP1 (facilitates MAPK3K1-mediated activation of JNK1), unique PACSIN2 (activated by PKC and/or CKII for endocytosis); unique VPS13C (necessary for proper mitochondrial function and maintenance of mitochondrial membrane potential; also involved in regulation of PINK1/PRKN-mediated mitophagy in response to mitochondrial depolarisation), unique VPS26C (involved in transport of lysosomal enzyme receptor IGF2R and transcytosis of plgR-plgA), unique VPS29 (involved in GLUT1 endosome-to-membrane trafficking in association with ANKRD27), up-regulated ARPC2 and 5 (which also promote DNA damage repair), up-regulated BIN2 (inhibits phagocytosis), up-regulated CPNE3 (may mediate interactions between integrins and extracellular ligands), up-regulated DYSF (whose increase may suggest Ca²⁺-triggered degranulation), absent TMED4 (involved in the ER stress response and possibly the heat-shock response and apoptosis regulation), and down-regulated ACTR1B and DCTN1 (also associated with MHC-I antigen processing).

Table 14 Dysregulated Proteins Relevant to Chromatin Architecture, RNA Processing, and Translation After 2 Weeks' ARV Therapy

Unique to Class		Up-regulated in Class		Absent from Class		Down-regulated in Class	
CTRL2	IRIS2	CTRL2	IRIS2	CTRL2	IRIS2	CTRL2	IRIS2
*CSRP1 FAM98B HNRNPH2 MECP2 RPS17 UPFI	ARS2 FARSB HAT1 HCFC1 LARS LUC7L2 NAP1L1 PRPF40A RPL13 RPL17 RPL23A RPRD1B RPS20 RPS24 SRSF2 SNRNP70 SMARCE1 SSRP1	EF1A3	DARS EF1A3 HIST1H1B HIST1H1C	EDC4 EIF3E FARSA FBL LUC7L2 NOP56 NOP58 PABPN1 PARP10 PARP10 QARS RPS13 RTCA RTRAF SAFB SNRPD1	PABPN1 PARP10 RPL7A	EIF4A1	H2AFY

Red text indicates an opposite-direction change. Selected changes in the IRIS2 class further support findings already discussed: unique RPRD1B and CSRP1 (associated with formation of HIV elongation complex containing Tat), unique IARS2 (links amino acids with nucleotide triplets in tRNA; thought to be among the first proteins that appeared in evolution), and unique SMARCE1 (associated with the AMPK pathway). *Although the function of LIM protein/transcription factor CSRP1 is largely unknown, it is prominently co-expressed with CSRP2 by smooth muscle cells, as well as in coronary arteries [14]. Together, they mediate serum response factor (SRF) binding of transcription factors GATA4 and GATA6 (implicated in embryonic cardiac development and function, although GATA4 may also “play a role in sphingolipid signalling by regulating the expression of sphingosine-1-phosphate degrading enzyme, sphingosine-1-phosphate lyase”, and GATA6 may also “regulate genes that protect epithelial cells from bacterial infection”). Inhibition of CSRP1 causes abnormal cell movement [14], but FOXO3-mediated transcriptional repression of CSRP1 also promotes longevity [15].

3.7. Head-to-head Comparison Reveals Additional Differences

Findings of the head-to-head comparison have been integrated throughout sections 3.1 through 3.6. For example, on direct comparison of CTRL2 and IRIS2 samples, certain ISGs (e.g. IFIT1, OAS2, GBP4) are unique to the latter class, as are: the rate-limiting enzyme TSTD1, inflammatory PRRs TLR-2 and FcγRI, regulatory receptor SIGLEC5, DNA-repair enzyme PARP1, AhR-enhancing AhR-interacting protein, and caspase 10, among others. Of particular interest, MMP8 levels are higher in IRIS2 than in CTRL2 sample (unadjusted p = 0.003), but while MMP9 levels trend higher, this is non-significant (unadjusted p = 0.171).

4. Integrated Models of CTRL2 & IRIS2 Neutrophil Immunometabolic Differences

Current study findings (as detailed above) are integrated with Chapter 1 literature to predict class-specific neutrophil immunometabolic functional profiles (fig.s 9 and 10).

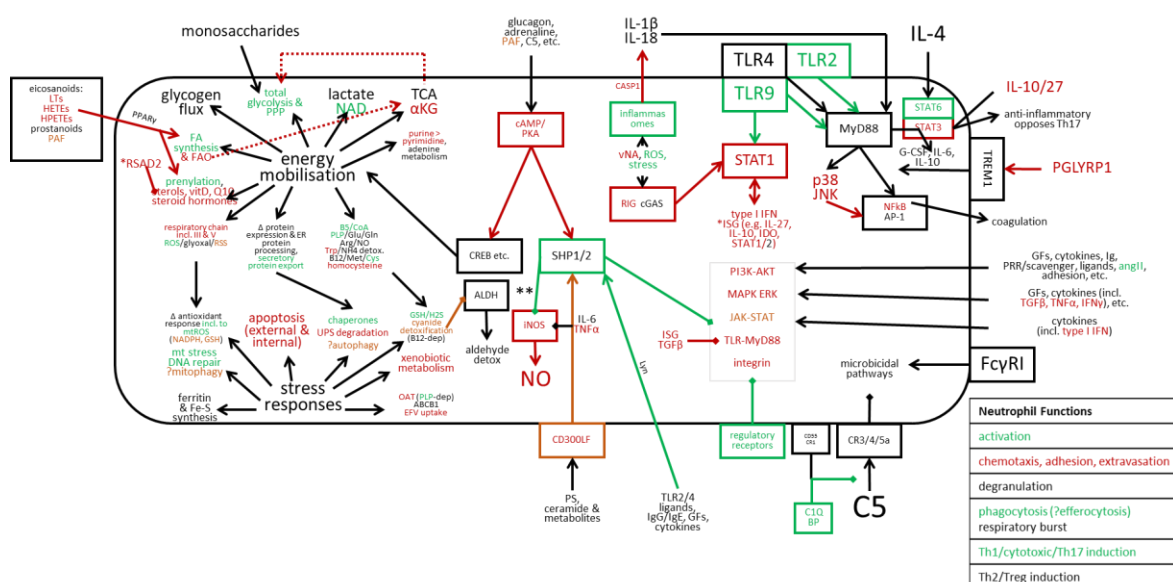


Figure 9 CTRL2 Neutrophil Integrated Functional Profile. Green indicates predicted up-regulation; red indicates predicted down-regulation. Abbreviations: **Lts** leukotrienes, **HETEs** hydroxyeicosatetraenoic acids, **HPETEs** hydroxyperoxyeicosatetraenoic acids, **PAF** platelet activating factor, **TCA** tricarboxylic acid cycle, **FA** fatty acid, **FAO** fatty acid oxidation, **vNA** viral nucleic acids, **vitD** vitamin D, **ROS** reactive oxygen species, **RCS** reactive carbonyl species, **RSS** reactive sulphur species, **ISG** interferon-stimulated genes, **Gf**s growth factors, **Ig** immunoglobulin, **PRR** pattern recognition receptor, **angII** angiotensin II, **GSH** glutathione, **UPS** ubiquitin-proteasome system, **Ag** antigen, **PLP** pyridoxal phosphate, **EFV** efavirenz, **NO** nitric oxide, **PS** phosphatidylserine. All remaining abbreviations are gene/protein names or accepted amino acid or metabolic intermediate/reducing equivalent abbreviations. Double asterisks indicate data deriving from literature rather than from the current study. The single asterisk indicates that RSAD2 is an antiviral ISG. Not pictured: potentially increased glucocorticoid (but decreased sex steroid) sensitivity.

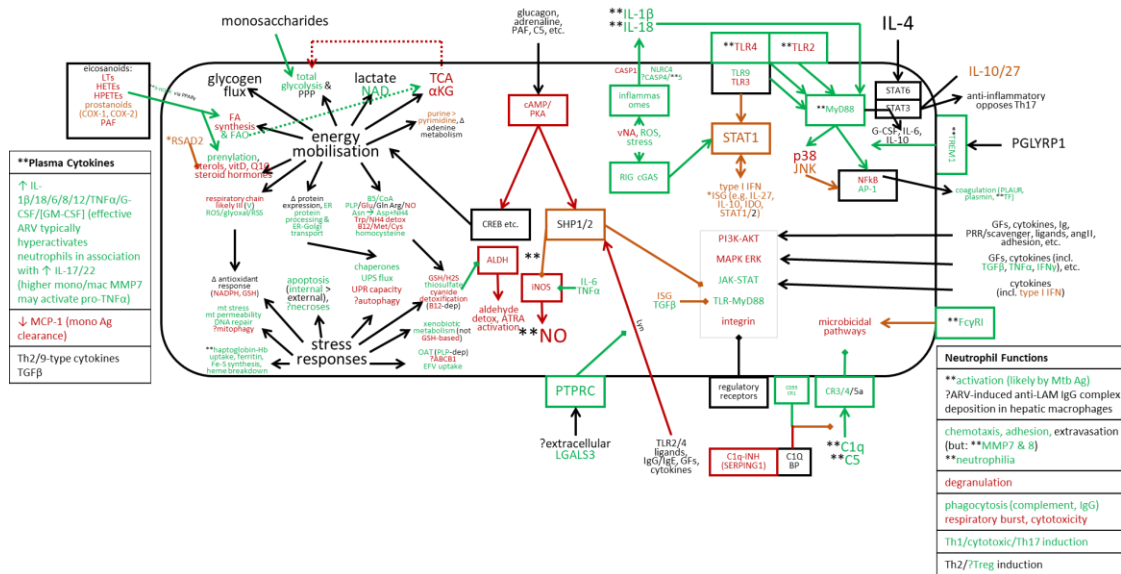
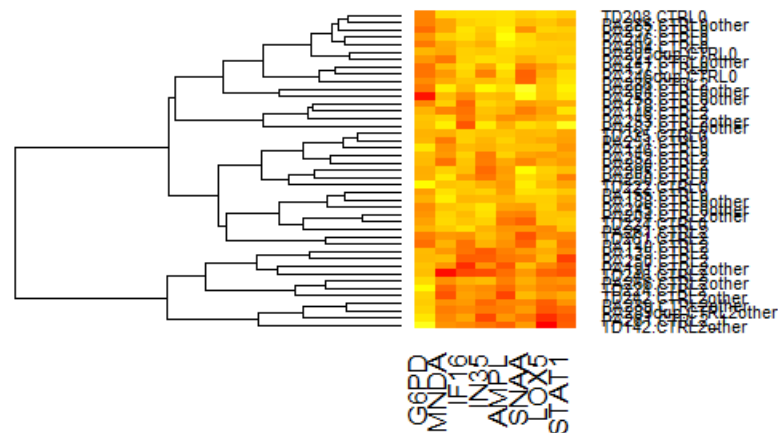


Figure 10 IRIS2 Neutrophil Integrated Functional Profile. Green indicates predicted up-regulation; red indicates predicted down-regulation. Abbreviations: **Lts** leukotrienes, **HETE**s hydroxyeicosatetraenoic acids, **HPETE**s hydroxyperoxyeicosatetraenoic acids, **PAF** platelet activating factor, **TCA** tricarboxylic acid cycle, **FA** fatty acid, **FAO** fatty acid oxidation, **vNA** viral nucleic acids, **vitD** vitamin D, **ROS** reactive oxygen species, **RCS** reactive carbonyl species, **RSS** reactive sulphur species, **ISG** interferon-stimulated genes, **Gf**s growth factors, **Ig** immunoglobulin, **PRR** pattern recognition receptor, **angII** angiotensin II, **GSH** glutathione, **UPS** ubiquitin-proteasome system, **Ag** antigen, **PLP** pyridoxal phosphate, **EFV** efavirenz, **NO** nitric oxide, **PS** phosphatidylserine. All remaining abbreviations are gene/protein names or accepted amino acid or metabolic intermediate/reducing equivalent abbreviations. Double asterisks indicate data deriving from literature rather than from the current study. The single asterisk indicates that RSAD2 is an anti-viral ISG. Not pictured: potentially decreased glucocorticoid (but increased sex steroid) signalling.

5. Biomarker Analysis

5.1. Dataset B: Distinguishing Between Partially ATT-treated Co-infected Patients at Baseline and After Two Weeks' ARV Therapy

As noted above, CTRL0 and CTRL2 neutrophil proteomes differ relatively subtly. Although the RandomForest algorithm identifies a number of SDE proteins able to discriminate between these classes in this cohort, discrimination is poor (fig. 11). These do not represent ISG, which are among the detectable/undetectable rather than SDE proteins. Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. Receiver-operator characteristic (ROC)-mediated performance evaluation for the top four candidate biomarkers (Table 15) is shown in fig. 12 and their suitability as biomarkers is evaluated in Table 16.



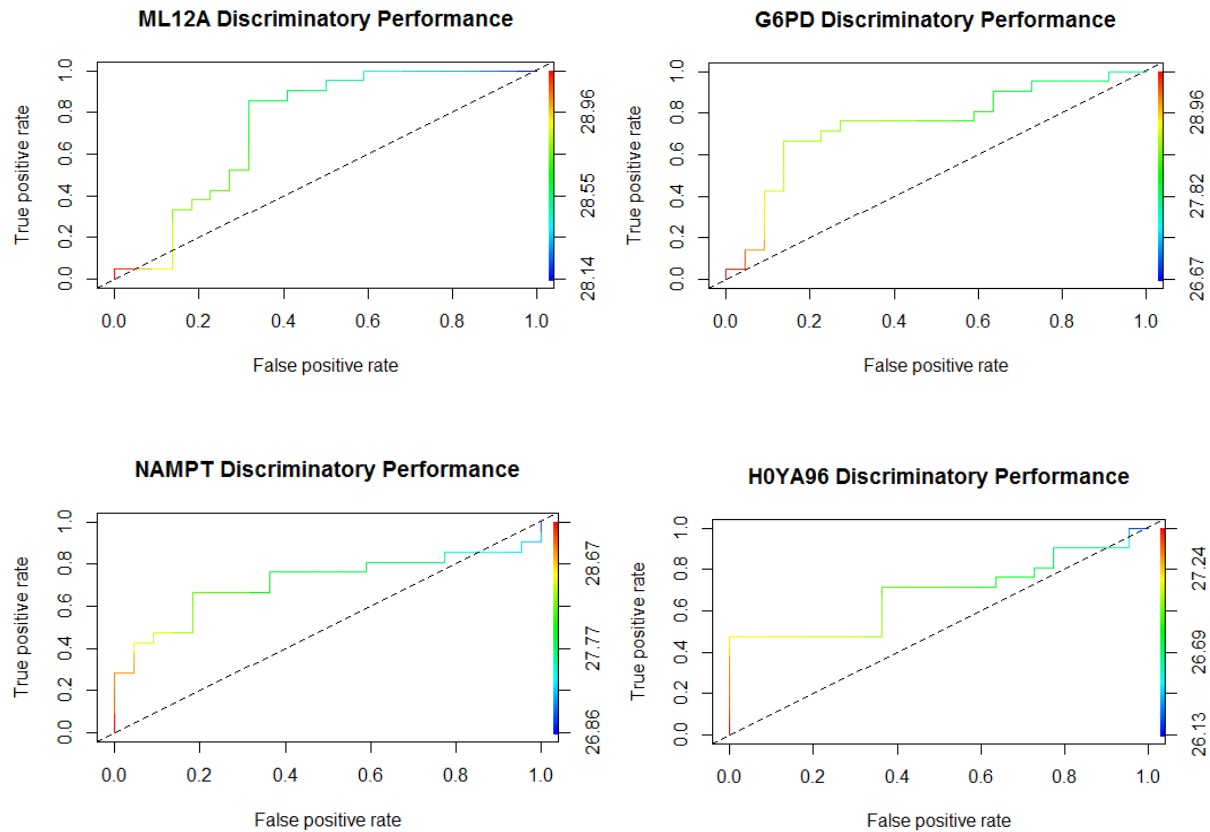


Figure 12 ROC Curves for Top Four Candidate Biomarkers

Table 15 Sensitivity and Specificity of Top Four Candidate Biomarkers

Candidate (UniProt ID)	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
MYL12A (ML12A)	86	68	28.60
G6PD	67	86	28.57
NAMPT	67	82	28.20
HNRNPD (H0YA96)	71	64	26.92

Table 16 Candidate Suitability as Biomarkers

Candidate (UniProt ID)	Function(s)	Available in Blood/ Urine	Association with Other Diseases	Suitable?
MYL12A (ML12A)	nonsarcomeric myosin regulatory light chain may be involved in DNA damage repair de-represses p53-driven apoptosis	both	sepsis cancer dysmegakaryopoiesis	N
G6PD	catalyses first step funnelling G-6-P into PPP major cellular mechanism generating NADPH	both	anemia cancer	Y
NAMPT	rate-limiting enzyme in NAD biosynthesis	both	infectious/inflammatory disease cancer	N
HNRNPD (H0YA96)	mRNA processing	both	oncogenesis	Y

As noted above, IRIS0 and IRIS2 neutrophil proteomes differ more significantly. The RandomForest algorithm identifies a number of SDE proteins able to discriminate relatively well between these classes in this cohort (fig. 13). Again, these do not represent ISG, which are among the detectable/undetectable rather than SDE proteins. Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. Receiver-operator characteristic-mediated performance evaluation for the top four candidate biomarkers (Table 17) is shown in fig. 14 and their suitability as biomarkers is evaluated in Table 18.

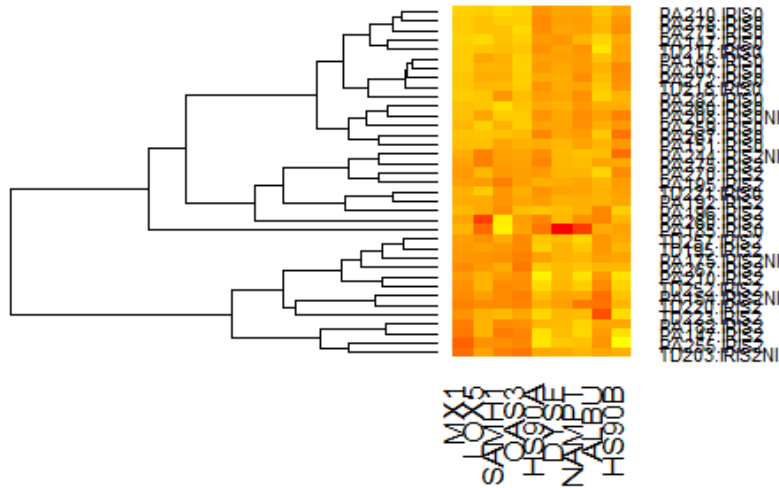


Figure 13 Heatmap Demonstrating IRIS Timepoint Stratification on the Basis of Candidate Biomarkers

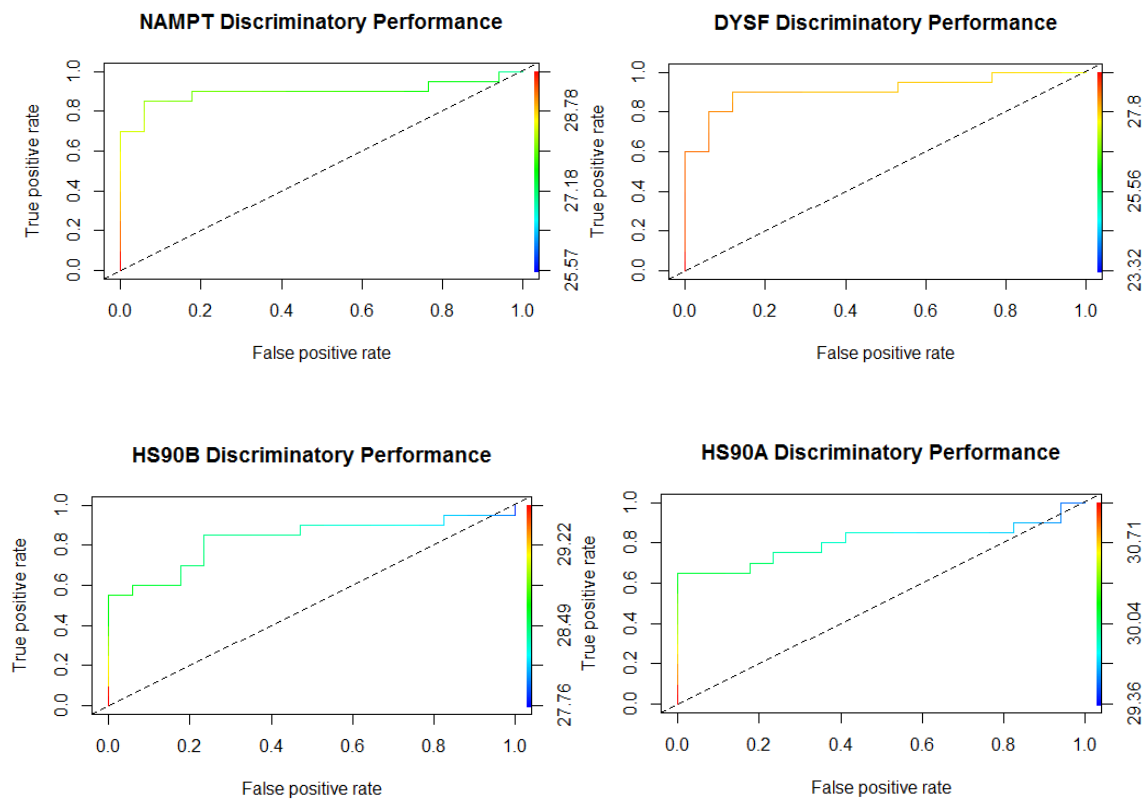


Figure 14 ROC Curves for Top Four Candidate Biomarkers

Table 17 Sensitivity and Specificity of Top Four Candidate Biomarkers			
Candidate (UniProt ID)	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
NAMPT	85	94	28.10
DYSF	90	88	28.01
HSP90AB1 (HS90B)	85	76	28.44
HSP90AA1 (HS90A)	75	76	29.95

Table 18 Candidate Suitability as Biomarkers

Candidate (UniProt ID)	Function	Available in Blood/Urine	Association with Other Diseases	Suitable?
NAMPT	rate-limiting enzyme in NAD biosynthesis	both	infectious/inflammatory disease cancer	N
DYSF	membrane fusion and resealing	both	muscular dystrophies	Y
HSP90AB1 (HS90B)	constitutive chaperone regulates transcription prevents SMAD3 ubiquitination and thus TGF β signaling inhibition main chaperone involved in phosphorylation (activation) of STAT1 during heat shock	both	cancer	Y
HSP90AA1 (HS90A)	inducible chaperone which regulates transcription binds LPS and mediates LPS-induced inflammation (incl. TNF secretion) prevents SMAD3 ubiquitination and thus TGF β signaling inhibition	both	sepsis cancer	N

Note that proteins up-regulated during inflammation or cellular transformation may reflect general cellular stress responses, and may thus be unsuitable as biomarkers.

5.2. Dataset B: Distinguishing Between ARV-exposed Patients Who Do and Do Not Develop TB-IRIS

As noted above, CTRL2 and IRIS2 neutrophil proteomes share some similarities (though they are not as similar as the CTRL0 and IRIS0 neutrophil proteomes), likely due in part to common changes induced by ARV therapy, but are also significantly different (fig. 15). Together, the first two components explain 42.4% of variance in the dataset.

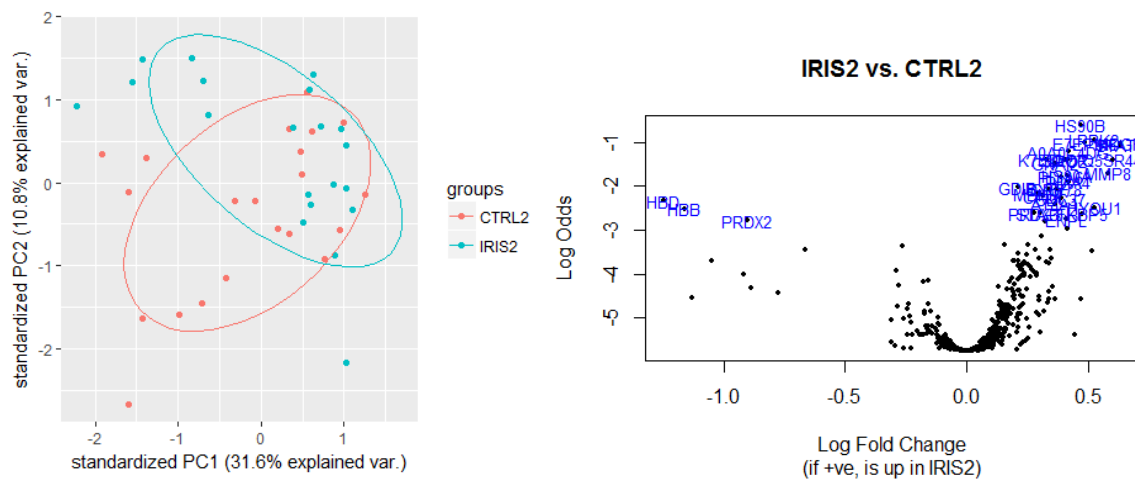


Figure 14 PCA Biplot and Volcano Plot Demonstrating Between-class Differences

The RandomForest algorithm identifies a number of SDE proteins able to discriminate between these classes in this cohort, but this discrimination is poor (fig. 15). Combining candidate biomarkers into a panel may improve discriminatory performance, however. Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. ROC-mediated performance evaluation for the top six candidate biomarkers (Table 19) is shown in fig. 17 and their suitability as biomarkers is evaluated in Table 20.

	mitogenic, transcriptional repressor			
CSE1L (XPO2)	returns importin- α from nucleus to cytoplasm after cargo import	both	cancer	Y
RAB31	roles in vesicle trafficking e.g. insulin-stimulated expression of GLUT4, internalization of EGFR, maturation of phagosome after Mtb uptake	blood	cancer	Y

All above biomarker candidates take into account only *quantifiable* proteins identifiable between the classes being compared. However, differentially *detectable/undetectable* proteins between classes may represent a more powerful way than SDE proteins to discriminate between classes. Furthermore, candidate biomarkers which discriminate between the CTRL2 and IRIS2 classes in a head-to-head comparison may be less useful for risk stratification than the differences arising between baseline and CTRL2 or IRIS2, especially given the significant proteome changes occurring between the IRIS0 and IRIS2 classes.

5.3. Dataset A: Distinguishing Between Partially ATT-treated Co-infected Patients at Baseline and After Two Weeks' ARV Therapy

As noted above, CTRL0 and CTRL2 neutrophil proteomes differ relatively subtly. The RandomForest algorithm identifies a number of SDE able to discriminate reasonably well between these classes in this cohort (fig. 18). These do not represent ISG, which are among the detectable/undetectable rather than SDE proteins. Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. Receiver-operator characteristic-mediated performance evaluation for the top three candidate biomarkers (Table 20) is shown in fig. 19 and their biomarker suitability is evaluated in Table 21.

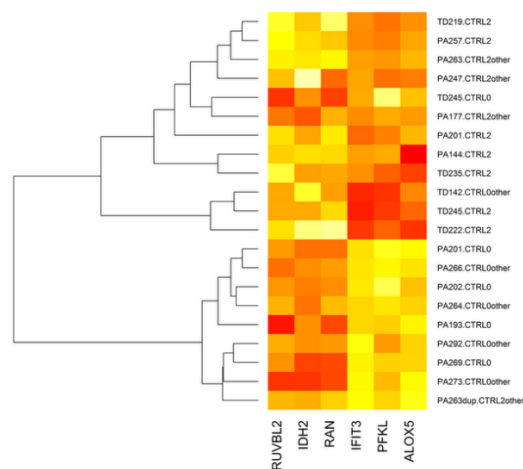


Figure 18 Heatmap Demonstrating Class Stratification on the Basis of Candidate Biomarkers

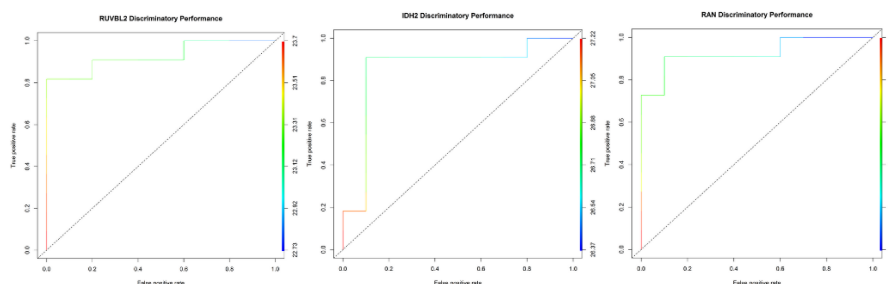


Figure 19 ROC Curves for Top Three Candidate Biomarkers

Table 20 Sensitivity and Specificity of Top Three Candidate Biomarkers

Candidate	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
RUVBL2	82	100	23.33
IDH2	91	90	26.71
RAN	91	90	26.97

Table 21 Candidate Suitability as Biomarkers

Candidate	Function	Available in Blood/ Urine	Association with Other Diseases	Suitable?
RUVBL2	chromatin remodelling	blood	cancer	Y
IDH2	mitochondrial citrate dehydrogenase	urine	neoplasia/cancer	Y
RAN	nuclear import/export	urine	neoplasia/cancer	Y

As noted above, IRIS0 and IRIS2 neutrophil proteomes differ significantly. The RandomForest algorithm identifies a number of SDE proteins able to discriminate well between these classes in this cohort (fig. 20). Again, these do not represent ISG, which are among the detectable/undetectable rather than SDE proteins. Discriminatory capacity of these candidate biomarkers must be validated in an independent cohort. Receiver-operator characteristic-mediated performance evaluation for the top three candidate biomarkers (Table 21) is shown in fig. 21 and their biomarker suitability is evaluated in Table 22.

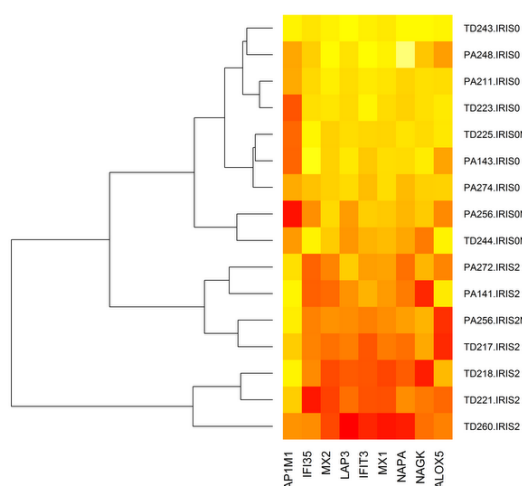


Figure 20 Heatmap Demonstrating Class Stratification on the Basis of Candidate Biomarkers

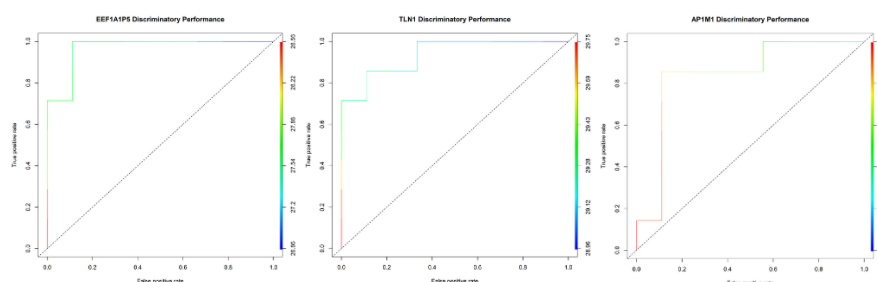


Figure 21 ROC Curves for Top Three Candidate Biomarkers

Table 21 Sensitivity and Specificity of Top Three Candidate Biomarkers

Candidate	Sensitivity (%)	Specificity (%)	Cut-off (log2LFQ)
EEF1A1P5	100	89	27.55
TLN1	86	89	29.23
AP1M1	86	89	22.90

Table 22 Candidate Suitability as Biomarkers

Candidate	Function	Available in Blood/ Urine	Association with Other Diseases	Suitable?
EEF1A1P5	loads tRNA into ribosomal A-site	blood (mRNA only)	none apparent	Y
TLN1	concentrates under contact sites	both	hepatocellular carcinoma	Y
AP1M1	component of Golgi AP-1 complex	blood (mRNA only)	TNBC tumour metastasis to CNS, Alzheimer's Disease, breast cancer progression	Y

It is notable that the identities of candidate biomarkers derived from datasets A versus B are different. As discussed in Chapter 3, dataset A is much smaller than dataset B, and therefore a less reliable source for identification of candidate biomarkers. However, dataset A also includes fewer "other"-designated CTRL samples and fewer non-INSHI-designated IRIS samples, and therefore may be a *more* reliable source for identification of truly discriminatory candidate biomarkers. Future work will evaluate the ability of biomarkers identified in one dataset to discriminate classes in the other dataset.

Appendix K Per-comparison Estimated Protein Fold-changes and Statistical Significance

Table 1 CTRL2 Changes Relative to CTRL0

Proteins	logFC	FC	Unadj. p-value	Adj. p-value
STAT1	-0,80403	0,572749	0.000138609396131418	0.0120590174634333
IN35	-0,65909	0,633276	0.000945360959544616	0.0685386695669846
MNDA	-0,33412	0,79327	0.00282544850930482	0.175581443078228
TCPE	0,455754	1,3715	0.00369950799851246	0.190895266409182
EF1A3	0,498621	1,412863	0.00448961816904344	0.190895266409182
PSME1	-0,27078	0,828873	0.00474649273415455	0.190895266409182
PSME2	-0,37582	0,770666	0.00529480936427938	0.190895266409182
RS3	-0,35927	0,779561	0.00566260735981889	0.190895266409182
PKN1	-0,33552	0,792498	0.00620318836962919	0.190895266409182
DHB4	-0,3808	0,768011	0.0062955391026597	0.190895266409182
STAT3	-0,31094	0,806117	0.00658259539342008	0.190895266409182
ML12A	0,197537	1,146739	0.00779830947323805	0.203554165155188
DJC13	-0,33593	0,792275	0.00795499036238667	0.203554165155188
ELMO1	-0,26788	0,830542	0.0100106256181754	0.241923452439238
G6PD	0,393824	1,313871	0.0113451490920768	0.259744202897549
ILEU	-0,23881	0,847445	0.014084708588529	0.30482664967684
A0A0C4DGS1	-0,32482	0,798398	0.014715769294744	0.30482664967684
VIME	0,225	1,168777	0.0169684909263698	0.321039990122611
HNRPU	-0,28048	0,823318	0.0182733225029858	0.321039990122611
CNDP2	-0,3179	0,802238	0.0188673015443481	0.321039990122611
TNPO1	-0,25635	0,837204	0.0200438409994391	0.321039990122611
TWF2	0,352208	1,276513	0.0200822219104741	0.321039990122611
PSA6	-0,35819	0,780142	0.0210449404352733	0.321039990122611
COPA	-0,25687	0,8369	0.0213390159151872	0.321039990122611
HNRPQ	-0,28966	0,818092	0.0214026660081741	0.321039990122611
NAMPT	0,350612	1,275102	0.0281341220698314	0.405262430833088
X6RJP6	0,386782	1,307473	0.0291393112249079	0.405262430833088
CALR	-0,31876	0,801761	0.030927519678017	0.405262430833088
TPP2	0,221322	1,165801	0.0319916444262483	0.405262430833088
E9PFN5	-0,24514	0,843733	0.0328469558074755	0.405262430833088
CLIC1	-0,20657	0,866594	0.0340056677075979	0.405262430833088
A0A024R617	-0,2432	0,844869	0.0348264102228087	0.405262430833088
ARF1	-0,18458	0,879903	0.0352988947059109	0.405262430833088
FMNL1	-0,214	0,862142	0.0354022353371433	0.405262430833088
AT1A1	-0,23399	0,850282	0.0365609357963807	0.407795053113477
PDIA6	-0,28205	0,822421	0.0377118770408037	0.408688091406562
XRCC6	-0,17881	0,883432	0.0410229238130695	0.408688091406562
NAGK	-0,24972	0,841058	0.0421815045071032	0.408688091406562
PDIA4	-0,29549	0,814796	0.0423604504606988	0.408688091406562
PRDX3	-0,23201	0,851448	0.0434319615081392	0.408688091406562
HDGF	-0,40235	0,756626	0.0436018490270819	0.408688091406562
H4	-0,22779	0,853943	0.0439215617757002	0.408688091406562
A0A087X0X3	-0,24061	0,846386	0.0441571041289848	0.408688091406562

F8W1A4	-0,26899	0,8299	0.0480172290136179	0.428346594457049
GSHR	0,330219	1,257205	0.0484354420586859	0.428346594457049
IF4A1	-0,1999	0,87061	0.0497586489380901	0.428346594457049
AMPL	-1,20021	0,435213	2.3464635016563e-06	0.0008522646953916
LOX5	-0,98294	0,505947	3.91845836961655e-06	0.0008522646953916
SNAA	-0,50146	0,706392	6.38493587108935e-05	0.00925815701307956
IF16	-0,83749	0,559617	8.59357888603609e-05	0.00934551703856425

Table 2 IRIS2 Changes Relative to IRIS0

Proteins	logFC	FC	Unadj. p-value	Adj. p-value
FKBP5	0,648886	1,567957	0.000272879978093183	0.016879575787764
HS90A	0,451421	1,367387	0.000349941544573922	0.0189405861000635
PSA6	-0,30311	0,810504	0.000531529574643143	0.0255724784244979
LOX5	-0,65311	0,635907	0.000633933487959915	0.0274493200286643
STAT1	-0,47226	0,720837	0.00073018610691638	0.0287427803904357
MARCS	1,030241	2,042365	0.00144577216050691	0.0490503410050385
SNAA	-0,62906	0,646597	0.00155038800799741	0.0490503410050385
IN35	-0,5833	0,667433	0.00158592326575182	0.0490503410050385
MMP8	0,661073	1,581258	0.00196514248289006	0.0567271130060932
HS90B	0,364516	1,28745	0.00210813771226482	0.0570514768381666
PRDX2	-1,12973	0,457	0.00226147564120478	0.0576011148612747
BGLR	-0,56005	0,67828	0.00283502882688197	0.0657668077401089
SH3L1	-0,54751	0,684199	0.0028858414481803	0.0657668077401089
ACLY	-0,26688	0,831117	0.00424839203773524	0.0919776876169679
PRDX6	-0,28418	0,82121	0.00467807222154689	0.0964573939014192
IL16	-0,40612	0,754652	0.00498259092753963	0.0980664487102119
GDIR2	0,545306	1,459329	0.00591673974211992	0.111378909290814
B3AT	-1,39791	0,379479	0.00617342684290887	0.111378909290814
DYSF	0,70218	1,626961	0.00700606686243539	0.121345078057381
PGM2	0,669114	1,590096	0.00800133118630098	0.133252938602628
RETN	1,237883	2,358522	0.00895675078855966	0.135717316090069
A0A087WUQ6	-0,49285	0,710622	0.00937068025385809	0.135717316090069
MOR192	-0,73205	0,602049	0.00959660365326005	0.135717316090069
SYDC	0,485406	1,39998	0.00994047583303499	0.135717316090069
DCTN2	-0,35785	0,780327	0.010148505577348	0.135717316090069
A0A0A0MTR7	-0,30597	0,808899	0.0103415166645218	0.135717316090069
CPNE3	0,353257	1,277441	0.010343352034578	0.135717316090069
E9PFN5	-0,25233	0,839539	0.0115956473660479	0.143809634407793
MMP9	0,590202	1,505457	0.0116243353447408	0.143809634407793
EF1A3	0,445424	1,361714	0.0130442498321512	0.156893338258929
PEBP1	-0,29269	0,816376	0.0147658587988864	0.170733577673165
K7ELL7	0,264237	1,201001	0.015927756075989	0.170733577673165
X6R433	0,217183	1,162462	0.0161834478792234	0.170733577673165
CDC37	0,330532	1,257477	0.0164140154374711	0.170733577673165
SPTB1	-1,69559	0,308728	0.0166260242163229	0.170733577673165
H12	1,835217	3,568252	0.0167481197373403	0.170733577673165
LMAN1	0,312609	1,241952	0.0169550666049564	0.170733577673165

PDC6I	-0,32598	0,797759	0.0176283379953806	0.171316885754411
S10A8	1,118915	2,171836	0.0178042952862551	0.171316885754411
A0A0C4DGS1	0,306489	1,236694	0.0194615378802437	0.183192302220555
CATC	-0,39484	0,760571	0.02069926482339	0.185466884123698
DOPD	-0,35944	0,779467	0.0210082167133674	0.185466884123698
SPTB2	-0,16837	0,88985	0.0210291988873423	0.185466884123698
HBA	-1,05595	0,48098	0.0220495826586205	0.185466884123698
PGM1	-0,27158	0,828411	0.0222065737335905	0.185466884123698
ANXA3	0,365324	1,288171	0.0224207633466801	0.185466884123698
C1TC	-0,26673	0,831204	0.0227014892807297	0.185466884123698
APMAP	0,301357	1,232303	0.0232082215665041	0.186095554412894
H15	1,727728	3,312058	0.0242189340187211	0.190669062365568
SPTA1	-1,21595	0,430489	0.0251472962376037	0.194442486980043
H2AY	-0,29426	0,815489	0.0272906545057015	0.207313217560855
CH60	0,331459	1,258285	0.0289395502902418	0.215711080526111
GBP5	0,761946	1,695777	0.0293925028892391	0.215711080526111
GNAI2	0,246298	1,186159	0.0302452625424129	0.218269978014413
ARPC5	0,826695	1,773617	0.0307661909293774	0.218389519220007
ACTY	-0,27662	0,825523	0.0325383985247139	0.221023223132924
S10AB	-0,57739	0,670176	0.032960300346139	0.221023223132924
XPO2	0,221255	1,165748	0.0329758148687139	0.221023223132924
CAND1	-0,27482	0,826553	0.0335862890134593	0.221023223132924
NAGK	-0,46564	0,724151	0.0336894520248799	0.221023223132924
MARE1	0,222165	1,166483	0.0354787771151995	0.226145098260109
PPIA	0,617422	1,534132	0.0355147036528577	0.226145098260109
ENPL	0,28109	1,215112	0.0372970626264543	0.234052581409488
APOBR	0,27308	1,208385	0.0388115020585938	0.240076862733873
CLIC1	-0,26438	0,832559	0.0398584687756375	0.243080520842973
HBB	-0,85434	0,55312	0.0417704209773087	0.251202670599648
CALX	0,318857	1,247342	0.0424994600147558	0.252085838169716
F5H0W4	0,39493	1,314879	0.0445279099958414	0.259186762485049
HSP74	0,1581	1,115817	0.0448937810308977	0.259186762485049
GTR3	0,288701	1,22154	0.0460118085336243	0.260241257222184
Q32Q12	0,841793	1,792276	0.0462784683743838	0.260241257222184
C9JGI3	-0,86197	0,550201	0.0492580279761085	0.27110018209441
ARPC2	1,539832	2,907606	0.0497688318985684	0.27110018209441
AMPL	-1,32781	0,398372	1.07376351318582e-08	1.5497986706982e-06
OAS3	-2,89058	0,134849	1.20369106428999e-11	5.21198230837566e-09
SAMH1	-0,99174	0,50287	3.60284349445251e-05	0.00390007808274484
MX1	-3,80506	0,071542	5.62555776047357e-11	1.21793325514253e-08
NAMPT	0,848401	1,800504	5.6456642565529e-05	0.00488914524617481
ALBU	-0,79096	0,577958	8.60166640110917e-05	0.00620753591946712

Table 3 IRIS2 Changes Relative to CTRL2

Proteins	logFC	FC	Unadj. p-value	Adj. p-value
HS90B	0,472747	1,387749	0.000816142567173899	0.0923231315825385
LRRK2	0,525299	1,439232	0.00122820917025523	0.0923231315825385

E7EPM6	0,487037	1,401564	0.00132834650801012	0.0923231315825385
HDGF	0,634501	1,552401	0.00135033480567164	0.0923231315825385
STAT1	0,630635	1,548247	0.00139060959825254	0.0923231315825385
A0A0C4DGS1	0,422381	1,340137	0.00169496806919218	0.0923231315825385
K7ELL7	0,329793	1,256833	0.00208712034951308	0.0923231315825385
Q5SR44	0,600196	1,515923	0.00212399971103137	0.0923231315825385
SQOR	0,405268	1,324335	0.00212734848867836	0.0923231315825385
XPO2	0,417263	1,335392	0.00214970588206185	0.0923231315825385
GNAI2	0,362859	1,285972	0.00233460792507568	0.0923231315825385
MMP8	0,58025	1,495109	0.00303327360075865	0.10412633110211
HS90A	0,417533	1,335642	0.00322423435224248	0.10412633110211
PDIA6	0,391306	1,311581	0.00335119226535525	0.10412633110211
PDIA4	0,41972	1,337668	0.00381587056136309	0.11066024627953
GDIB	0,210052	1,15673	0.004481492012709	0.11462500667728
DYSF	0,337693	1,263734	0.00469344414990083	0.11462500667728
GRP78	0,369125	1,291569	0.00474310372457711	0.11462500667728
MDHM	0,292221	1,224524	0.00529421649777678	0.121209693501731
CALX	0,312481	1,241841	0.00569160617770106	0.123792434364998
CDC37	0,392246	1,312435	0.00610254322312095	0.126409823907505
HBD	-1,24529	0,421823	0.00653916047996151	0.129297036762875
AT8A1	0,366909	1,289587	0.00713012563530166	0.134852376145923
HYOU1	0,52675	1,44068	0.00780230238476156	0.141416730723803
HBB	-1,16128	0,447117	0.00814994422270163	0.141809029475008
PRDX3	0,279713	1,213953	0.009093403472763	0.143852076606786
STAT3	0,305918	1,236205	0.00918843730222966	0.143852076606786
FKBP5	0,475316	1,390223	0.00925944401147126	0.143852076606786
ENPL	0,412575	1,33106	0.0108464298515767	0.161957836127251
PRDX2	-0,899	0,536258	0.0113032846022368	0.161957836127251
DJC13	0,323769	1,251596	0.0115418228044707	0.161957836127251
BST1	0,416261	1,334464	0.0142916871241042	0.194277621843292
RAB31	0,310307	1,239972	0.0173289895001034	0.228427588865
HSP7C	0,251461	1,190412	0.0220622028844857	0.279339582440563
HSP74	0,158519	1,116141	0.0226889533874549	0.279339582440563
TBA1B	-0,2625	0,833642	0.0231177585468052	0.279339582440563
HNRPQ	0,341753	1,267296	0.025870261380845	0.291197693230701
MOR192	-0,66308	0,631528	0.0260924293839545	0.291197693230701
CEAM1	0,284442	1,217939	0.0261073793930974	0.291197693230701
E7ER45	0,51594	1,429926	0.0268766318955943	0.292283371864588
COPA	0,21259	1,158766	0.028822047101886	0.303959907658463
AT1A1	0,249633	1,188905	0.0293478531532309	0.303959907658463
ATPA	0,296353	1,228036	0.0330010904142366	0.312371991324244
GDIR2	0,351612	1,275985	0.0332552554749465	0.312371991324244
HBA	-1,04678	0,484049	0.0352139320374444	0.312371991324244
EF1G	0,21112	1,157587	0.0355769149786426	0.312371991324244
XRCC6	0,19471	1,144494	0.0357799818866509	0.312371991324244
COPB	0,199384	1,148208	0.0359604197450182	0.312371991324244
ITB2	0,239095	1,180252	0.0367357120692135	0.312371991324244
CD97	0,326928	1,254339	0.0370986204742539	0.312371991324244

GANAB	0,231278	1,173875	0.0381073470858219	0.312371991324244
CH60	0,340904	1,26655	0.038638909274558	0.312371991324244
H3BQZ7	0,230296	1,173076	0.0386817044922863	0.312371991324244
RS3	0,2553	1,193584	0.0390538151456682	0.312371991324244
HNRPU	0,233468	1,175658	0.0394953092478929	0.312371991324244
TNPO1	0,21365	1,159618	0.0409745295439881	0.318284291993479
A0A087X0X3	0,263721	1,200572	0.0423305563884624	0.323048982964582
MNDA	0,226001	1,169588	0.0446864925067128	0.331221952369716
VAPA	0,268425	1,204492	0.0449243567581914	0.331221952369716
ILF3	0,223004	1,167161	0.045946191621449	0.333109889255505
NAGK	-0,28858	0,818708	0.0477202606945271	0.337589078974099
NLRC4	0,278429	1,212874	0.0481161445894119	0.337589078974099

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Chapter 6

Partial Hypothesis Validation via Live-cell Fluorescence Microscopy:

Efavirenz Alters Neutrophil Organelle Morphology

"Equipped with our five senses – along with telescopes and microscopes and mass spectrometers and ... detectors sensitive to the entire electromagnetic spectrum – we explore the universe around us and call the adventure science."

- Edwin Hubble

Abstract

Background Discovery proteomic data generated from TB-HIV co-infected patient-derived neutrophils (Chapter 5) suggests that two weeks of antiretroviral (ARV) therapy dysregulates mitochondrial and protein-folding homeostasis in both controls and TB-IRIS cases. These features also differentiate cases from controls prior to and during ARV therapy, suggesting that case-derived neutrophils are more vulnerable to ARV-induced perturbation. Efavirenz (EFV) is the basis of South Africa's first-line ARV regimen. EFV significantly alters function (including mitochondrial) of various cell types, but its impact on neutrophils is unknown. Neutrophils possess a complex mitochondrial network, and oxidative phosphorylation accounts for a dynamic proportion of ATP production, dependent on functional context. Mitochondrial dysfunction compromises generation of ATP (required by multiple processes, including protein folding) and metabolic reducing equivalents (required for macronutrient metabolism and anti-oxidant capacity), while increasing generation of reactive oxygen species. Indeed, EFV (especially in combination with INH) is associated with induction of oxidative stress. It is also known that EFV induces activation of both mitochondrial and endoplasmic reticulum (ER) adaptive stress responses (including autophagy and – in cases of supra-maximal stress - cell death). Furthermore, *Mycobacterium tuberculosis* (*Mtb*) and host responses to it (e.g. IFN γ) also induce mitochondrial and ER stress responses. **Methods** We therefore examined the effects of EFV on live *ex vivo* neutrophils derived from healthy (TB-HIV-) human volunteers, using fluorescence microscopy. Neutrophils - in the presence of autologous peripheral blood mononuclear cells (PBMC) and plasma (i.e. approximating *in vivo* immunological conditions) - were stained for mitochondria and lysosomes (using probes which, in combination, can detect mitophagy), as well as for misfolded proteins (as a proxy for ER stress). Neutrophils were then exposed to *in vivo*-comparable concentrations of EFV $\pm$ whole *Mtb* H37Rv lysate. **Results** In agreement with proteomic data-driven hypotheses and the literature, EFV rapidly altered architecture of mitochondria, lysosomes, and aggresomes in a manner suggesting mitochondrial, lysosomal, and protein folding stress (with modulation of such changes by pre-exposure to *Mtb* antigens), as well as suggesting commitment to cell death. **Conclusions** Additional investigations are required to confirm the exact nature of EFV-induced organellar stress responses in neutrophils, and to determine whether these responses initiate positive feedback inflammatory cycles which may contribute to TB-IRIS pathogenesis. To the best of our knowledge, this is the first study investigating the effects of EFV on neutrophils, and the first study investigating the effects of EFV on lysosomes.

6.1. Background

South Africa employs an efavirenz (EFV)-based first-line antiretroviral (ARV) therapy regimen [1]. Indeed, the regimen utilised by clinical studies from which the current cohort originates consisted of EFV, emtricitabine (FTC), and tenofovir (TDF). Although not statistically significant, it is interesting to note that of three cohort participants receiving an EFV-lacking and one receiving a TDF-lacking alternate ARV regimen, none developed TB-IRIS (Chapter 2).

First-line ARVs activate general cellular stress responses [2] and dysregulate many cellular processes (including mitochondrial [3] [4] [5] [6], redox homeostasis [7], phagocytosis, and amyloid- β production [8]). Such alterations have been demonstrated in various cell types (e.g. hepatocytes, renal epithelia, and neurons), but ARV effects on leukocytes (especially neutrophils) are less well known. Specific effects of EFV on various cell types include induction of mitochondrial dysfunction, oxidative stress (especially in combination with isoniazid [INH]), altered Ca²⁺ homeostasis and autophagic flux, endoplasmic reticulum (ER) stress, elevated pro-inflammatory cytokine levels, and CB1 cannabinoid receptor interference [9] [10] [11] [2] [4] [5] [12].

6.1.1. Mitochondria & EFV

The major cellular ATP source is mitochondrial oxidative phosphorylation (oxphos) [13] [14], which also generates metabolic reducing equivalents. Neutrophils exhibit a complex mitochondrial network and likely dynamically alter the glycolytic:oxphos energy metabolism ratio, depending on functional state requirements [15]. Multiple cellular functions (including protein folding) require ATP, and both cellular macronutrient metabolism and antioxidant capacity depend on availability of reducing equivalents (the latter also depends on availability of reduced glutathione [GSH]).

Mitochondrial functions other than energy metabolism include roles in intracellular signalling, Ca^{2+} and redox homeostasis, autophagy, cell death, and innate immunity [16] [17] [18] [19] [20]. For example, intracellular signalling molecules of mitochondrial origin include " Ca^{2+} , reactive oxygen species (ROS), pro-apoptotic molecules, ATP/ADP/AMP," and reducing equivalents (NAD^+/NADH) [21] [22] [23]. During normal cellular activation, signal transduction-increased cytoplasmic Ca^{2+} is taken up by mitochondria, and higher mitochondrial matrix Ca^{2+} levels enhance oxphos-mediated ATP production to support the increased cellular activity [24] [24].

Unsurprisingly, then, mitochondrial stress and dysfunction compromise generation of ATP and reducing equivalents, as well as causing increased production of mitochondrial (mt)ROS and efflux of Ca^{2+} from mitochondria into the cytosol. Of note, redox stress feeds back to further damage mitochondria. Oxphos dysfunction impairing ATP synthesis typically leads to AMP/ADP-mediated AMPK activation to induce mitochondrial biogenesis and autophagy [25]. Such adaptive stress responses can combat a certain level of mitochondrial stress/damage, but supra-maximal stress leads to mitochondrial initiation of cell death [9]. Of the first-line ARVs, EFV exhibits the most convincing mitotoxic effects: it damages mtDNA, inhibits mitochondrial complex I [10] and oxphos-mediated ATP production [26], enhances mtROS production [9], alters mitochondrial architecture [4], and depolarises the mitochondrial membrane.

6.1.2. Autophagy, Lysosomes, & EFV

Autophagy is a common stress response, during which lysosomal degradation of specific autophagosomal cargo eliminates defective/unnecessary cellular molecules/organelles and augments energy production; autophagy of dysfunctional mitochondria is called mitophagy [27] [28] [29]. Autophagy is crucial to housekeeping quality control, maintenance of homeostasis, and survival [30]. Both endocytic/phagocytic and autophagic vacuoles require lysosomes for hydrolase-mediated cargo degradation (including for intracellular pathogen killing and antigen presentation) [31]; lysosomal membrane transporters subsequently translocate degradation products to the cytosol [32] [33].

Lysosomes are thus the major effectors of macromolecule degradation and recycling [34] [35], but play additional roles in Ca^{2+} homeostasis, "plasma membrane repair, exosome release, cholesterol homeostasis, cell adhesion and migration, cell death (including apoptosis), metabolic signalling, and gene regulation" [32] [33] [36]. Lysosome membrane permeabilisation (e.g. in response to oxidative stress) potentiates apoptosis triggered by classical stimuli (e.g. p53 activation, death receptor ligation, oxidative stress, or lack of growth factors) [33], though it can also independently initiate apoptosis [37]. Permeabilisation mechanisms are incompletely understood, but may - at least in part - be related to significant lysosomal-mitochondrial cross-talk [34]. For example, mitochondrial degradation elevates autophagolysosomal iron content and ROS production (via Fenton chemistry) [38]. This may damage compartment membrane lipids, resulting in permeabilisation and leakage of cathepsins into the cytosol [39] [40]. In this way, lysosomal enzymes may causally link autophagy and apoptosis [37].

In vitro, moderate EFV (a non-nucleoside reverse transcriptase inhibitor (NNRTI)) levels (25 μM , equivalent to 7.89mg/L) induce autophagy as a cellular survival mechanism, but high levels (50 μM , equivalent to 15.79mg/L) actually suppress autophagy resolution (possibly via impairing lysosome function), resulting in stress and an increased likelihood of apoptosis [9] [26] [4]. Nucleoside reverse transcriptase inhibitors

(NRTIs), at least, are known to concentrate in monocytes, producing four-fold higher levels compared to those reached within T-cells [41]. EFV pharmacokinetics (PK) and *in vivo* plasma concentration exhibit large inter-individual variation [42]. Typically a 600mg daily dose results in attainment of plasma C_{max} within 1-2hrs, producing plasma concentrations of 3.2-28.5 μ M (equivalent to 1-9mg/L), though concentrations of over 25mg/L have been reported in some patients (while the suggested therapeutic range for EFV is *much* lower than either, at 1-4 μ g/mL) [43] [44] [45]. To date, it appears that no studies have examined the effect of EFV on lysosome function and fate.

6.1.3. The ER, Protein Quality Control, & EFV

Similarly to mitochondria, an ER network extends throughout the neutrophil interior. Functions of the ER include protein-folding quality control (to avert toxic build-up of incorrectly-folded proteins [46]), Ca^{2+} homeostasis (the ER is the major organelle responsible for regulating this, though mitochondria and lysosomes also contribute), lipid biosynthesis [47], modulation of steroid hormone activation [48], and cytochrome p450 (CYP450) oxidase-mediated (NADPH-dependent) drug detoxification (hepatic CYP450s are located in the smooth ER) [49]. Unsurprisingly, many ER functions are Ca^{2+} -dependent [50].

During translation, amino acid sequence thermodynamic interactions drive spontaneous nascent protein folding into a tertiary structure (some proteins also become part of functional complexes, assuming quaternary structure) [51]. Many proteins (including secretory glycoproteins) are – via targeting by the signal recognition particle (SRP) - co-translationally translocated into the smooth ER for protein folding quality control and processing (including proteolytic cleavage and post-translational modification such as glycosylation); many other proteins are post-translationally targeted to the ER in an SRP-independent manner [46] [52].

Protein function and trafficking require correct folding, but approximately 30% of newly-synthesised proteins fold along incorrect pathways (e.g. due to high levels of protein synthesis, incomplete translation, mutations/damage, or altered microenvironment pH/ionic balance/etc.) [51] [53]. The three major protein quality control systems (also noted to exhibit dysregulation in Chapters 4 and 5) are fold-supporting chaperones (including ER-residents), the protein-degrading ubiquitin-proteasome system (UPS), and autophagy (which can degrade larger structures including protein complexes and aggregates, organelles, and even pathogens [54]) [51]. Permanently misfolded glycoproteins (unable to be corrected by chaperones) are exported from the ER lumen to the cytosol for UPS degradation [50]. “Up to 80-90% of all intracellular proteins are degraded via the UPS” [55] [51] and approximately 20% of newly-synthesised “polypeptides are immediately [targeted] for degradation” [51]. Damaged/misfolded proteins not yet rescued by chaperones or eliminated by the UPS often exhibit anomalous surface hydrophobic/polar patches that thermodynamically drive aggregation (including in the ER and cytoplasm); aggregates can become sequestered in structures called aggresomes [51] [53] [56] [54]. Aggresome cargo remains subject to the actions of chaperones (e.g. for disaggregation) and UPS members, but the last resort for clearance of irreversibly misfolded/abnormal proteins (i.e. those not prevented, corrected, or eliminated by the other mechanisms) is autophagy of aggregates/aggresomes (‘aggrephagy’) [51] [56] [54]. However, aggresomes sometimes become irreversibly sequestered within the cell [51] [56] [54], or are exocytosed.

Furthermore, ER stress and accumulation of toxic unfolded proteins in the ER lumen (proteotoxic stress) can trigger cell death [52]. Various proteins sense ER stress (and intracellular accumulation of improperly-folded proteins) and execute a programme called the unfolded protein response (UPR), comprising: regulated IRE1-dependent mRNA decay (RIDD: degradation of mRNAs proximal to the ER) [57], a pause in protein translation, increased synthesis of ER membrane lipids for lumen expansion, increased ER chaperone expression, ER-associated protein degradation (ERAD: “retrotranslocation, polyubiquitination, and cytoplasmic proteasomal degradation of misfolded proteins from the ER”), activation of autophagy, and enhanced antioxidant defence [52]. The UPR can also be triggered by hypo/hyperglycemia and fatty acid overload, and thus also regulates glucose metabolism and lipid synthesis, integrating information to arrive at cell death decisions [48].

Indeed, both the UPR and autophagy represent major *semi-specific* cellular stress responses to diverse stimuli (including protein misfolding, Ca^{2+} imbalance, oxidative stress, and glucose starvation/lack of ATP). While such responses are typically adaptive (favouring continued cell functioning and survival), UPR over-responsiveness is detrimental (e.g. RIDD “impairs synthesis of secreted and membrane proteins [impacting] immune function” [57]). Similarly, prolonged or supra-maximal stimuli can result in autophagy-dependent cell-death [51]. Failure to correct an ER malfunction and misfolded protein overload results in apoptosis, in part through activation of a series of ER chaperones that participate in cell death induction [46] [52]. Additionally, severe ER stress produces ROS-mediated ERK inhibition, inhibiting autophagic flux and aggravating ER stress, thereby favouring cell death [58]. Furthermore, ER stress-induced Ca^{2+} release can cause cytosolic Ca^{2+} overload, thus impacting mitochondrial and lysosomal functions (including death pathways mediated by these organelles) [51]. Intracellular amyloid- β accumulation (e.g. due to protein-folding stress) itself impairs mitochondria, increasing ROS generation [59].

EFV is associated with ER stress (altering ER morphology) and the UPR, and elevates cytosolic Ca^{2+} concentration (in part due to release of mitochondrial Ca^{2+}) [9] [2] [23]. However, EFV-induced ER stress is distinct from that induced by standard pharmacological ER stressors [2]. Smoking is also known to induce the UPR, and *Mycobacterium tuberculosis* (*Mtb*) antigens are known to induce ER stress (via TLR-2/4 and ROS/ Ca^{2+}). Interestingly, EFV-induced ER stress and the UPR are exacerbated by ethanol and hepatitis [60].

6.2. Experimental Rationale: Hypotheses Derived from Proteomic Data

Discovery proteomic data regarding neutrophils derived from TB/HIV-coinfected patients suggests that ARV treatment dysregulates mitochondrial and protein-folding homeostasis (Chapter 5). Especially in the IRIS group, two weeks of ARV perturbs intracellular levels of proteins in a manner suggesting:

(a) Disturbed mitochondrial energy metabolism and function. For example: down-regulated TYMP, (required for maintenance of mtDNA levels), absent respiratory complex IV subunits MT-CO3 and COX6B1, unique PPA2 (which uses pyrophosphates as an ATP-alternate energy source and is required to maintain mitochondrial membrane potential), unique HEBP2 (which – especially during oxidative stress and prior to necrosis – enhances mitochondrial inner and outer membrane permeabilisation), absent DDI2 (involved in NRF1-mediated mitochondrial biogenesis), and up-regulated HSPD1 (which participates in protein-folding quality-control for proteins imported into the mitochondrion under stress conditions).

(b) Altered protein dynamics and ER stress. For example: down-regulated LAP3 (a peptidase normally involved in cellular protein processing and turnover, as well as in glutathione metabolism), up-regulated FKBP5 (involved in protein-folding quality control), unique SPCS3 (a component of the complex which removes the signal targeting nascent proteins to the endoplasmic reticulum [ER]), unique SSR1 (involved in nascent protein translocation into the ER), and up-regulated members of the CANX/CALR protein-folding quality-control cycle (such as a subunit of glucosidase II, and CANX itself).

(c) Increased susceptibility to pro-inflammatory forms of cell death. For example: unique HMOX2 (which catabolises heme and thus increases available iron), unique FTH1 (involved in intracellular iron sequestration), unique GPX4 (which counters ferroptosis resulting from iron-dependent lipid-ROS accumulation), and up-regulated NLRC4 (an inflammasome sensor capable of activating CASP4/5 and pyroptosis).

Furthermore, dysregulation of proteins belonging to related pathways differentiates CTRL from IRIS neutrophils, both prior to and after two weeks of ARV treatment (Chapters 4 and 5). For example, at baseline (prior to ARV exposure), neutrophils from the IRIS (relative to the CTRL) group already exhibit alterations in levels of proteins suggestive of mitochondrial stress, ATP depletion, and poorer antioxidant capacity.

Taken together, these findings suggest that IRIS-group neutrophils exhibit a metabolically-exhausted phenotype less capable of responding adaptively to stressors, and may thus be more sensitive to ARV-induced functional (including mitochondrial) perturbations and inflammatory forms of cell death. This perhaps contributes to the vulnerability of these individuals to developing TB-IRIS.

6.3. Research Question Based on Proteome Data-driven Hypotheses

Does EFV - in the presence or absence of *Mtb* antigens - alter neutrophil mitochondrial function (inducing dysfunction and mitophagy) and/or ER function (altering protein folding dynamics)?

In order to investigate this question, we developed a fluorescence microscopy assay to observe the effects of EFV on mitochondrial morphology and misfolded protein structures in live primary human neutrophils.

6.4. Assay Development

Ex vivo human systems “represent a ... model for disease states” [61]. Peripheral blood mononuclear cell (PBMC) culture is the commonest system for functional leukocyte characterisation, though cell isolation introduces additional technical variability [62] and isolated PBMC exhibit skewed leukocyte ratios (whole blood contains 60-70% neutrophils, 20% lymphocytes, and 4-6% monocytes, while isolated PBMC comprise largely lymphocytes, along with 10-15% monocytes) [63] [64].

We therefore initially attempted to develop a diluted whole blood microscopy assay (Appendix L) in order to achieve an experimental context more closely resembling the *in vivo* circulatory environment (i.e. containing soluble plasma factors and correct leukocytes ratios, both of which significantly impact neutrophil function) [61] [63] [64] [65]. Ex vivo whole blood culture – including for leukocyte response characterisation – has been in use since the 1960s [66] [67] [68] [69]. It is simple (heparinized blood is diluted with tissue culture medium and then stimulated), low-cost, reproducible (avoiding technical variability associated with leukocyte isolation and storage), requires lower blood volumes than for isolation, and leads to higher cell viability as well as a more native immunological functional profile (including less culture vessel adherence-induced artificial activation) [61] [70] [71] [62] [72]. Cell activities seen in whole blood resemble cell activities *in vivo* to a much greater degree, and donor-specific whole-blood cultures perform consistently over time (i.e. exhibit low within-subject variability) [62] [63]. Whole-blood leukocyte culture may thus be the most appropriate system in which to study ex vivo responses of immune cells to exogenous stimuli, and indeed is currently used largely for this purpose [61].

In order to examine mitochondrial and protein-folding homeostasis, we selected three relevant fluorescent probes appropriate for concurrent use in live leukocytes (fig. 1).

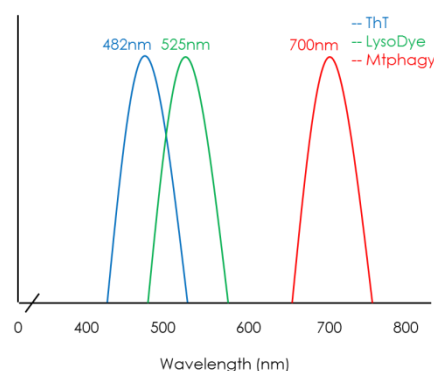


Figure 1: Fluorescent Probe Emission Spectra. Thioflavin T (ThT; Calbiochem) is a benzothiazole exhibiting enhanced fluorescence on binding β -amyloid fibrils [73] [74]. Its fluorescence level directly correlates with the UPR, indicating ER-accumulating misfolded proteins (ER stress) [75]. Mtphagy and LysoDye are provided in a Mitophagy Detection Kit (Dojindo Molecular Technologies), although mitochondrion- and lysosome-specific target molecules are not specified. Mtphagy becomes covalently immobilised and accumulates in intact mitochondria, producing weak fluorescence until mitochondrial compartment acidification (e.g. via fusion of mitochondrial autophagosomes to lysosomes during mitophagy) intensifies fluorescence [76] [77]. Co-localisation of brightly-fluorescing mitochondria with LysoDye-stained lysosomes supports conclusions of mitophagy.

During preliminary assay development and optimisation, we determined that diluted whole blood auto-fluorescence and erythrocytes did not interfere with confocal microscopy-based high-resolution visualisation of unstained and ThT-stained leukocytes. Indeed, no whole blood auto-fluorescence [78] (deriving from serum proteins and circulating leukocytes) was detected in emission channels of this probe (Appendix L). Thus, live-cell microscopy of ThT-stained diluted whole blood is a viable method to assay the impact of stimuli on leukocyte protein folding/ER stress. ThT is inexpensive, water-soluble, compatible with live cells, and easy to use, and the assay is relatively rapid (~1hr for phlebotomy, dilution, plating, stimulation, and staining, plus ~1.5hrs for imaging).

However, we determined that other fluorescent probes of interest (LysoTracker Red dnd-99, Mtphagy, and LysoDye) become sequestered by whole blood plasma proteins and consequently fail to stain their intracellular targets (Appendix L). Rather than being performed in diluted whole blood, then, assays employing such probes require leukocyte (PBMC and neutrophil) isolation and recombination prior to staining, re-addition of autologous plasma for provision of soluble factors present in whole blood, and, finally, exposure to stimuli of interest. Indeed, we observed that Mtphagy and LysoDye successfully stain mitochondria and lysosomes (respectively) of isolated recombined PBMC and neutrophils, when probes are added prior to autologous plasma (whose presence nonetheless somewhat reduces probe fluorescence intensity) (Appendix L). Thus, live-cell microscopy of Mtphagy/LysoDye co-stained isolated leukocytes in the presence of autologous plasma is a viable method to assay the impact of stimuli on leukocyte mitophagy. Mtphagy and LysoDye probes are expensive, DMSO-soluble, compatible with live cells, and easy to use, and the assay is relatively long (~6hrs for phlebotomy, cell isolation, dilution, plating, and staining, plus ~1.5hrs for imaging).

Finally, optimised assay parameters were combined to examine the effects of EFV $\pm$ *Mtb* antigens on neutrophil mitophagy and protein folding/ER stress. Since relatively high PBMC concentrations make it difficult to clearly visualise neutrophils (Appendix L) without adding an explicit (cell viability-compromising) nuclear stain, only isolated neutrophils (but containing up to 20% naturally “contaminating” PBMC) were used in the final assay (i.e. circumventing the need for recombination of isolated PBMC and neutrophils in the correct ratio). An appropriate (*in vivo*-comparable) *in-well* EFV concentration was determined from EFV PK literature (*vide supra*, Section 6.1.), in which the EFV plasma level typically ranges from 1-9mg/L (with average C_{max} ranging from 1.89mg/L for normal metabolisers to 2.53mg/L for slow metabolisers), but achieves levels as high as 25.26mg/L. Additionally, EFV concentrates in leukocytes, and its metabolism may be impaired under inflammatory conditions. So as to mimic the *in vivo* situation likely established during inflammation (and given our interest in the cytotoxic effects of EFV), we aimed for the higher end of the normal EFV plasma level range, at 8mg/L (8ng/ μ l). Additionally, to activate healthy volunteer leukocytes in an *in vivo*-similar manner, we employed *Mtb* antigens (5 μ g/ml whole *Mtb* H37Rv lysate in PBS).

6.5. Materials & Methods

6.5.1. Stock Preparation

Fluorescent probe stocks were pre-prepared and stored according to manufacturer instructions (Table 1). Aqueous solutions used during preparation included PBS and culture-grade DMSO (both Sigma-

Aldrich). EFV was supplied by the National Institutes of Health (NIH) in powder form, made up to 10µg/ul in culture-grade DMSO, and stored at -20°C.

Table 1: Fluorescent Probe Stocks

Probe	Starting Mass	Solvent Volume	Stock Concentration	In-well Concentration	Storage Conditions
ThT (0.22µm filtered)	30mg	30mL (PBS)	3.14mM	5-10µM	foil, 4°C
Mtphagy	5µg	50µL (DMSO)	100µM	100-200nM	foil, -20°C
LysoDye	30µg	55µL (DMSO)	1mM	1-2µM	foil, -20°C

6.5.2. Healthy Volunteer Phlebotomy and Neutrophil Isolation

Five healthy volunteers (HV1-5) underwent phlebotomy on three different days (HV1 on a single occasion, HV2 and 3 on a single occasion, and HV4 and 5 on a single occasion). Within two hours, venous blood underwent PBMC and neutrophil isolation as previously described (Chapter 2, Appendix E), with the following alterations. After erythrocyte lysis, neutrophils were resuspended in an appropriate volume of RPMI to facilitate differential counting (ABX Pentra 60, HORIBA) of a 200ul aliquot (Table 2). To eliminate lysis buffer residue, neutrophils were then made up to 50ml RPMI before repeating the prior centrifugation step. Based on differential counts, neutrophils were resuspended in appropriate volume of PBS to achieve a concentration of 1M cells/ml.

Table 2: Isolated Neutrophil Differential Counts (x10⁶/ml)

Healthy Volunteer	WCC	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)	Total Neutrophils (x10 ⁶)	Required PBS Dilution Volume (ml)
HV1 (RI) M, 34	9.3	6.4 (68.6)	2.45 (26.3)	0.18 (1.9)	0.3 (3.2)	0 (0)	in 2ml: 12.8	12.8
HV2 (Ke) F, 34	7.4	6.32 (85.8)	0.67 (9.1)	0.02 (0.3)	0.35 (4.7)	0.01 (0.1)	in 5ml: 31.6	31.6
HV3 (CI) F, 27	4.4	4.33 (97.9)	0.04 (1)	0.01 (0.2)	0.04 (0.9)	0 (0)	in 30ml: 129.9	129.9
HV4 (Br) F, 32	13.7	unreadable	0.33 (2.4)	0.08 (0.6)	unreadable	0.01 (0.1)	in 6ml: 79.65	79.65
HV5 (BM) M, 29	12.2	unreadable	0.32 (2.6)	0.1 (0.8)	unreadable	0.01 (0.1)	in 6ml: 70.64	70.64

WCC: white cell count. M: male. F: female. Unreadable values result from neutrophil percentages beyond the linear range of the differential cell counter, but are calculated by subtracting other cell type percentages from 100% and multiplying the WCC by this fraction.

6.5.3. Neutrophil Plating, Treatment Conditions, and Microscopy

Neutrophils in PBS were plated in microscope-compatible four-compartment tissue-culture petri dishes (Cell View 35mm, Lasec), limiting the number of investigable conditions to four per volunteer:

- (1) unstained control (cells + autologous plasma)
- (2) vehicle-only control (cells + probes + autologous plasma + DMSO)
- (3) EFV experiment (cells + probes + autologous plasma + EFV in DMSO)
- (4) EFV + *Mtb* antigens (cells + probes + autologous plasma + *Mtb* antigens in PBS + EFV in DMSO).

Each component was plated at room temperature, in the dark, at the appropriate volume per well (Table 3), in the following order: neutrophils, Mtphagy, LysoDye, ThT, autologous plasma, *Mtb* antigens, and EFV (or DMSO). Plating order of the final three components reflects the *in vivo* exposure sequence. DMSO concentration of the vehicle-only condition was matched to that of the experimental conditions (as determined by EFV concentration), such that final in-well DMSO concentration for conditions 2 through 4 was 0.28%.

Table 3a: Calculations for In-well Probe and Reagent Concentrations

Probe/Reagent	Stock Concentration	Desired Concentration	Dilution Factor	Volume (µl) to add to 500µl Well
ThT	3.14mM	5µM	312.5	1.6

Mtphagy	100µM	100nM	1000	0.5
LysoDye	1mM	1µM	1000	0.5
EFV	10µg/ul	8ng/ul	1250	0.4
Mtb lysate in PBS	0.67mg/ml	5µg/ml	135.14	3.7

Table 3b: Volume (µl) of Each Component to Add per Well (i.e. per Condition)

Reagent	(1) unstained control	(2) vehicle-only control	(3) EFV experiment	(4) EFV + Mtb experiment	Final In-well Concentration
1M/ml neutrophils in PBS	450	447	447	443.3	1M/ml
ThT	-	1.6	1.6	1.6	5µM
Mtphagy	-	0.5	0.5	0.5	100nM
LysoDye	-	0.5	0.5	0.5	1µM
plasma	50	50	50	50	10%
EFV	-	-	0.4	0.4	8ng/ul
DMSO	-	0.4	-	-	0.28%
Mtb lysate	-	-	-	3.7	5µg/ml
TOTAL VOLUME (µl)	500	500	500	500	-

Incubation time for each component: Mtphagy (10min), LysoDye (10min), ThT (10min), autologous plasma (0min), Mtb lysate (10min), EFV/DMSO (30min).

Petri dishes were covered in foil and incubated in a humidified atmosphere (30°C, 5% CO₂) for 30min, followed by an additional 30-45min equilibration in the microscope incubator. All incubation steps were performed in the dark.

6.5.4. Confocal Microscopy Image Acquisition

Imaging was performed using a Zeiss LSM 880 confocal microscope with Airyscan, Zen software (version 2.3, Service Pack 1), and a 63x oil-immersion objective (optical magnification x2). The unstained control formed the basis for selection of imaging parameters. Excitation laser wavelengths were as follows: 405nm (ThT), 561nm (Mtphagy), and 488nm (LysoDye). Emission range wavelengths were as follows: 400-519nm (blue), 655-748nm (red), and 499-566nm (green). Although no excitation and little emission wavelength overlap exists between probes (Table 4), channels were captured individually and sequentially. Although both LysoDye and ThT (partially) fluoresce in the green spectrum, the latter did not produce interfering fluorescence when exciting the former. Additionally, a differential interference contrast image was captured using transmitted photo-multiplier tube white light. Electronic magnification during acquisition: low (1x), medium (2x), and high (3x).

Table 4: Excitation and Emission Wavelength Ranges (nm) for Selected Fluorescent Probes

Probe	Excitation (recommended)	Emission (recommended)	Recommended Filter	References
ThT (blue)	405 (458) maximum: ~385 (free) ~437 (bound)	400-519 (480-520) maximum: ~445 (free) ~485 (bound)	Ex. 460-490nm band-pass Em. 520nm	[79] [75]
Mtphagy (red)	561 (530)	655-748 (700)	Ex. 500-560nm Em. 670-730nm	[77]
LysoDye (green)	488 (398)	499-566 (525)	Ex. 350-450nm Em. 500-560nm	

6.5.5. Image Analysis

Fluorescence micrographs were visually inspected prior to quantitative analysis. Raw image files were imported into Fiji Image_J [80] [81] and masks were set based on size and shape to include all neutrophils (HV1: n=9, HV2: n=3, HV3: n=8, HV4: n = 8) while excluding other cell types and fragments. Average cell fluorescence analysis (as a proxy for number of bright features) and - using the BioVoxxel plugin [82] - speckle inspection (counting of small, bright subcellular features - i.e. mitochondria, lysosomes, and aggresomes) were performed. Fluorescence minimum-intensity thresholding was applied to the lowest-intensity condition image per volunteer in order to create within-subject speckle-selection masks (Table 5), and speckle size was limited to 4-10 pixels to exclude false positive features (maximum thresholding intensity was held constant at 255). Two-tailed t-tests were used to compare quantitative imaging data between conditions (control versus EFV, and EFV versus EFV + Mtb). Due to a

lack of individual-channel baseline determination during microscopy image capture, formal co-localisation analysis was not performed. However, visual inspection suggests that very few features co-localise regardless.

Table 5: Minimum-intensity Thresholds for Speckle Inspection

	HV1 (Ri)	HV2 (Ke)	HV3 (Br)	HV4 (BM)
Mitochondria	131	81	53	143
Lysosomes	39	35	150	88
Aggresomes	129	115	188	121

6.6. Results

6.6.1. Visual Inspection: Organelle Number, Size, Shape, Fluorescence, Distribution, & Co-localisation

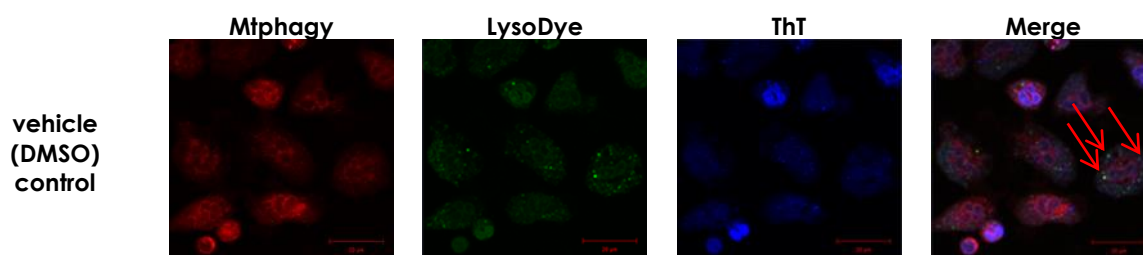
Only images for HV1-4 are included, as HV5 had recently recovered from infection and exhibited numerous circulating immature granulocytes (inconsistent with the other HVs).

6.6.1.1. Control Condition (Fig. 2-5, top row)

Mtphagy dye demonstrates a fused network of many low-intensity fluorescent mitochondria extending throughout the cytoplasm, resembling published micrographs of neutrophil mitochondria visualised by alternative probes. A few medium-intensity fluorescent individual tubular or globular mitochondria are visible ($\leq 1\mu\text{m}$ diameter). Mitochondrial fluorescence is most intense perinuclearly, possibly reflecting higher mitochondrial density or differing chemical conditions (e.g. pH) in this region, probe binding to ectopic/non-specific nuclear envelope targets, or sharing of probe targets via the inter-connected intracellular membranous network. HV3 mitochondrial fluorescence intensity is particularly low.

LysoDye demonstrates two lysosome subsets: many low-intensity fluorescent smaller ($<1\mu\text{m}$) puncta scattered (or perhaps forming a network) throughout the cytoplasm, and a few - sometimes more peripherally-situated - intensely-fluorescent larger ($\sim 1\mu\text{m}$) cytoplasmic puncta. Low-intensity perinuclear fluorescence may reflect the presence of lysosomes in this region, probe binding to ectopic/non-specific nuclear envelope targets, or sharing of probe targets via the inter-connected intracellular membranous network. HV3 and HV4 lysosomal fluorescence intensity is particularly high and low, respectively. Lysosomes appear not to co-localise with mitochondria.

ThT also demonstrates two staining patterns: many low-intensity fluorescing smaller ($<1\mu\text{m}$) puncta scattered (or perhaps forming a network) throughout the cytoplasm, and a few - sometimes more peripherally-situated - intensely-fluorescent larger ($\sim 1\mu\text{m}$) cytoplasmic puncta (likely aggresomes). The low-intensity network extending throughout the cytoplasm - as well as intense perinuclear fluorescence - may reflect improperly-folded proteins within the ER network (known to extend throughout the cytoplasm and to have intimate contacts with the nuclear envelope). Alternatively, less intense fluorescence may represent staining of non-target molecules, or sharing of probe targets via the inter-connected intracellular membranous network. HV3 aggresome fluorescence intensity is particularly low. Aggresomes largely appear not to co-localise with lysosomes (except occasionally, indicated by red arrows, especially in HV3) and are likely distinct from them.



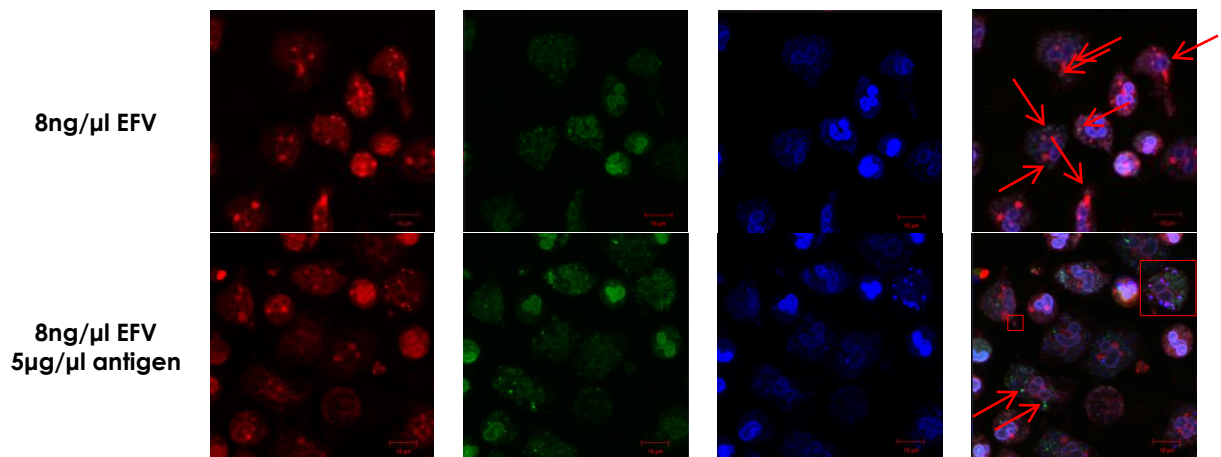


Figure 2: Fluorescence Micrographs of HV1 Neutrophils Exposed to Control and Experimental Conditions (500x)

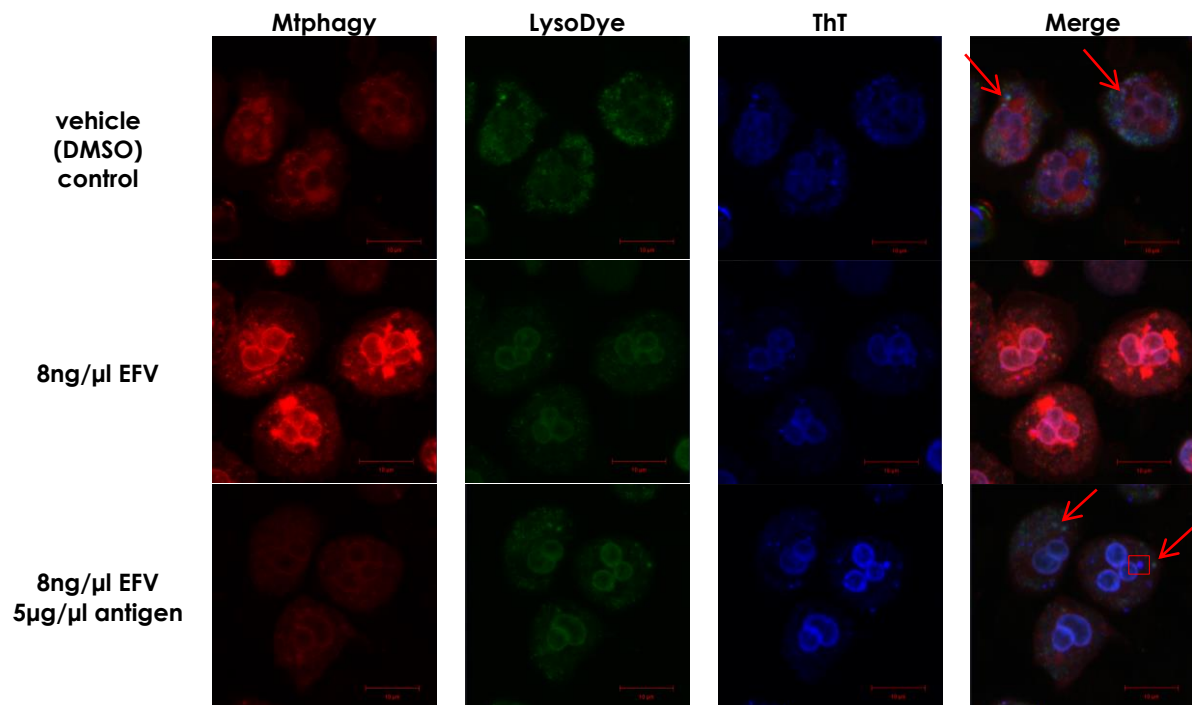
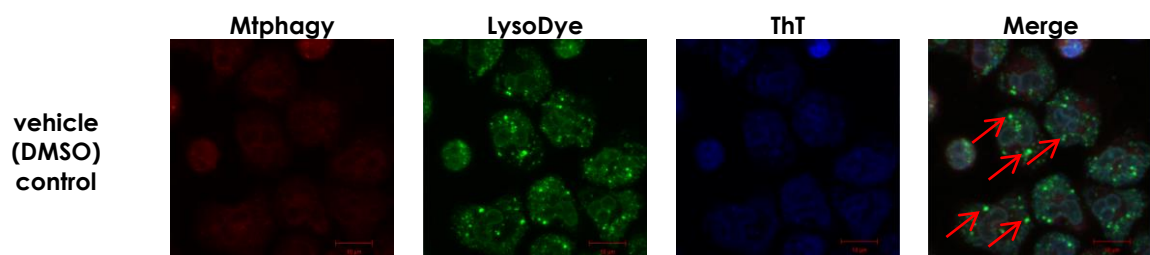


Figure 3: Fluorescence Micrographs of HV2 Neutrophils Exposed to Control and Experimental Conditions (1000x)



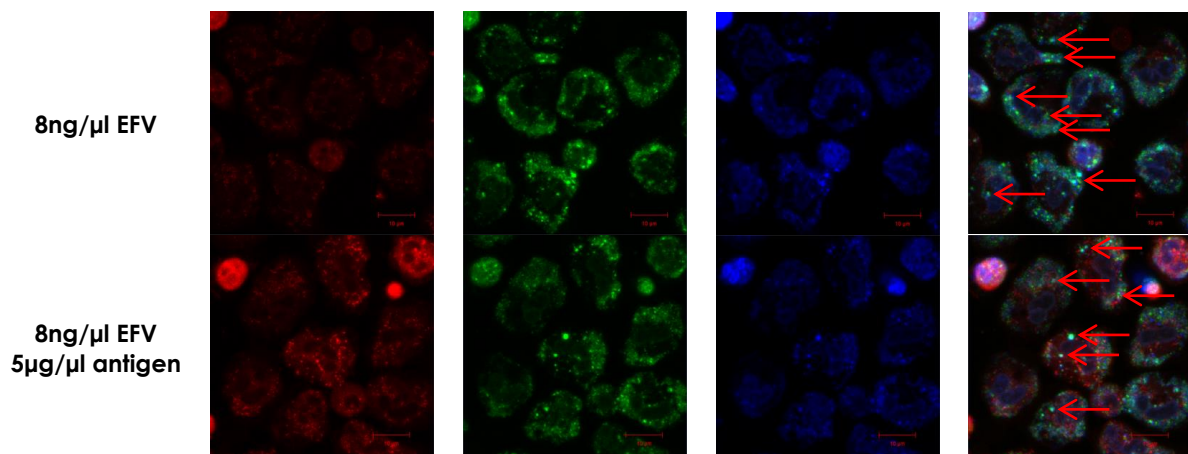


Figure 4: Fluorescence Micrographs of HV3 Neutrophils Exposed to Control and Experimental Conditions (650x)

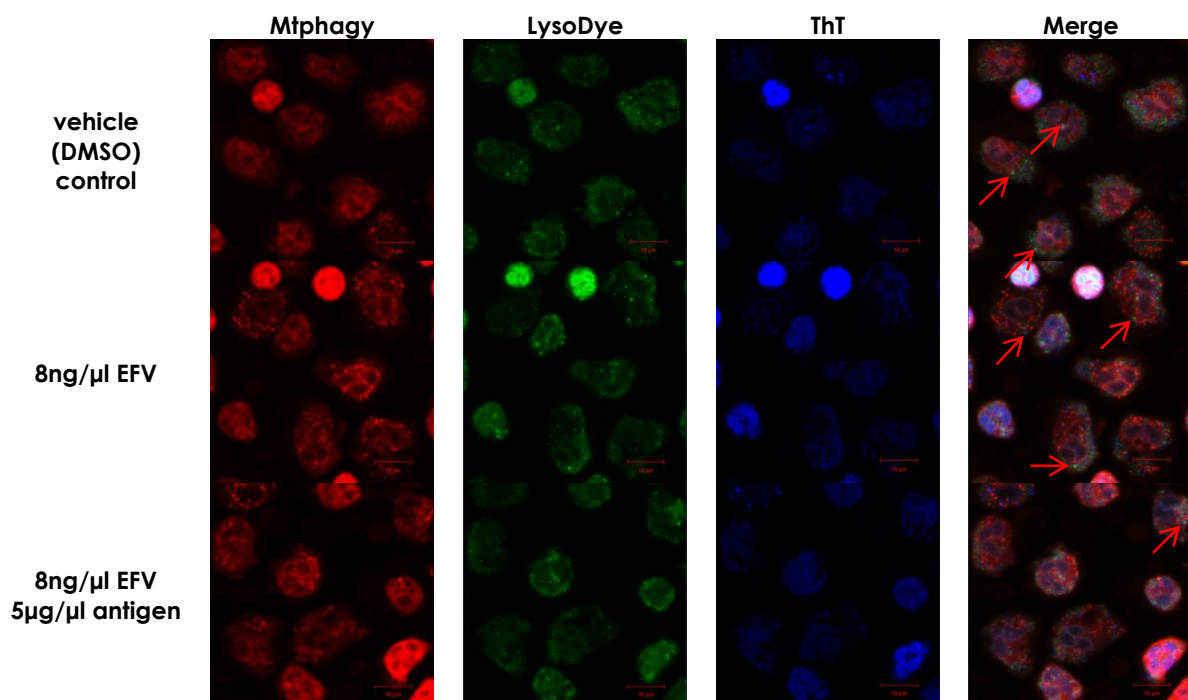


Figure 5: Fluorescence Micrographs of HV4 Neutrophils Exposed to Control and Experimental Conditions (650x)

6.6.1.2. Experimental Conditions (Fig. 2-5, middle and bottom rows)

(a) EFV induces mitochondrial stress (partially mitigated by pre-exposure to Mtb antigens)

Relative to control, EFV enhances overall mitochondrial network fluorescence intensity, including – in HV1 and HV2 – enhancing nuclear envelope region fluorescence. Furthermore, EFV induces formation of: (a) large (~2-3μm), intensely fluorescent, perinuclear globular structures suggestive of swollen mitochondria (HV1 and 2) or (b) many short (~1μm), intensely fluorescent, scattered/peripheral tubular

structures suggestive of mitochondrial fragmentation (HV3 and 4). No obvious apparent co-localisation of mitochondria with either lysosomes or aggresomes occurs. Overall, findings suggest that EFV induces mitochondrial stress (along a functional continuum: less severe/earlier fragmentation versus more severe/late swelling, likely due to a 30min longer EFV exposure prior to imaging on the first day of microscopy). The lack of obvious apparent co-localisation of intensely-fluorescent mitochondrial structures with lysosomes suggests that mitophagy is *not* occurring, but definitive conclusions are elusive (*vide infra*: Section 6.7).

Relative to EFV-only, pre-exposure to *Mtb* antigens prior to EFV treatment prevents EFV-induced enhancement of overall mitochondrial network fluorescence intensity (including enhancement of the nuclear envelope region fluorescence observed in HV1 and 2). Furthermore, it results in a decreased frequency of the EFV-induced intensely-fluorescent globular structures seen in HV1 and 2 (although it is plausible that HV1 neutrophils exposed to both *Mtb* antigens and EFV may actually be undergoing cell death). In agreement with this idea, pre-exposure to *Mtb* antigens prior to EFV treatment enhances EFV-induced mitochondrial fragmentation in HV3, leading to an increased number of intensely-fluorescent tubular fragments (including enhancement of nuclear envelope region fluorescence), while no effect is apparent in HV4 (apart from a subtle decrease in mitochondrial fragment fluorescent intensity). Again, no obvious apparent co-localisation of mitochondria with either lysosomes or aggresomes occurs, except for minor apparent co-localisation of mitochondria and aggresomes in HV1 (indicated by a red box).

Overall, findings suggest three equally-plausible effects of *Mtb* antigen pre-exposure: pre-induction of adaptive stress responses which lessen susceptibility to EFV cytotoxic effects (favouring cell survival – as suggested by some mitochondrial features more closely resembling those found under control conditions), synergistic cytotoxicity with EFV (favouring cell dysfunction and death, as suggested by HV1 results), and/or frustration of adaptive EFV-induced stress responses such as mitophagy (also favouring cell dysfunction and death). The pattern of more extensive mitochondrial fragmentation observed in HV3 could also occur in any one of these cases. Supra-maximal stress leading to (potentially pro-inflammatory) cell death (e.g. pyroptosis or secondary necrosis) *in situ* could slow antigen clearance and may contribute to local inflammation via various mechanisms.

(b) EFV may induce lysosome permeabilisation and aggrephagy (both partially mitigated by pre-exposure to Mtb antigens)

Relative to EFV-induced *mitochondrial* changes, lysosomal and aggresomal changes are less visually apparent, and more difficult to interpret in context of the (sparse) existing literature. However, findings suggest that:

- EFV-induced cell stress (e.g. oxidative stress resulting from mitochondrial dysfunction) leads to lysosomal membrane permeabilisation and dispersal of lysosome contents (including probe targets), causing diminished fluorescence intensity.
- *Mtb* pre-exposure induces lysosomal biogenesis (in at least two HVs) and may prevent lysosomal membrane permeabilisation (in at least three HVs).
- EFV may increase protein synthesis (and perhaps misfolding) throughout the ER network, but opposes formation (or favours resolution) of large aggresomes. Since large aggresomes are present at baseline (control), resolution (e.g. aggrephagy) seems the more likely explanation, as is supported by generally higher numbers of apparent lysosome-aggresome co-localisation events (relative to control) in at least three HVs.
- Only at later/more severe stages of mitochondrial dysfunction (HV1 and 2) does *Mtb* antigen pre-exposure oppose responses to EFV (e.g. aggrephagy – supported by generally fewer apparent lysosome-aggresome co-localisation events, relative to EFV-only, in at least three HVs).

For analysis of lysosome and aggresome results, please see Appendix O. Overall, these additional findings are consistent with the idea that EFV induces mitochondrial, lysosomal, and ER stress (along with certain relevant stress responses, such as mitochondrial fission and aggrephagy). Furthermore, *Mtb*

antigen pre-exposure may induce at least lysosomal stress responses which increase resilience to subsequent EFV exposure, but may also interfere with EFV-induced adaptive stress responses once they become supra-maximal (maladaptive). This is in agreement with concepts regarding opposition of stress response-induced apoptosis by *Mtb*. Additional low-/high-magnification images in Appendix N.

6.6.2. Quantitative Analysis Supports Qualitative Visual Observations

Relative to both other conditions, EFV exposure results in higher average and maximal Mtpagy fluorescence intensity per neutrophil (due to an increase in the number of intensely-fluorescent subcellular mitochondrial structures), but lower average and maximal LysoDye or ThT fluorescence intensity per neutrophil (due to a decreased in the number of intensely-fluorescent subcellular lysosomal or aggresomal puncta) (Table 6a-c). Differences are less marked for lysosome and aggresome (relative to mitochondrial) observations.

Table 6a1: Mtpagy Fluorescent Intensity

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle	41.84 (±12.13)	155.22 (±43.43)	51.88 (±9.73)	156.00 (±26.21)	23.98 (±1.36)	126.86 (±15.85)	57.00 (±10.14)	223.25 (±25.40)
EFV	59.27 (±19.20)	233.7 (±31.70)	95.13 (±12.48)	*255.00 (±0.00)	21.61 (±1.65)	137.00 (±18.14)	62.82 (±12.09)	244.86 (±19.24)
EFV + Mtb	37.37 (±10.32)	175.89 (±43.22)	30.82 (±2.68)	92.67 (±12.01)	40.52 (±8.34)	219.50 (±32.11)	79.07 (±34.02)	26.62 (±15.93)

*Maximal intensity.

Table 6a2: Significance (p-values) of Mtpagy Fluorescent Intensity Differences between Conditions

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle vs. EFV	0.03	0.0005	0.01	0.02	0.02	0.31	0.30	0.08
vehicle vs. EFV+Mtb	0.41	0.33	0.06	0.04	0.0007	0.00002	0.12	0.05
EFV vs. EFV+Mtb	0.007	0.005	0.002	0.002	0.0003	0.00007	0.24	0.85

In three of four volunteers, average intensity differs significantly ($p < 0.05$) between vehicle and EFV conditions. In three of four volunteers, average intensity does not differ significantly ($p > 0.05$) between vehicle and EFV+Mtb conditions. In three of four volunteers, average and maximal intensity differs significantly ($p < 0.05$) between EFV and EFV+Mtb conditions.

Table 6b1: LysoDye Fluorescent Intensity

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle	17.97 (±2.98)	129.67 (±68.38)	26.62 (±2.35)	132.33 (±47.69)	39.59 (±4.96)	*255.00 (±0)	28.33 (±6.14)	132.00 (±32.45)
EFV	15.42 (±4.52)	74.56 (±21.86)	17.34 (±1.28)	83.67 (±7.37)	36.86 (±5.37)	252.17 (±5.23)	29.11 (±12.47)	168.86 (±41.08)
EFV + Mtb	12.45 (±3.02)	86.44 (±34.85)	20.99 (±1.11)	101.33 (±17.01)	31.77 (±6.62)	214.5 (±49.12)	*255.00 (±0)	*255.00 (±0)

*maximal intensity

Table 6b2: Significance (p-values) of LysoDye Fluorescent Intensity Differences between Conditions

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle vs. EFV	0.20	0.05	0.008	0.22	0.40	0.24	0.88	0.08
vehicle vs. EFV+Mtb	0.001	0.12	0.04	0.38	0.02	0.05	1.93E-12	1.36E-05
EFV vs. EFV+Mtb	0.12	0.4	0.02	0.21	0.14	0.07	5.54E-09	0.001

Average and maximal intensity do not differ significantly ($p > 0.05$) between vehicle and EFV conditions. In all four volunteers, average intensity differs significantly ($p < 0.05$) between vehicle and EFV+Mtb conditions. Average and maximal intensity do not differ significantly ($p > 0.05$) between EFV and EFV+Mtb conditions.

Table 6c1: ThT Fluorescent Intensity

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle	87.87 (±1.41)	135.67 (±10.57)	91.11 (±2.96)	163.67 (±15.28)	157.62 (±0.97)	247.00 (±6.86)	93.12 (±6.44)	160.88 (±39.80)
EFV	118.66 (±2.85)	174.6 (±11.88)	102.64 (±0.49)	158.00 (±2.00)	101.05 (±1.25)	150.67 (±7.03)	65.23 (±1.35)	100.43 (±5.80)
EFV + Mtb	94.78 (±4.11)	164.11 (±34.21)	76.70 (±0.29)	120.67 (±6.03)	73.89 (±1.42)	117.5 (±6.05)	89.92 (±2.59)	136.25 (±14.68)

Table 6c2: Significance (p-values) of ThT Fluorescent Intensity Differences between Conditions

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle vs. EFV	9.60E-14	7.84E-07	0.02	0.56	3.72E-15	9.45E-11	3.04E-06	0.003
vehicle vs. EFV+Mtb	0.0008	0.04	0.01	0.03	6.23E-21	4.63E-14	0.22	0.14
EFV vs. EFV+Mtb	6.95E-10	0.40	2.11E-06	0.005	1.55E-13	3.41E-06	1.21E-10	0.0001

In all volunteers, average intensity differs significantly ($p < 0.05$) between vehicle and EFV conditions. In three of four volunteers, average and maximum intensity differ significantly ($p < 0.05$) between vehicle and EFV+Mtb conditions. In all and three of four volunteers, average and maximal intensity respectively differ significantly ($p < 0.05$) between EFV and EFV+Mtb conditions.

Further supporting these findings, EFV exposure – relative to both other conditions - results in a higher number of Mtpagy-stained speckles per neutrophil, but a lower number of LysoDye- or ThT-stained speckles per neutrophil (Table 7a-c).

Table 7a1: Average Number of Mtpagy-stained Bright Speckles per Neutrophil

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle	2.22 (± 4.84)	14	19.33 (± 4.04)	24	6 (± 3.46)	10	6.5 (± 5.55)	16
EFV	2.6 (± 1.96)	6	33 (± 7.21)	41	25.67 (± 11.25)	42	10.57 (± 6.02)	23
EFV + Mtb	1.33 (± 1.87)	6	0.33 (± 0.58)	1	25.62 (± 6.82)	32	9.88 (± 6.56)	20

Table 7a2: Significance (p-values) of Per-neutrophil Mitochondrial Speckle Number Differences between Conditions

	HV1	HV2	HV3	HV4
vehicle vs. EFV	0.83	0.06	0.007	0.20
vehicle vs. EFV+Mtb	0.62	0.01	2.22E-05	1.28
EFV vs. EFV+Mtb	0.17	0.01	0.99	0.83

Despite the trends visible in Table 7a1, in three of four volunteers, average per-neutrophil speckle number does not differ significantly ($p > 0.05$) between conditions. Given visible micrograph differences, this is likely related to speckle inspection parameters being too stringent (i.e. excluding larger true speckles).

Table 7b1: Average Number of LysoDye-stained Bright Speckles per Neutrophil

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle	11.89 (± 5.84)	21	43.67 (± 5.13)	48	3.57 (± 2.15)	7	2.62 (± 2.97)	7
EFV	3.5 (± 4.38)	14	22.33 (± 6.03)	28	4.67 (± 3.72)	12	3 (± 3.05)	8
EFV + Mtb	4 (± 5.54)	17	25.67 (± 19.35)	48	2.88 (± 2.35)	7	4.25 (± 4.33)	12

Table 7b2: Significance (p-values) of Per-neutrophil Lysosomal Speckle Number Differences between Conditions

	HV1	HV2	HV3	HV4
vehicle vs. EFV	0.003	0.01	0.54	0.81
vehicle vs. EFV+Mtb	0.01	0.24	0.55	0.40
EFV vs. EFV+Mtb	0.83	0.80	0.33	0.53

Despite the trends visible in Table 7b1, in all volunteers, average per-neutrophil speckle number does not differ significantly ($p > 0.05$) between conditions (with the exception of two volunteers, for whom there is a significant ($p < 0.05$) difference in speckle number between the control and EFV conditions). Given visible micrograph differences, this is likely related to speckle inspection parameters being too stringent.

Table 7c1: Average Number of ThT-stained Bright Speckles per Neutrophil

	HV1		HV2		HV3		HV4	
	ave.	max.	ave.	max.	ave.	max.	ave.	max.
vehicle	16.33 (± 7.52)	29	32.33 (± 13.80)	8	37.57 (± 8.54)	49	10.87 (± 5.31)	38
EFV	30 (± 18.52)	62	86.67 (± 11.37)	96	0 (± 0)	0	0 (± 0)	0
EFV + Mtb	6.67 (± 15.54)	48	0.33 (± 0.58)	1	0 (± 0)	0	1.88 (± 1.36)	3

Table 7c2: Significance (p-values) of Per-neutrophil Aggresomal Speckle Number Differences between Conditions

	HV1	HV2	HV3	HV4
vehicle vs. EFV	0.05	0.006	2.42E-05	0.08
vehicle vs. EFV+Mtb	0.12	0.06	2.42E-05	0.14
EFV vs. EFV+Mtb	0.008	0.006	no difference (div. by zero)	0.006

Trends in Table 7c1 appear to be opposite for HV1 and 2 versus HV3 and 4, obscuring true differences. Given visible micrograph differences, this is likely related to speckle inspection parameters being too stringent.

6.7. Discussion

Neutrophil fluorescence microscopy demonstrates that EFV rapidly impacts mitochondrion, lysosome, and ER/aggresome morphology (changes indicating altered functions and protein expression) in ex vivo human neutrophils derived from healthy volunteers, with partial alteration of effects by pre-exposure to Mtb antigens.

6.7.1. Mitochondria

It is known that EFV (which is generally cytotoxic) inhibits oxphos-mediated ATP production, enhances mtROS production, depolarises the mitochondrial membrane, induces release of mitochondrial Ca^{2+} into the cytosol, and results in damaged and dysfunctional mitochondria. As we have also seen, severely dysfunctional mitochondria are usually eliminated via mitophagy (which may be induced by EFV levels comparable to those used in the present study, unless EFV accumulation in neutrophils produces high enough levels to frustrate mitophagy), but supra-maximal mitochondrial damage/dysfunction induces cell death.

In agreement with the literature, results of the present study suggest that EFV induces mitochondrial stress, with shorter and longer durations of exposure causing fragmentation and swelling, respectively. In both SH-SY5Y cells and primary neurons, EFV diminishes ATP generation, concomitantly with mitochondrial fragmentation and depolarisation, and up-regulation of autophagy [26]. It is likely that similar events accompany EFV-induced mitochondrial stress in neutrophils. While lower levels of EFV would likely induce an adaptive stress response favouring continued cell function and survival, a supra-maximal EFV dose (or prolonged exposure in the presence of other xenobiotic or infectious stressors) may overwhelm/over-activate stress response pathways, favouring cell dysfunction and death. In the present study, increased Mtpagy fluorescent intensity and perinuclear aggregation of swollen mitochondria both suggest that mitophagy is occurring (despite the apparent absence of co-localisation with lysosomes). However, swelling (a late-stage mitochondrial response to severe stress) suggests that the point-of-no-return for commitment to cell death has also been passed.

Although the apparent lack of mitochondrion-lysosome co-localisation observed in the present study suggest that mitophagy is *not* occurring, definitive conclusions are elusive. While autophagosome-lysosome fusion (i.e. co-localisation) is thought to be required for acidification-mediated enhancement of Mtpagy fluorescent intensity, the observed increased intensity may instead reflect an increased local density of probe targets, or perhaps lysosome-independent mitochondrial acidification (e.g. dysfunction of inner membrane oxphos complexes leads to retention of protons in the mitochondrial matrix). While functional (coupled) mitochondria maintain a matrix pH alkaline relative to the cytosol, mitochondrial membrane depolarisation leads to matrix acidification [83] [84] [85]. Anaerobic respiration-mediated lactic acid accumulation in the cytosol may also contribute to mitochondrial matrix acidification [83]. Certain pharmaceutical compounds (e.g. isoflurane) are known to depolarise the mitochondrial membrane and induce mitochondrial matrix acidification [86], as does ischemia (including ischemia-reperfusion), with acidification interfering with mitochondrial pyruvate dehydrogenase function, enhancing oxidative stress, retarding carbon-energy metabolic recovery, and increasing mitochondrial susceptibility to stress [83].

Results of the present study suggest that 8ng/ μl EFV represents supra-maximal mitochondrial stress and results in induction of primary human neutrophil cell death. Since TB-associated liquefaction may be due to co-ordinated monocyte and neutrophil death in affected organs, this suggests that EFV-induced sudden, massive apoptosis/necrosis may occur, impairing antigen clearance and boosting local – and thus systemic – inflammation.

Furthermore, macrophage *Mtb* infection is also known to alter mitochondrial architecture and function, for example inducing a Warburg-like shift away from oxphos (towards increased lactate production) [87]. Virulent *Mtb* (e.g. H37Rv) increases mitochondrial membrane potential and cellular ATP level, inhibiting apoptosis and instead favouring necrosis [88], while avirulent *Mtb* suppresses stress-induced mitochondrial responses [89]. As mentioned in Chapter 4, H37Rv infection of macrophages alters the levels of various mitochondrial proteins [89] in ways that oppose mitochondrial stress and apoptosis (e.g. up-regulating ACSL1, VDAC2, ATP50, PRDX1, and ATAD3A, and down-regulating ATP5A1/B, OAS2, PRDX3, HADHA, and SQDRL), concomitantly favouring *Mtb* survival [89]. *Mtb* triggers mitochondrial ROS generation via TNF-mediated activation of the RIP1K/3K pathway, ROS are able to induce autophagy, and excessive mtROS are known to trigger necrosis [90]. Furthermore, *Mtb* translocation from the

phagosome to the cytosol co-occurs with elevated mtROS production, required for induction of autophagy (microbicidal) and necrosis (which ultimately increases *Mtb* burden and impairs host survival) [91]. Simultaneously, however, *Mtb* enhanced intracellular survival (*eis* gene (which acetylates DUSP16 to dephosphorylate JNK) and virulence factor mannosylated glycoprotein phosphoribosyltransferase both suppress microbicidal autophagy through inhibition of all-source host ROS generation [90].

In the present study, observed effects of *Mtb* antigen exposure prior to EFV treatment may be interpreted in three possible ways: (a) pre-induction of adaptive stress responses to lessen susceptibility to EFV cytotoxic effects and favour cell survival, (b) synergistic cytotoxicity with EFV to favour cell dysfunction and death, and/or (c) frustration of adaptive EFV-induced stress responses such as mitophagy (also favouring cell dysfunction and death). Both (a) and (c) are consistent with antigen pre-exposure-mediated limiting of swollen mitochondria observed in neutrophils from two volunteers (in agreement with literature regarding *Mtb* H37Rv limiting all-source ROS generation and inhibiting apoptosis in favour of necrosis), while the pattern of more extensive mitochondrial fragmentation observed in a single volunteer could occur in any one of the above cases. Supra-maximal stress leading to (potentially pro-inflammatory) cell death (e.g. ferroptosis, pyroptosis, necroptosis, or secondary necrosis) *in situ* could slow antigen clearance and may contribute to local inflammation via various mechanisms.

6.7.2. Lysosomes

It appears that no studies to date have examined the effect of EFV on lysosomes. Results of the present study suggest that EFV-induced cell stress (possibly oxidative stress resulting from mitochondrial dysfunction) leads to lysosomal membrane permeabilisation and dispersal of lysosome contents (including probe targets), causing diminished LysoDye fluorescence intensity. Such permeabilisation would favour cell death. In a single volunteer, EFV appears to induce lysosomal fission or biogenesis.

Regarding lysosomal effects of *Mtb* antigens, subversion of phagocyte function (e.g. "avoiding delivery to or directly targeting lysosomes") is a common strategy of many intracellular pathogens [92], (including *Mtb*). Unsurprisingly, lysosomes are required for phagosomal and autophagy-mediated *Mtb* destruction, but *Mtb* products prevent phagosome maturation and fusion to lysosomes. As mentioned above, *Mtb* translocation from the phagosome to the cytosol co-occurs with elevated mtROS production; *Mtb*-mediated elevation in mtROS production can increase cytosolic ROS levels, leading to oxidation and permeabilisation of lysosomes, followed by cell death (necrosis induction by an elevated *Mtb* load has also been associated with lysosomal permeabilisation) [91]. However, also as mentioned, certain *Mtb* genes suppress microbicidal autophagy through inhibition of all-source host ROS generation [90]. Furthermore, LPS-induce macrophage AGS3 expression suppresses mTOR nutrient-sensing activity and leads to TFEB-mediated enhancement of lysosomal function [92].

In agreement with the literature, results of the present study suggests that pre-exposure to *Mtb* antigens prior to EFV treatment apparently enhances lysosomal fission/biogenesis in neutrophils from at least two volunteers, but may also prevent lysosomal membrane permeabilisation (in neutrophils from at least three volunteers) – perhaps through antigen-mediated suppression of ROS production.

6.7.3. The Endoplasmic Reticulum

It is known that EFV induces ER stress/the UPR and alters ER morphology. Autophagy is typically enhanced by ER stress, but – at least in mouse endothelia – EFV induces ER stress while concomitantly suppressing autophagy [93]. Little direct information is available regarding the effect of EFV on aggresome formation or clearance, but logic dictates that ER stress would co-occur with increased aggresome formation (and perhaps clearance, up to a point). In agreement with the literature, findings

of the present study suggest that EFV may increase protein synthesis (and perhaps misfolding) throughout the ER network. However, the present study also suggests that EFV appears to oppose formation (or favour resolution) of large aggresomes (it may also induce a subtle increase in the number of *smaller* misfolded protein puncta). Since large aggresomes are present at baseline (control), resolution (e.g. aggrephagy) seems the more likely explanation, as is supported by generally higher numbers of apparent lysosome-aggresome co-localisation events (relative to control) in neutrophils from at least three volunteers. This is consistent with the levels of EFV used in the current study being comparable to those known to induce autophagy.

Mycobacterial infection, too, is known to impact ER stress [46]. *Mtb* antigens ESAT-6, 38kDa antigen, and HBHA induce ER stress via TLR-2/4 and ROS/Ca²⁺, and IFN γ (the predominant cytokine present during active TB) as well as oxidants cause both mitochondrial and ER stress. Avirulent *Mtb*-infected THP-1 macrophages exhibit lower levels of ATP2A2 (which transports Ca²⁺ into the ER), elevated cytosolic Ca²⁺, and induction of apoptosis-favouring PC/PE expression; virulent *Mtb*-infection regulates proteins involved in cholesterol homeostasis (EST-1, AMRP) and inhibits apoptosis [46]. Virulent *Mtb* infection also up-regulates expression of endoplasmic reticulum chaperones (buffers increased Ca²⁺ to inhibit calpain/caspase activity), PDIA4, CANX (opposes accumulation of misfolded proteins and thus UPR-mediated apoptosis), PPIB, GLU2, ERLN2, and cathepsin Z [46].

Findings of the present study suggest that it is only when EFV-induced mitochondrial dysfunction is severe (i.e. mitochondria have become swollen) that pre-exposure to *Mtb* H37Rv (classified as a virulent strain) antigens opposes responses to EFV (e.g. aggrephagy – supported by the presence of a few larger intensely-fluorescent aggresomal puncta and generally fewer apparent lysosome-aggresome co-localisation events, relative to EFV-only, in neutrophils from at least three volunteers). In agreement with literature, this suggests that *Mtb* antigens induce ER stress (and, at least during severe mitochondrial stress, apparently enhance aggresome formation, opposing the effect of EFV).

6.8. Causal Inferences & Therapeutic Targeting in Inter-linked Systems

Since the mitochondria, lysosomes, and ER all represent major cellular stimulus-integration-and-response-pathway-initiation hubs, complex bi-directional links (both physical and functional) exist between these organelles, energy/macromolecule metabolism, Ca²⁺ and redox homeostasis, stress responses (including autophagy), cell death, and inflammation (fig. 6). Such inter-linking complicates causal inference and identification of potential therapeutic interventions which are both safe and effective (Chapter 7).

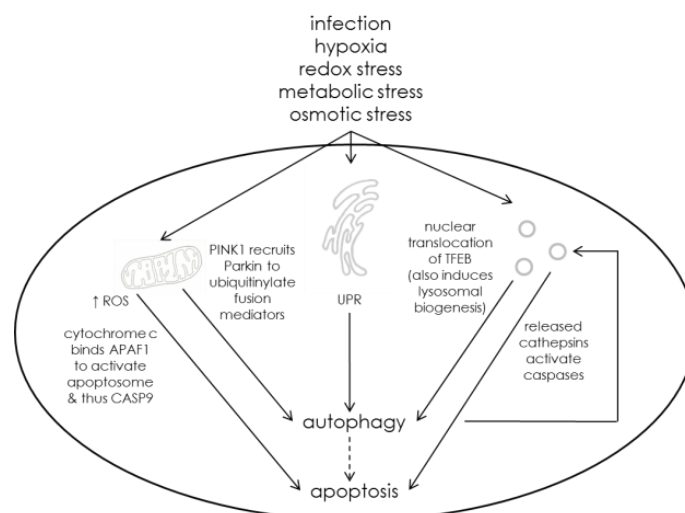


Figure 6: Key Molecules in Stimulus Integration by & Cross-talk between Mitochondria, Lysosomes, and the ER.

Functional overlaps exist at multiple levels between mitochondria, lysosomes, and the ER: shared stressors, shared/interacting response pathways and effectors, shared production/regulation of small inorganic molecules (e.g. Ca^{2+} , ROS), and shared/interacting cellular outcomes (e.g. autophagy, cell death). Additionally, intimate physical contact sites exist for molecule sharing. During stress conditions, all three organelles produce more ROS and release Ca^{2+} into the cytosol [94]. ROS not only mediate oxidative damage of biomolecules, but also influence transcription factor (including NFkB, Nrf2, and FOXO3), Ub-proteasome, metabolic enzyme, and autophagy functions. For example, redox stress induces an Nrf2-mediated stress-response, including heightened expression of SQSTM1/p62, favouring autophagy (including mitophagy and aggrephagy). Organelle dysfunction, ROS-mediated damage, and protein aggregate accumulation progressively limit capacity of the cell's stress-response systems to recover homeostasis. For example, impaired oxphos leads to a shortage of NAD^+ (normally required for redox homeostasis) and ATP (required for various cellular functions, including biomolecule phosphorylation and ATP-dependent chaperone activity). Ca^{2+} is a key second messenger in many signalling pathways (including elevated cytosolic Ca^{2+} being able to activate apoptosis or necrosis). Furthermore, many chaperones are also Ca^{2+} -regulated. Bcl-2 family proteins modulate both mitochondrial and ER Ca^{2+} dynamics [95]. Two master cellular nutrient/energy/metabolic state sensors and stress response regulators - mTOR and AMPK - function from within the lysosomal membrane [96]. Although AMPK activation (e.g. by increased energy requirements/decreased ATP availability, or increased cytosolic Ca^{2+}) inhibits mTOR to support autophagy, it also opposes aggresome formation (via lowering SQSTM1/p62 levels); perhaps an appropriate response to a state of energy/substrate starvation rather than biomolecule (e.g. protein) over-abundance. Simultaneously, mTOR inhibition activates TFEB to induce CLEAR network gene expression (supporting autophagosome formation and lysosome biogenesis) [96], and opposes initiation of protein translation [97]. In an AMPK-independent mechanism, infection-mediated increased AGS3 expression directly suppresses mTOR nutrient-sensing, also causing TFEB-mediated induction of CLEAR network gene expression. Additionally, both Ca^{2+} and ROS can induce the CLEAR network: high ROS levels activate lysosomal TRPML1 channels, facilitating Ca^{2+} release and calcineurin-dependent TFEB-mediated induction of autophagy and lysosome biogenesis [96]. TRPML1 is also a prerequisite for mitophagic clearance (concomitant with efficient removal of excess mtROS) of extensively-damaged mitochondria [96]. Interestingly, cell death causality can work in any direction: stressors may cause any of the above three organelles to trigger apoptosis, but - whichever is the earliest trigger - cell death pathways mediated by the others can be activated downstream to amplify death signalling (i.e. to ensure commitment to death) [98] [99] [100] [101] [102]. This incredible degree of functional inter-linking likely reflects: (a) cellular integration of multiple external and internal stimuli to determine appropriate cellular responses at any given time, (b) induction of a fairly general/overlapping adaptive stress response regardless of the specific stressor (i.e. each stimulus invokes most of the cell's major stress response pathways, although each response is somewhat specialised to combat a certain type of stress), and (c) convergence of many stress responses (e.g. in the case of prolonged/supra-maximal or maladaptive positive feedback cycle-inducing stressors) on cell death.

Thus, mitochondria, lysosomes, and the ER all act as nutrient/energy-sensing cellular hubs, integrating information from multiple metabolic pathways to determine ultimate appropriate cellular responses and fates. Mounting evidence associates aging and disease with the above cellular processes, and their pharmacological modulation for the purposes of health optimisation and disease prevention/cure are under intense scrutiny. Pharmacological targeting of key nodes in these interconnected networks "has high therapeutic potential", but it is crucial that these mechanisms (including their regulation, interactions, and functions) be more completely understood prior to more specifically-targeted pharmacological modulation, to prevent unforeseen consequences given system complexity [103].

For example, lysosomal dysfunction can impair mitophagy (and concomitant removal of excessive ROS), causing cellular dysfunction that can hypothetically be ameliorated by "correction of the primary lysosomal defect OR neutralisation of mitochondrial oxidative stress" [104]. However, it is important to clearly identify the most proximal pathology-causing deranged step or component of an effector pathway "before attempting to intervene" [105] [51]. Especially in this type of interconnected and inter-dependent system (where many branches involve second messengers common to multiple signalling pathways, and where most branches can also lead to cell death), the problem of multiple off-target effects can lead to significant unintended consequences. Conceptually, for example, preventing apoptosis would *only* be beneficial in the case of simultaneous alleviation of cell stress and induction of adaptive biological programmes which maintain normal cellular function. Similarly, pharmacological autophagy induction would only be beneficial if it were the early steps of the autophagy pathway that were compromised (if later steps, such as endolysosomal degradation, were deficient, autophagy induction may "further overload the cellular degradation system") [105]. Response inter-dependence, too, can give rise to unintended consequences (e.g. suppressing/enhancing function of the Ub-proteasome invokes enhanced/suppressed aggrephagy, and vice versa). Too, the anti-oxidant N-acetyl-cysteine exacerbates HeLa cell death induced by low concentrations of the ER stress inducer DTT (which transiently activate JNK and p38), but rescues cells when DTT concentrations are high (which persistently activates JNK and p38 while inhibiting ERK) [58].

One benefit of such interconnectedness, however, is emergence of a new therapeutic paradigm (particularly evident in the anticancer chemotherapeutic field), whereby a drug does not specifically target a dysregulated pathway, but acts as a cell stressor which leverages certain subsequently-induced adaptive compensatory mechanisms to combat the pathology of interest. For example, tunicamycin-induced mild ER stress provokes an adaptive autophagy response, producing neuroprotective effects in a Parkinson's Disease (PD) model [105]. In addition, a new therapeutic

paradigm is also perhaps required for drugs which impact multiple key pathways to produce an overall therapeutic effect. For example, inhibition of mTORC1 by the immunosuppressant rapamycin impacts mitochondria, lysosomes, and protein synthesis (among other effects) [106].

6.9. Potential Relevance to TB-IRIS Pathogenesis

Since the present study utilised neutrophils from TB/HIV-uninfected healthy volunteers and did not incorporate anti-mycobacterial antibiotics (Appendix O), no direct logical inferences may be drawn regarding involvement of EFV in TB-IRIS pathogenesis. However, various potential mechanisms plausibly link nucleoside (e.g. TDF) and non-nucleoside (e.g. EFV) reverse transcriptase inhibitor-, *Mtb*-, and anti-mycobacterial drug-induced cellular changes to positive-feedback pro-inflammatory cycles, and may thus be relevant to TB-IRIS pathogenesis. Indeed, multiple mitochondrial-, lysosomal-, and ER-mediated responses to cell stress (including various forms of cell death) converge on innate-type inflammation (Chapter 7).

6.9. Conclusions

Exposure of primary human neutrophils to an *in vivo*-comparable EFV concentration apparently rapidly induces changes suggestive of mitochondrial, lysosomal, and ER stress (along with certain relevant stress responses, such as mitochondrial fission and aggrephagy). Such changes in organellar architecture require altered protein expression, and reflect functional alterations. Furthermore, *Mtb* antigen pre-exposure may induce at least lysosomal stress responses which increase resilience to subsequent EFV exposure, but may also interfere with EFV-induced adaptive stress responses once they become supra-maximal (maladaptive). This is in agreement with concepts regarding *Mtb*-mediated inhibition of (e.g. EFV-mediated) mtROS production; consequent lysosome permeabilisation and autophagy; and stress response-induced apoptosis (in favour of pro-inflammatory necrosis/pyroptosis).

6.10. Study Strengths, Shortcomings, and Future Directions

While every effort was made to optimize study design, execution and interpretation revealed potential shortcomings and raised additional questions.

Instead of a potentially non-representative cell line or animal model, the present study utilised the specific cell type of interest (live primary human neutrophils) in the presence of other circulating autologous leukocytes and serum soluble factors to mimic *in vivo* conditions as closely as possible. To our knowledge, this is the first study to investigate the effect of EFV on human neutrophils. However neutrophils were derived from TB/HIV-uninfected healthy volunteers (rather than TB-IRIS patients), and the present study did not investigate the effect of anti-mycobacterial antibiotics, such that no *direct* logical inferences may be drawn regarding involvement of EFV in TB-IRIS pathogenesis. While culture dish well number limited the number of examinable conditions in the present study, it would be informative to include additional conditions, e.g. *Mtb* antigens in isolation, other ARVs, anti-mycobacterial antibiotics, and positive controls for mitochondrial fragmentation/mitophagy, lysosomal biogenesis, and ER/protein-folding stress.

While every effort was made to control experimental conditions apart from exposures of interest, microscopy setup and dependence on availability of microscopy unit personnel made it difficult to ensure a consistent time-interval between cell stimulation and microscopic observation on different days. This resulted in observing earlier and later effects of EFV exposure (e.g. on mitochondrial stress responses). An ideal future experiment would image live cell responses to EFV $\pm$ *Mtb* antigens over-time, in order to observe the course of mitochondrial (and other organelle) changes in real time. An alternate strategy to partially validate the immunometabolic aberrations suggested by proteomic

results would be use of an instrument capable of measuring healthy volunteer live neutrophil metabolic flux (e.g. ratios of glycolysis to mitochondrial respiration/fatty acid oxidation) before and after exposure to contextually-appropriate stimuli.

While ThT and LysoDye emission wavelengths nearly overlap, their maxima differ by a not insignificant 40nm. Results which may at first glance appear to be due to overlapping wavelengths actually seem (based on careful image inspection) to represent staining of independent structures in close proximity. Given the close co-operation and apposition of the lysosomal, ER, and mitochondrial membranous networks, this is not unlikely. It is also known that protein aggregates preferentially bind mitochondria as well as trafficking into lysosomes (or lysosome-continuous membranous compartments). These speculations remain to be empirically verified. Although little apparent co-localisation of mitochondria and lysosomes was observed, induction of mitophagy by EFV could be more thoroughly investigated by demonstrating the presence of autophagic vacuoles and autophagy marker proteins. It must further be determined what dosages of EFV induce autophagy/mitophagy rather than frustrating autophagic/mitophagic flux (thereby resulting in autophagic stress, cell damage, and apoptosis).

Caveats regarding study conclusions: the effects of EFV may represent only general xenobiotic induction of cell stress rather than an NNRTI-specific phenomenon. Additionally, *in vivo* effects on viral load (in TB/HIV-coinfected patients rather than healthy volunteers) may actually induce an *adaptive* return towards baseline cellular function, despite xenobiotic stress. Nevertheless, it remains plausible that neutrophil stress induction by EFV contributes to the pathogenesis of TB-IRIS, and future studies may confirm the existence of specific and targetable mechanistic links.

Appendix L Method Development: Live-cell Fluorescence Microscopy Optimisation

L.1. Whole Diluted Blood: Confocal Two-colour Proof-of-Concept

As a proof of concept, we successfully applied fluorescence microscopy to a simple whole blood assay. We determined that confocal fluorescence microscopy is capable of visualising (with high resolution) unstained leukocytes and GFP-labelled *M. smegmatis* in whole blood (diluted 1 in 20 with PBS), without significant interference from background autofluorescence in the relevant emission spectra channels (not shown). However, the fluorescent lysosome probe (LysoTracker Red dnd-99) added to the diluted whole blood did not successfully stain its target organelle, likely due to sequestration by plasma proteins.

L.2. Whole Diluted Blood: Non-confocal Three-colours for Mitophagy/ER Stress

After obtaining the appropriate probes for detection of mitophagy (Mtphagy and LysoDye) and β -amyloid/ER stress (Thioflavin T (ThT)) in live cells, we again applied fluorescence microscopy to a simple whole blood assay assessing low, medium, and high concentrations of ThT (5, 10, and 20 μ M), Mtphagy (100, 200, and 400 μ M), or LysoDye (1, 2, and 4 μ M) individually, as well as a combination of the middle concentration of all three probes. We determined that a non-confocal inverted fluorescence microscope (axioscope) is capable of visualising (with poor resolution) stained leukocytes in whole blood (diluted 1 in 20 with PBS), without significant interference from background autofluorescence in the relevant emission spectra channels (not shown). However, high numbers of erythrocytes interfered with leukocyte visualisation, and only the ThT signal could be detected (at all concentrations, singly or in combination with other probes) in areas apparently co-localising with leukocytes. Neither Mtphagy nor LysoDye successfully stained their target organelles (at any concentrations tested), likely due to sequestration by plasma proteins (specified by the probe manufacturer to interfere with staining). Thus, while the ER stress assay may employ whole diluted blood, the mitophagy assay will require leukocyte isolation prior to staining, re-addition of autologous serum, and addition of appropriate stimuli.

L.3. Whole Diluted Blood: Confocal Imaging Improves Resolution of ThT Visualisation

To determine whether confocal imaging could provide improved resolution of ThT stained leukocytes in diluted whole blood, we imaged varying whole blood/PBS dilutions (1 in: 25, 50 and 100) stained with the middle concentration (10 μ M, as above) of ThT. Venous blood was obtained from a single healthy volunteer (female, 32) into an Na-heparin vacuette, and 2ml was diluted in 48ml warmed PBS to attain a 1 in 25 dilution, which was agitated gently but thoroughly for cell dispersal before plating. Of this, 5ml was taken forward into 5ml PBS to obtain a 1 in 50 dilution, which was mixed thoroughly before plating. Of this, 5ml was taken forward into 5ml PBS to obtain a 1 in 100 dilution, which was mixed thoroughly before plating. Each dilution was plated in glass-bottom culture dishes (NEST Scientific) in the dark: 996.8 μ l diluted blood + 3.2 μ l of 3.14mM ThT stock (swirled to mix). Dishes were covered in foil and incubated (30°C, 5% CO₂) for 30min prior to imaging. Imaging (fig. 1) was performed on a Zeiss LSM 880 with Airyscan confocal microscope, using Zen software (version 2.3 Service Pack 1), the 10x objective (maximal magnification, limited by NEST dishes), a 405nm laser for ThT excitation, and an emission range of 400-519nm (blue). Additionally, a differential interference contrast image (transmitted light) was captured using transmitted photo-multiplier tube white light.

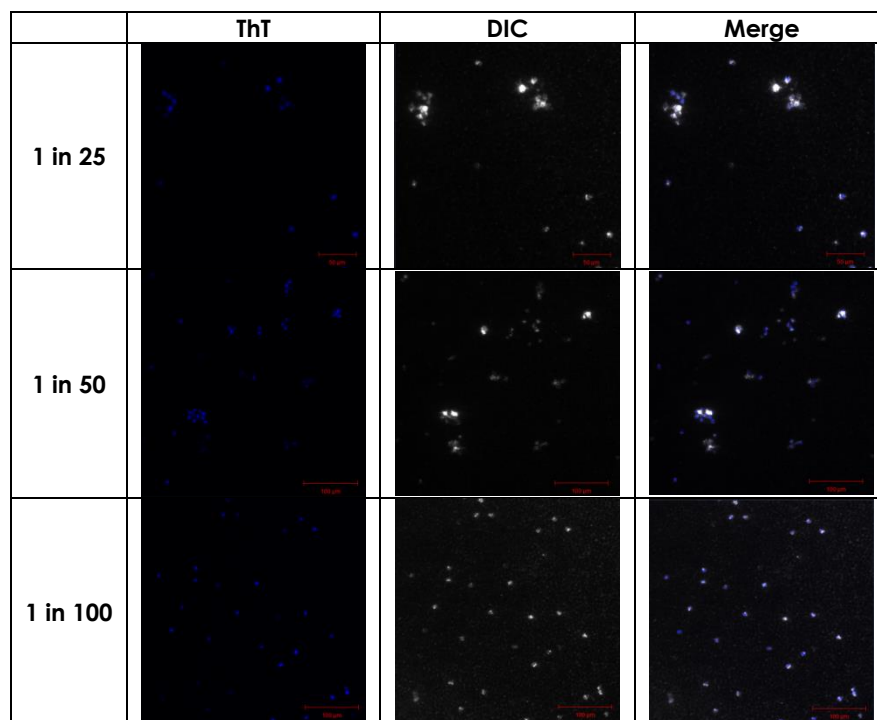


Figure 1: ThT-stained Leukocytes in Various Whole Blood Dilutions (100x)

The above methods facilitate high-resolution visualisation of ThT-stained leukocytes, despite a high number of erythrocytes, with no interfering autofluorescence in the ThT emission range/channel. ThT successfully stains leukocytes in all tested whole blood dilutions, appearing to target discrete subcellular structures which are most likely aggregates. Varying the dilution factor had little impact on erythrocyte density (and thus leukocyte visibility), due to an artefact arising from plated cells tending to congregate at plate well edges (i.e. different dilutions of whole blood generate a similar microscopic appearance). Thus, live-cell microscopy of ThT-stained whole blood appears to be a viable assay for stimuli which may impact protein folding/ER stress. ThT is inexpensive, water-soluble, compatible with live cells, and easy to use. The assay approach is relatively rapid (~1 hr for phlebotomy, dilution, plating, stimulation, and staining, plus ~1.5 hr for imaging).

L.4. Whole Diluted Blood: Confocal, Isolated Cells with Autologous Plasma, Two-colour

To determine whether isolated leukocytes (instead of diluted whole blood) can be successfully stained with MitoTracker and LysoTracker prior to re-addition of autologous plasma, we obtained 16ml healthy volunteer (male, 34) venous blood into Na-heparin vacutainers prior to Ficoll-based PBMC and dextran-based neutrophil isolation (as described in Chapter 2, with the following modifications). After the Ficoll spin, autologous plasma was reserved. Instead of cryopreservation, PBMC (live yield: 91.2×10^6 , 49% of total yield) were resuspended in PBS to 300 000/ml and 3M/ml. After discarding the supernatant post-erythrocyte lysis, neutrophils (total yield: 38.4×10^6) were resuspended in 50ml RPMI, spun at 500g for 5min, the supernatant was discarded, and neutrophils were again resuspended in PBS to 1M/ml and 10M/ml (to ~preserve *in vivo* ratios when plating). Both low and high cell concentrations were plated (in the dark) in two separate four-compartment tissue-culture petri dishes (Cell View 35mm, Lasec), using four identical conditions: 249ul of each cell type per well, $\pm$ 56ul autologous plasma to an in-well concentration of 10%, both conditions unstained, or stained with 1ul each of both probes (for a final in-well medium concentration, as above: 200nmol/L MitoTracker and 2umol/L LysoTracker), and swirled to mix. After probe addition, plates were covered in foil and incubated (30°C, 5% CO₂) for 20min to allow the probes to accumulate in mitochondria and lysosomes prior to addition of autologous plasma and a

further 30min incubation, prior to imaging. Imaging (fig.s 2 and 3) was performed with a Zeiss LSM 880 microscope, with Airyscan confocal, 63x oil objective (optical magnification x2), excitation lasers 561nm (red, Mtphagy) and 488nm (green, LysoDye), emission bands 655-748nm and 499-566nm. The negative control (no stain, no plasma; not shown) did not exhibit any background autofluorescence in these emission ranges, and was used as the basis on which microscopy parameters were developed. Microscopy software was Zen 2.3 Service Pack 1.

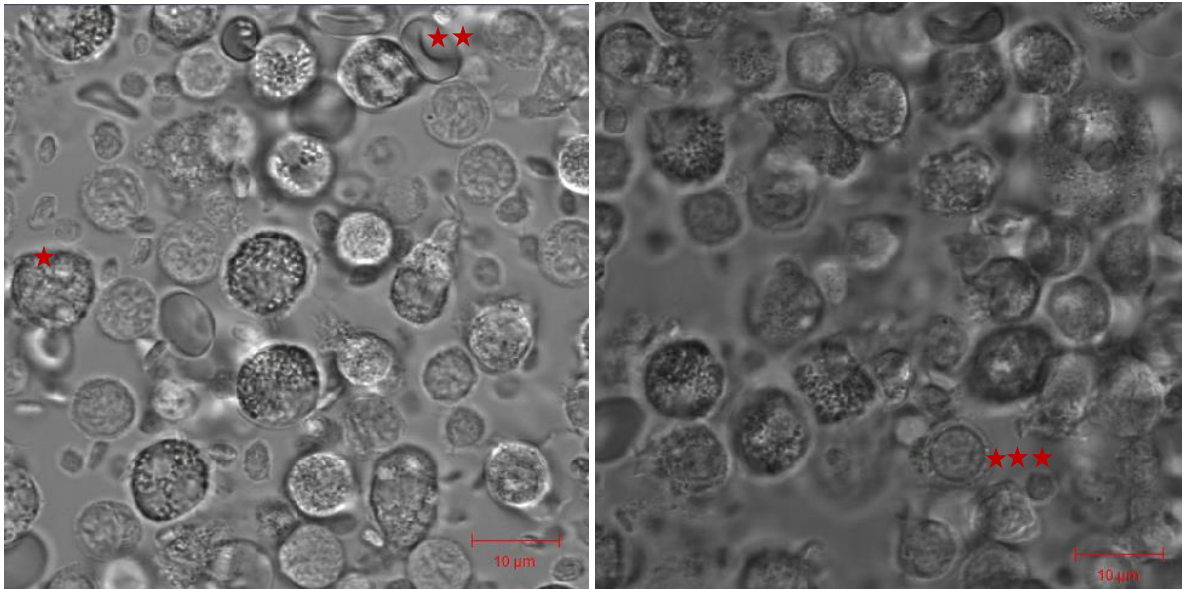


Figure 2: DIC Imaging of Recombined Isolated PBMC (3M/ml) and Neutrophils (10M/ml) (1500x) Large granular cells are polymorphonuclear lymphocytes (visible polymorphic nucleus *indicated). Although every effort was made to remove erythrocytes, some (**indicated) are still present. A probable lymphocyte (small, less granular, rounded nucleus) is also ***indicated.

	Mtphagy	LysoDye	Merge
low cell concentration - plasma			
low cell concentration + plasma			
high cell concentration - plasma			

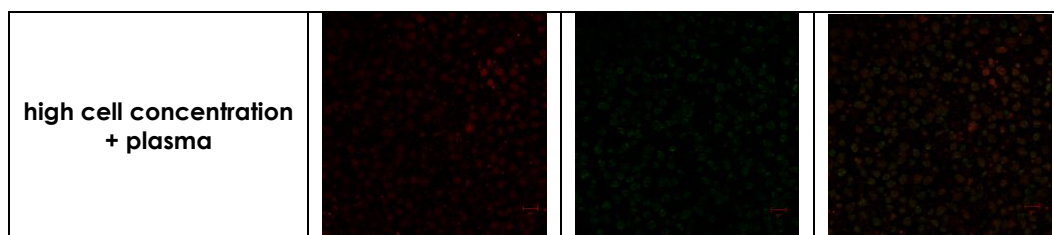


Figure 3: Mtphagy and LysoDye Co-stained Isolated Leukocytes $\pm$ Autologous Plasma (650x and 65x)

The above methods facilitate high-resolution visualisation of Mtphagy and LysoDye co-stained isolated leukocytes plated at both low and high concentrations, with no interfering autofluorescence in these emission ranges. Mtphagy and LysoDye successfully stain leukocyte mitochondria and lysosomes, though subsequent addition of autologous plasma appears to result in less bright fluorescence. Very bright Mtphagy/LysoDye fluorescence may also be a result of over-staining; the “low” in-well probe concentrations will be used in the final experiment. More red (mitochondrial) than green (lysosomal) staining can be observed overall, and there is apparent co-localisation of these structures in certain cells (suggesting mitophagy), especially in the low cell concentration without plasma (perhaps such cells experience relatively more ex vivo stress). Relatively more green appears to co-localise with red than the other way around, likely due to relatively more red than green staining. Thus, live-cell microscopy of Mtphagy/LysoDye co-stained isolated leukocytes in the presence of autologous plasma appears to be a viable assay for mitophagy. Relatively high concentrations of PBMC make it difficult to visualise neutrophils, so isolated neutrophils (with up to 20% “contaminating” PBMC) only will be used in the final assay. Mtphagy and LysoDye probes are expensive, DMSO-soluble, compatible with live cells, and easy to use. The assay approach is relatively slow (~6hrs for phlebotomy, cell isolation, dilution, plating, staining, plus ~1.5hr for imaging). The assay is apparently ready for use to examine the effects of EFV $\pm$ Mtb antigens on mitophagy.

Appendix M Microscopy Results: Impact of EFV ± *Mtb* Antigens on Lysosomes and Aggresomes

*M.1. EFV induces a reduction in lysosome fluorescence intensity (partially mitigated by the presence of *Mtb* antigens)*

Relative to control, EFV induces no obvious change in lysosomal number, size, or punctate shape (except in HV3 neutrophils, which exhibit more numerous, smaller lysosomes under this condition). Similarly, no obvious consistent change in lysosomal localisation occurs, although intensely-fluorescent lysosomes appear to occur more peripherally (at least in HV3 and 4). Overall, EFV exposure results in a decrease in lysosomal fluorescence intensity (except in HV3, whose neutrophils exhibit the opposite response), with nuclear envelope region fluorescence exhibiting no change (HV1 and 4), enhancement (HV2), or diminishment (HV3). Little to no obvious apparent co-localisation of lysosomes with aggresomes (indicated by red arrows) is observed under this condition, except in HV3 in which such apparent co-localisation is very common (an individual- rather than condition-specific effect, as it persists across all conditions for HV3). It is difficult to formulate a consistent interpretation explaining these findings. However, it is possible that cell stress (e.g. oxidative stress resulting from mitochondrial dysfunction) leads to lysosomal membrane permeabilisation, leading to dispersal of lysosome contents (including probe targets) and diminished fluorescence intensity. Additionally, HV3 neutrophils may experience higher levels of protein misfolding, leading to aggrephagy and lysosomal biogenesis.

Relative to EFV-only, pre-exposure to *Mtb* antigens prior to EFV treatment apparently induces production of a greater number of smaller lysosomes (HV1 and 4), though no obvious change in lysosome number, shape, or size is noted for HV2 and 3. Similarly, no obvious change in lysosomal localisation occurs, and – at least in HV2 - intensely-fluorescent lysosomes appear to continue occurring more peripherally. *Mtb* antigen pre-exposure appears to prevent the overall suppression in fluorescent intensity induced by EFV (again, except in HV3), but does not affect nuclear envelope region fluorescence. Only minor apparent co-localisation of lysosomes with aggresomes (indicated by red arrows) is observed under this condition (again, except in HV3, in which such apparent co-localisation is common). It is again difficult to formulate a consistent interpretation explaining these findings. However, findings suggest that *Mtb* pre-exposure induces lysosomal biogenesis (in at least two HVs) and may prevent lysosomal membrane permeabilisation (in at least three HVs).

M.2. EFV induces a reduction in the number of larger intensely-fluorescent aggresomes

Relative to control, EFV induces a subtle increase in the number of (especially smaller) puncta, which may also be more dispersed (i.e. there are fewer intensely-fluorescent peripheral puncta, except in HV3, and ThT staining appears more dispersed throughout the cytoplasm), but there is no change to punctate shape. Overall, fewer larger intensely-fluorescent puncta are observed (though in HV1 smaller puncta remain intensely fluorescent). Only certain cases (HV1 and 2) exhibit enhanced nuclear envelope region fluorescence. No obvious apparent co-localisation of aggresomes with mitochondria is observed under this condition. Overall, findings suggest that EFV may lead to increased protein synthesis (and perhaps misfolding) throughout the ER network, but opposition of formation (or resolution) of large aggresomes. Since large aggresomes are present at baseline (control), resolution (e.g. aggrephagy) seems the more likely explanation, as is supported by generally higher numbers of apparent lysosome-aggresome co-localisation events (relative to control) in at least three HVs.

Relative to EFV-only, pre-exposure to *Mtb* antigens prior to EFV treatment apparently does not impact the number, size, shape, fluorescent intensity of aggresomal puncta, or fluorescent intensity of the nuclear envelope region. Only certain cases (HV1 and 2) exhibit a few larger intensely-fluorescent puncta. Little to no obvious apparent co-localisation of aggresomes with mitochondria is observed under this condition. Overall, findings suggest that it is only at the later/more severe stages of mitochondrial dysfunction (HV1 and 2) that *Mtb* antigen pre-exposure opposes the responses to EFV (e.g. aggrephagy – supported by generally fewer apparent lysosome-aggresome co-localisation events, relative to EFV-only, in HV1, 3, and 4), perhaps via mechanisms similar to those discussed for mitochondria (*vide supra*).

Appendix N Additional Microscopy Results

Medium (2x) electronic magnification images were considered most representative of EFV-induced organelle changes, and are therefore included in the main text body (*vide supra*). An exception is HV2, whose representative low (1x) electronic magnification images were included in the main text body for a more close-up organelle view (to discern ultrastructural characteristics). Remaining low to high (3x) electronic magnification images (fig.s 4-14) are included here (including for post-infectious HV5). Although optical magnification includes a 2x lens and 63x oil-immersion lens (in combination with electronic magnification), all magnifications reported here are calculated based on scale bar (automatically embedded by image acquisition software). Lowest-magnification images provide a good overview of overall cell behaviour per condition.

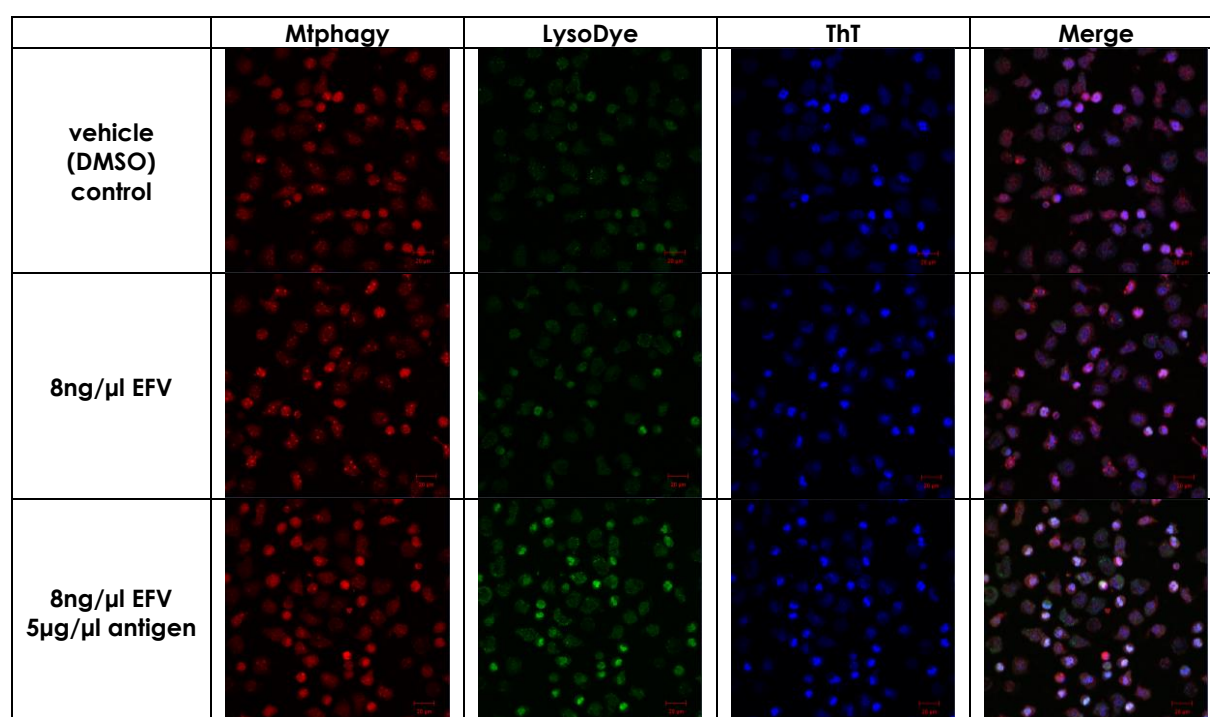
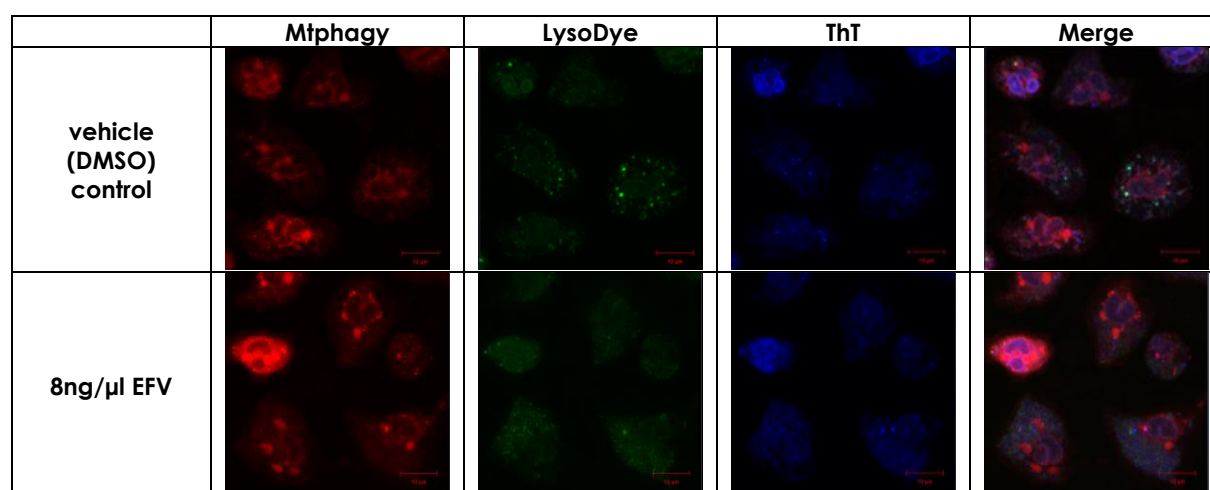


Figure 4: Fluorescence Micrographs of HV1 Neutrophils
Exposed to Control and Experimental Conditions (low magnification; here 200x)



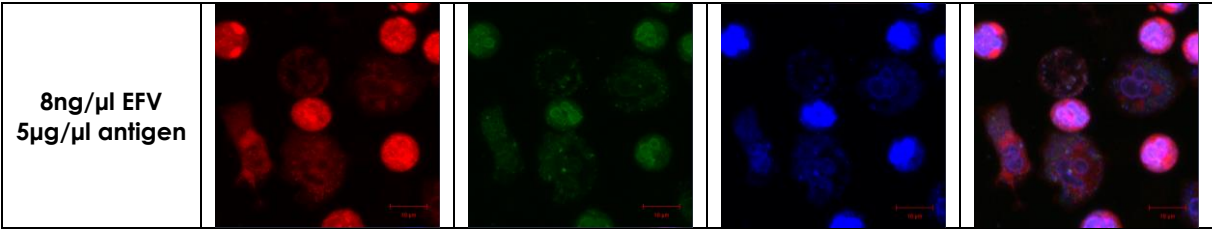


Figure 5: Fluorescence Micrographs of HV1 Neutrophils
Exposed to Control and Experimental Conditions (high magnification; here 800x)

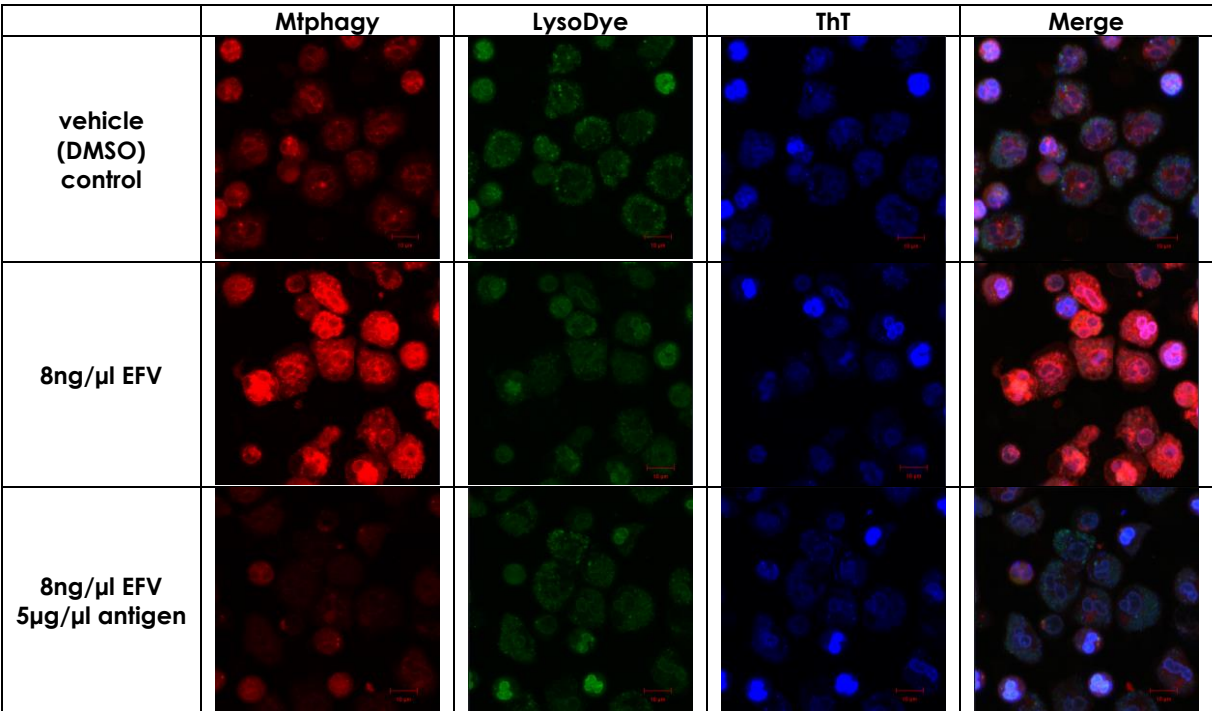
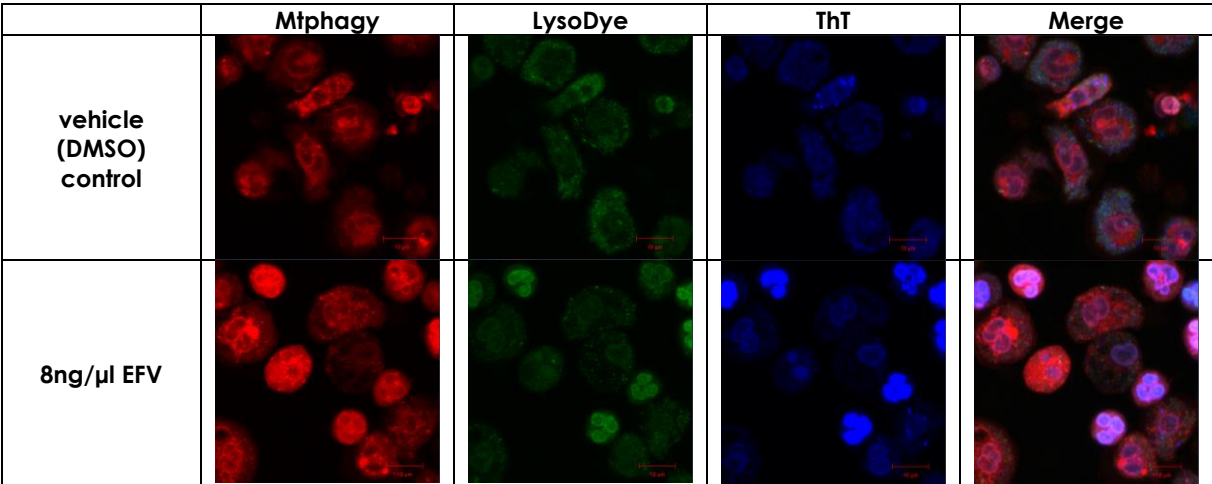


Figure 6: Fluorescence Micrographs of HV2 Neutrophils
Exposed to Control and Experimental Conditions (low magnification; here 600x)



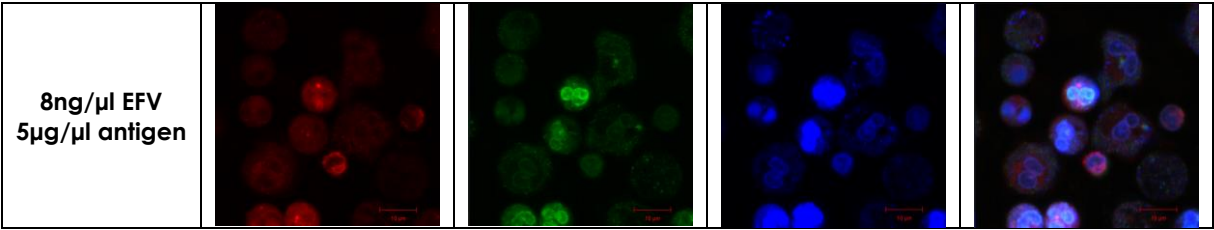


Figure 7: Fluorescence Micrographs of HV2 Neutrophils
Exposed to Control and Experimental Conditions (medium magnification; here:800x)

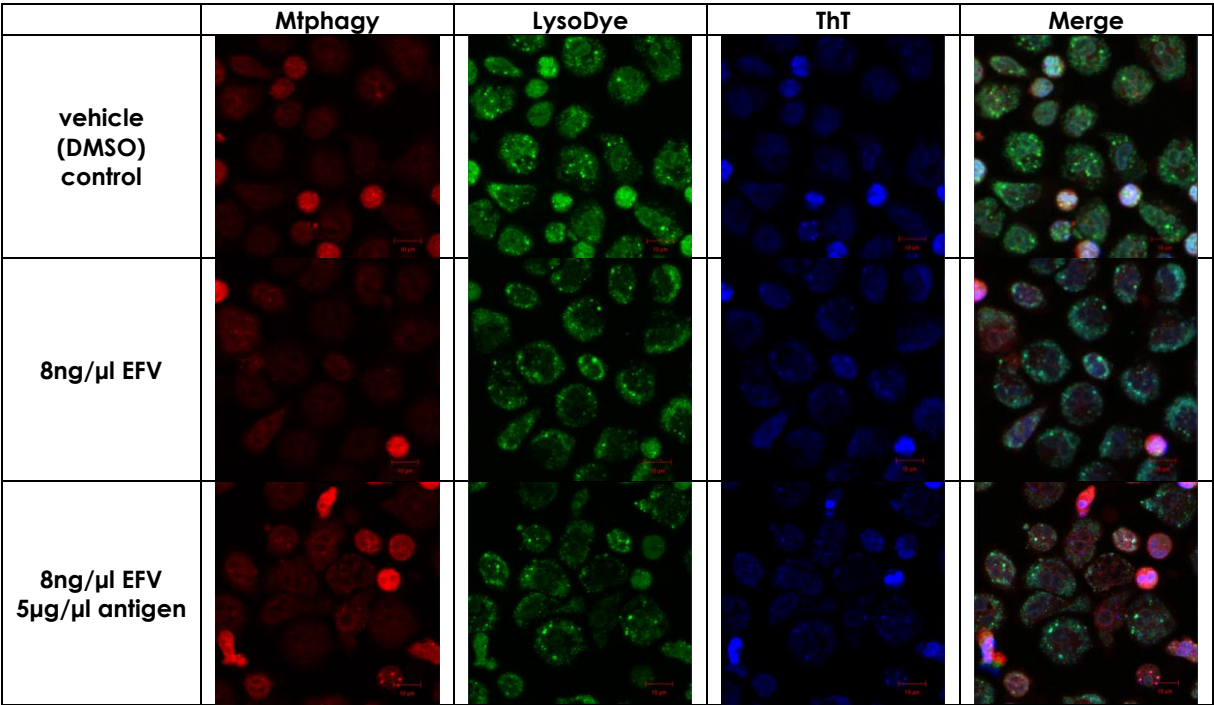


Figure 8: Fluorescence Micrographs of HV3 Neutrophils
Exposed to Control and Experimental Conditions (low magnification; here 600x)

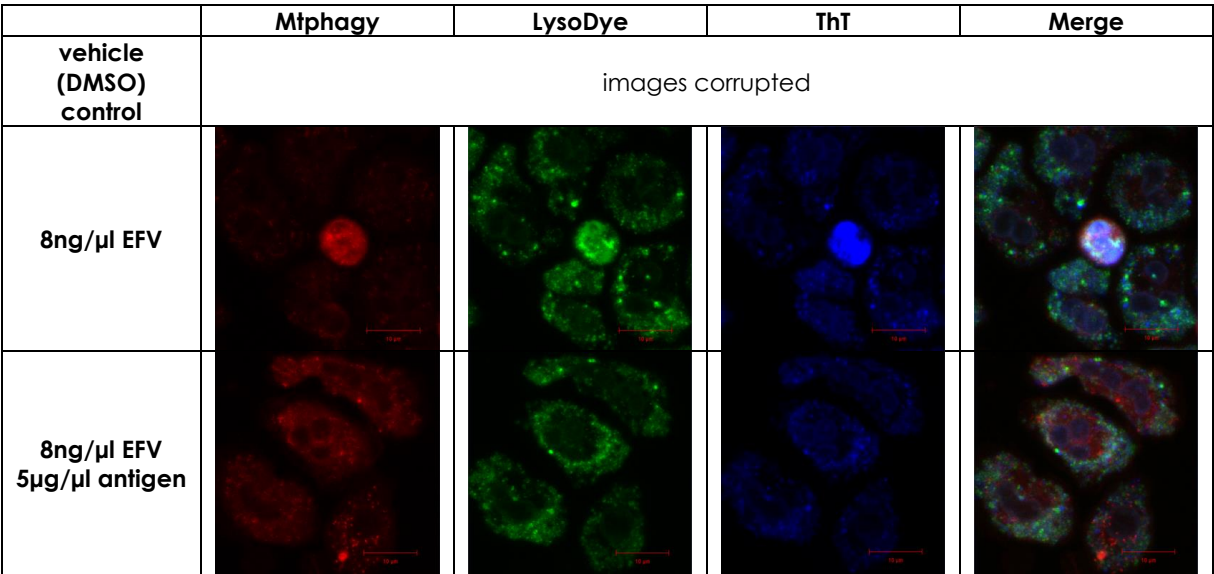


**Figure 9: Fluorescence Micrographs of HV3 Neutrophils
Exposed to Control and Experimental Conditions (high magnification; here 1100x)**

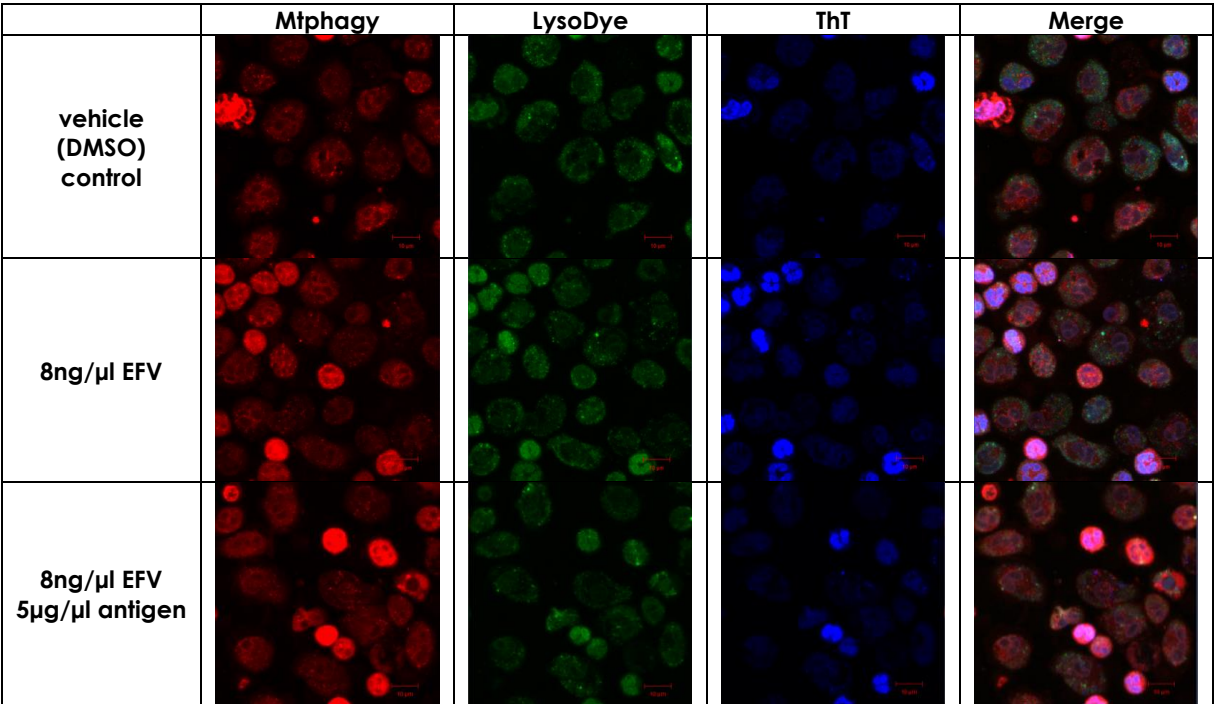


**Figure 10: Fluorescence Micrographs of HV4 Neutrophils
Exposed to Control and Experimental Conditions (low magnification; here 550x)**

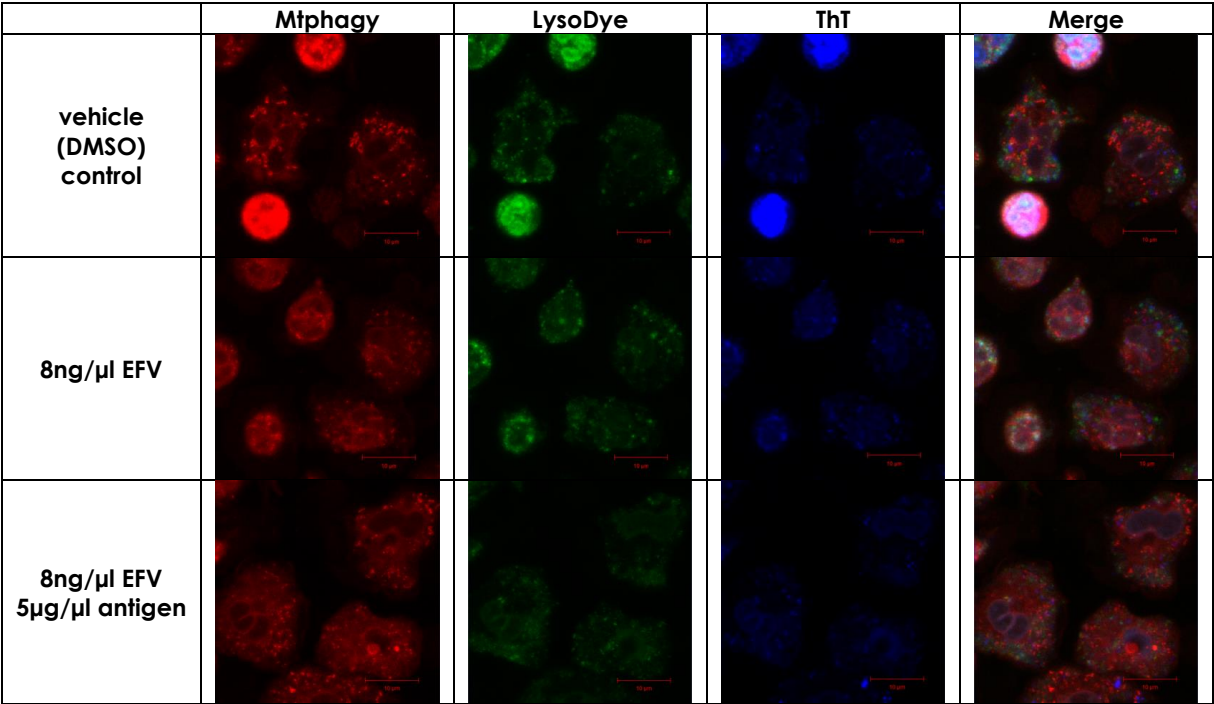


**Figure 11: Fluorescence Micrographs of HV4 Neutrophils
Exposed to Control and Experimental Conditions (high magnification; here 1100x)**

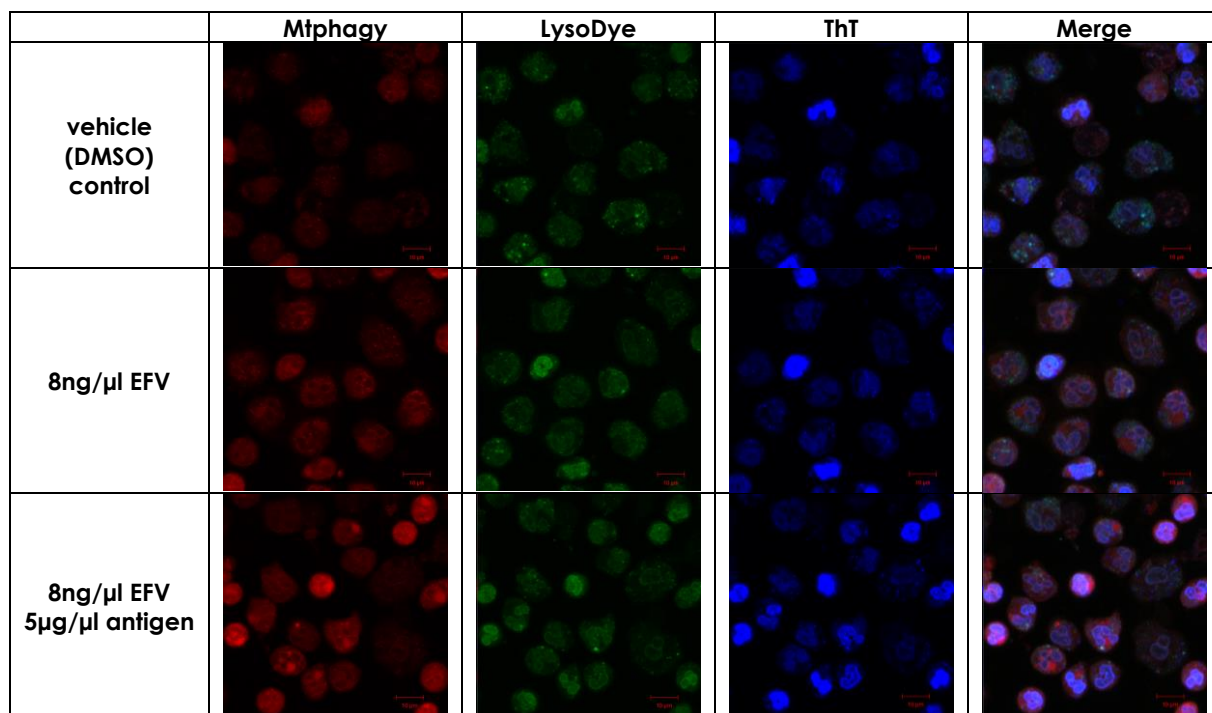


**Figure 12: Fluorescence Micrographs of HV5 Neutrophils
Exposed to Control and Experimental Conditions (low magnification; here 550x)**

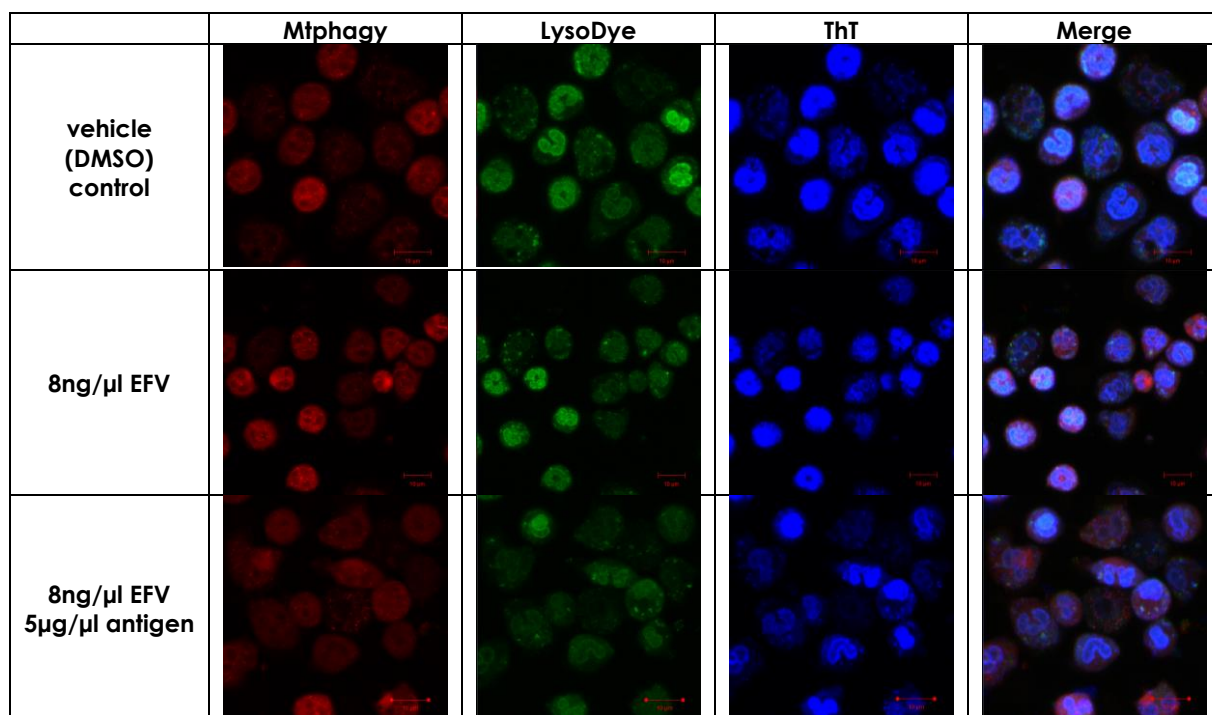


**Figure 13: Fluorescence Micrographs of HV5 Neutrophils
Exposed to Control and Experimental Conditions (medium magnification; here 800x)**

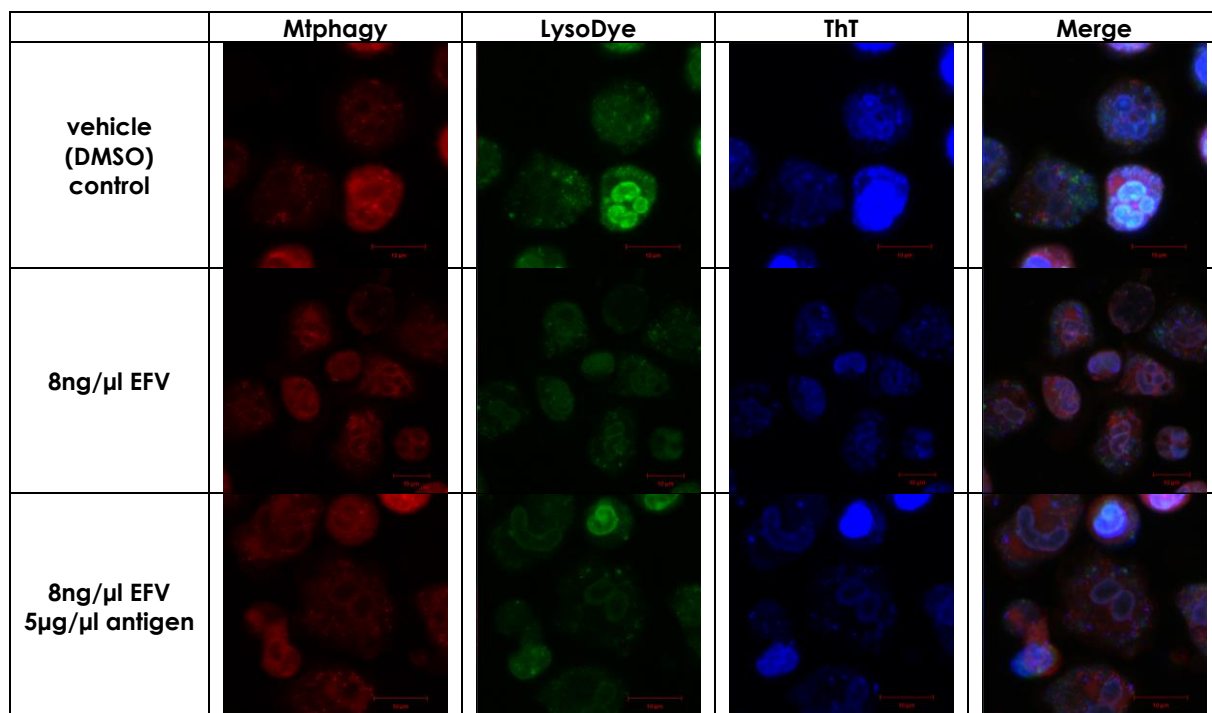


**Figure 14: Fluorescence Micrographs of HV5 Neutrophils
Exposed to Control and Experimental Conditions (high magnification; here 1100x)**

Appendix O Findings in Context: Anticipated Anti-mycobacterial Antibiotic Effects

While the effect of anti-mycobacterial antibiotics was not investigated by the present study, literature regarding the cellular effects of these is included as it is relevant to the *in vivo* situation in which TB-IRIS arises, and must be considered during hypothesis generation.

Mitochondria

It is known that INH is toxic to mitochondria [107]. At least in human HepG2 cells, INH lowers the number of mitochondria, causes mitochondrial fragmentation, down-regulates expression of mitochondrial COX IV, lowers membrane potential, induces mitochondrial swelling, possibly increases the likelihood of apoptosis, and impairs mitochondrial biogenesis through down-regulation of SIRT1, PGC1 α , and NRF1 [107]. In response to INH-induced stress, p38 MAPK signalling partially recovers mitochondrial biogenesis [107]. Both INH and PZA enhance NOX2- and mitochondrion-mediated ROS generation in Mtb infected cells, thereby directly promoting autophagy [90]. Taken together, these effects suggest that the presence of anti-mycobacterial antibiotics may enhance EFV mitochondrial toxicity.

Lysosomes & Autophagy

An early clinical study demonstrated that rifampicin and INH significantly elevated plasma levels of predominantly lysosomal liver enzymes (e.g. β -glucuronidase, and β -N-acetylgucosaminidase), suggesting an increase in "synthesis and release" [108]. A more recent study [109] reports that rifampicin and INH co-treated rats exhibit elevated liver lysosomal enzyme activity (mediated by oxidative stress, including lipid peroxidation). Indeed, rifampicin and INH are synergistically hepatotoxic, perhaps via rifampicin-mediated induction of CYP450, which results in conversion of INH-derived toxic hydrazine (which also depletes GSH reserves) to a hepatotoxic compound [109]. Recall that one xenobiotic detoxification system is conjugation of an acetyl group (donated by acetyl-CoA) by N-acetylase (induced by ethanol) to drugs such as INH and hydralazine [49].

Kim et al. (2012) demonstrated that both INH and PZA significantly elevate all-source ROS in Mtb-infected macrophages, with bacterially-generated hydroxyl radicals triggering autophagy (which was required for drug efficacy, and which "dampened Mtb-induced" macrophage pro-inflammatory responses) [110]. Similarly, Zhang et al. (2016) demonstrate that INH treatment of human HepG2 cells induces mTOR pathway inhibition-associated autophagy and (both extrinsic and intrinsic pathway) apoptosis (with both autophagy and p38 MAPK signalling opposing apoptosis via down-regulation of CASP-3 and -9 and PARP pathway responses) [111]. INH also induces autophagy in *M. marinum*-infected (but not uninfected) murine RAW 264.7 macrophages, in a ROS generation-dependent manner [112] [113]. In addition, INH did not impair lysosomal degradation [111]. Similarly, a study screening the effect of common anti-tuberculous drugs on the activity of purified brain CTSB, L, and H (the major lysosomal cysteine proteases) demonstrated that while rifampicin inhibited these enzymes, INH only weakly inhibited them (PZA and streptomycin had no effect, despite streptomycin being lysosomotropic and rapidly accumulating in lysosomes) [114].

Many substances (e.g. metal ions, organic molecules, metabolites, certain drugs) are lysosomotropic, and their accumulation can alter lysosomal functions [114]. Interestingly, ethambutol (at least in rat retinal cells) impairs autophagic flux, and (in mammalian cell lines) accumulates in autophagosomes and enlarged and de-acidified lysosomes exhibiting concurrent Zn²⁺ accumulation, suggesting that this drug impairs autophagy through lysosome neutralisation [115]. Taken together, these effects suggest that at least the major anti-mycobacterial antibiotics (e.g. INH, PZA) likely *enhance* autophagy and lysosomal function.

The ER & Protein Homeostasis

Rifampicin treatment of L02 cells up-regulates GRP78 (a UPR hallmark) in a dose- and time-dependent manner, as well as activating expression of eIF2 α [116]. An early study [117] demonstrated via electron microscopy that INH-treated young rats underwent proliferation of hepatic rough ER (RER), as well as exhibiting significant depletion of blood and liver GSH (and related thiols), with concomitant elevation

of glutathione reductase activity and "significant decreases in glutathione peroxidase and catalase activity, indicative of a diminished capacity to handle H₂O₂ and lipid peroxides" (aggravated by moderate to severe PEM). Similarly, Zodape (2011) observed via liver histopathology that INH-treated rats exhibited RER proliferation [118]. It has also been shown that after INH treatment of mice the Chinese herb DMB exerts a hepatoprotective effect, with simultaneous down-regulation of the UPR (GRP78) and the ATF4-CHOP pathway, suggesting that INH-induced ER stress contributes to hepatotoxicity [119]. Taken together, these effects suggest that anti-mycobacterial antibiotics likely induce ER stress and the UPR.

Putting It All Together

It is evident from the literature that EFV, *Mtb*, and anti-mycobacterial antibiotics exert both synergistic and opposing effects on mitochondrial, lysosomal, and ER functions (consequently modulating autophagy and cell death). In a very rough manner, we can integrate existing information regarding these effects to hypothesise what may happen in the presence of all three agents (Table 1).

Table 1 Effects of EFV, *Mtb*, and INH on Organelle Functions

	mitochondria	lysosomes	ER	Autophagy	Cell Death	References
EFV	↓ oxphos, ATP ↑ ROS ↓ polarisation Ca ²⁺ release	↑ ROS-mediated permeabilisation	↑ ER stress, UPR Δ morphology	↑/↓ autophagy, mitophagy, aggregophagy	n/a	[4] [5] [2] [9] [10] [11] [12] [26] [27] [28] [30] [29] [60]
<i>Mtb</i>	↓ oxphos ↑/↓ ROS ↑ lactate, ATP ↑ polarisation ↑ membrane integrity Δ protein expression ↓ fragmentation	↑ ROS-mediated oxidation & permeabilisation ? ↑ CLEAR network induction	↑ ER stress	↑/↓ autophagy, aggregophagy	virulent <i>Mtb</i> : ↓ apoptosis (including mitochondrially-mediated) ↑ necrosis (including lysosomal-mediated)	[90] [46] [111] [87] [89] [91] [108] [110] [114] [115]
INH	mitotoxic ↓ mtCOX IV expression ↑ fragmentation, swelling ↓ mitochondrial biogenesis	(with PZN) ↑ all-source ROS (with rifampicin) ↑ enzyme expression & activity ethambutol neutralises lysosome pH	↑ RER proliferation (? lumen expansion) rifampicin ↑ UPR ? ↑ ER stress Zidovudine-induces steatosis is associated with ER stress & fat accumulation [?]	↑ autophagy (mTOR inhibition- & ROS-mediated) PZN also ↑ autophagy	↑ apoptosis	[107] [111] [108] [109] [110] [115] [116] [117] [118] [112]
Synergies	↓ mitochondrial oxphos	↑ ROS-mediated lysosomal damage	↑ ER stress, UPR	n/a	n/a	
Oppositions	ROS, fragmentation, membrane potential, mitophagy	n/a	auto/aggregophagy	autophagy	apoptosis	

Increased ROS levels stimulate autophagy.

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Chapter 7

Discussion and Conclusions:

Integrating Context, Literature, Proteomic Findings, and Microscopy Findings to Generate Hypotheses Regarding TB-IRIS Pathogenesis, Risk Stratification, Prevention, and Therapeutics

"When you understand something, you're able to perceive its structure: its connections, relationships, and significance relative to everything else. Context ... is the field of these connections [that] we plug new information into. When something is contextualised, we suddenly get it. It feels 'meaningful' because it fits into the [framework] we already know and understand. The more you understand information in this way (the more connected and contextualised it becomes), the more it starts to morph and grow into knowledge. It self-organises ... horizontally, across subjects, and ... vertically ... [becoming] both deeper and higher. When it does, we talk of 'deep understanding'."

- David McCandless, Knowledge is Beautiful

Abstract

Background TB-IRIS arises against a complex biological background featuring the modulation of host immunity by interacting bacterial and viral infections and their multi-component pharmacological therapies. This complicates prediction and understanding of the system, especially disentanglement of cause and effect. **Methods** The complex immunological context within which TB-IRIS arises is reviewed - including host responses to and modulation by Mtb, HIV, as well as host and pathogen responses to and modulation by ATT and ARV therapy – and this biological system is viewed through the lens of Complexity Theory. First we integrate existing literature findings to generate the most parsimonious model able to explain these findings, highlighting remaining questions regarding TB-IRIS predisposition and pathogenesis. Then we summarise the immunometabolic changes suggested by neutrophil proteomic findings, and provide contextualising information demonstrating the bi-directional links between metabolic and immune components implicated by proteomic findings. This includes discussing feedback circuits between immune components implicated in TB-IRIS pathogenesis (focusing on type I and II IFNs, inflammasomes/IL-1 β /PGE2, neutrophils, IL-12/Th1/IFN γ , NO, and IL-17). In light of this context, we examine whether proteomically-suggested metabolic and immune changes are in agreement, and extrapolate hypotheses regarding potential driving factors (causes rather than effects). We then examine whether literature-derived hypotheses and neutrophil proteomic results agree, and whether live-cell microscopy findings are congruent with potentially the most controversial hypothesis implied by our model of TB-IRIS (i.e. that initiation of ARV therapy may contribute to the metabolic stress implicated in TB-IRIS predisposition and pathogenesis). Based on this model, we attempt to predict existing compounds which may be repurposed for safe and effective prophylaxis/therapy of TB-IRIS. Throughout, we link findings to the immunological context of co-infection (reviewed at the start of this chapter). Finally, we discuss overall project outcomes (including candidate biomarkers), strengths, weaknesses, and future directions. **Results** Complexity of the biological system within which TB-IRIS arises complicates understanding of the system, disentanglement of cause and effect, prediction of system behaviour, and ability to meaningfully perturb the system in a prophylactic/therapeutic manner. Systems biology approaches are likely best-suited to facilitating future healthcare innovation, including improved understanding, prevention, and treatment of complex (multifactorial) disorders such as TB-IRIS. Existing literature suggests a biologically compelling model partially explaining TB-IRIS predisposition and pathogenesis, and supported and extended by neutrophil immunometabolic changes suggested by proteomic findings. Highlights of the extended model include more advanced baseline TB/HIV, including higher type I IFN (and possibly Th2-type cytokine) levels, with consequent impaired myeloid-mediated Mtb antigen clearance and depletion of cellular adaptogens. The resultant abnormal immunometabolic state produces myeloid cells less able to counteract metabolic stress and primed for less-restrained inflammation. Introduction of mitotoxic ARV drugs and rapid lifting of HIV-mediated immune embargoes escalates myeloid metabolic (including oxidative) stress and overactivation (including via NLR4, CASP4/5, TLR/IL-1R-MyD88-NF κ B, and MAPK-AP1 signalling), producing – instead of Mtb clearance – inflammatory cell death with release of immune-activating and tissue-damaging host- and Mtb-derived molecules. Reactivation of Mtb lymphocyte memory responses likely only produces clinically-apparent inflammation (TB-IRIS) in when multiple simultaneous but incompatible immune programmes (e.g. overzealous myeloid activity, Th1, Th2, Th17, and T_{reg}) coexist. Live-cell microscopy partially validates that efavirenz induces metabolic stress in healthy volunteer neutrophils. Based on this model of TB-IRIS predisposition and pathogenesis, various strategies and existing compounds with the potential for safe and effective TB-IRIS prophylaxis/therapy are identified (e.g. glutathione, vitamins B-complex and A/D/E, rapamycin, and metformin), with the aim of returning the system towards normal functioning, rather than further perturbation. **Conclusions** Appropriate clinical cohorts were assembled and well-suited systems biology research and statistical techniques were selected in order to generate hypotheses regarding TB-IRIS predisposition and pathogenesis. Methods were optimised and applied for: neutrophil isolation from clinical samples, lysate preparation, HPLC-MS/MS-based proteomics, and computational differential proteomic profiling (encompassing protein identities, relative quantities, and selected post-translational modifications). Literature and proteomic findings were integrated to generate a parsimonious model largely able to explain TB-IRIS predisposition and pathogenesis, and the most controversial arising hypothesis was partially validated by orthogonal methods. Candidate risk-stratification and diagnostic biomarkers were identified, as were strategies and compounds with the potential for TB-IRIS prophylaxis/therapy. Regarding future directions, research techniques are available to validate all other major hypotheses implied by our model, biomarker/biomarker panel performance may be assessed in a larger independent cohort, and strategy/compound safety and efficacy in preventing/treating TB-IRIS may be assessed first pre-clinically and then (for any showing promise) via clinical trials. To the best of our knowledge, this is the first study to deeply interrogate neutrophil metabolic function, including regarding how this relates to TB-IRIS predisposition and pathogenesis.

7.1. Immunological Context of TB-IRIS

Findings regarding tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) immunopathogenesis are best interpreted in context of the immunological background against which it arises [1]. While a comprehensive review of immunology is beyond the scope of any thesis, we consider the most relevant broad principles governing immunity, host responses to tuberculosis (TB), and the impact on these by human immunodeficiency virus (HIV) infection, anti-tubercular therapy (ATT), and antiretroviral (ARV) therapy.

7.1.1. General Immune Paradigms

Immunity has two major functions: tolerance and defence. These require discrimination between normal- and abnormal/non-self, with recognition mediated by *cognate* receptor-ligand interactions. Based on receptor specificity, activation threshold, and capacity for memory, immune cells (leukocytes) are traditionally categorised as members of the (evolutionarily ancient) innate or (more recent, having arisen ~500 million years ago) adaptive systems.

The innate system employs a finite set of genetically-encoded pattern-recognition receptors (PRR), which recognise intra- and extracellular conserved molecular signatures present during infection or host stress/damage. Signatures comprise pathogen-associated molecular patterns (PAMPs), intracellular homeostasis-altering molecular processes (HAMPs), and host-derived damage/danger-associated molecular patterns (DAMPs) or alarmins. The latter three are derived from stressed/damaged host cells (e.g. abnormally altered structure/levels, present in the incorrect biological compartment, or secreted to alert other cells to abnormality) rather than directly from pathogens. Innate cells are often stationed in sentinel positions and are capable of rapid responses, but generally not of memory.

Adaptive system receptors, on the other hand, are derived through variable recombination of pre-existing genetic building blocks, providing a massive repertoire sufficient to cover the entire potential antigenic universe. Each receptor specificity recognise a tiny epitope on pathogen- or altered host-specific biomolecular antigens (proteins, lipids, polysaccharides, or combinations). Each individual T- and B-cell exhibits T-cell receptors (TCRs) or B-cell receptors (BCRs) with a single specificity. There are in excess of 10^{12-16} TCR and 10^{8-11} BCR/immunoglobulin (Ig) specificities, many of which are expressed but never utilised. Evolution of such an energy-expensive approach is one of many examples reflecting the importance of immunity to survival. Adaptive cells have more stringent activation requirements and respond more slowly than innate cells, but are capable of memory. Memory facilitates more rapid and powerful secondary responses on re-exposure, but also creates the potential for severe chronic pathology. As an (inevitable) exception to the rule, some leukocytes express both innate *and* adaptive receptors, and some innate cells do exhibit memory (and metabolic reprogramming producing memory-like responses).

During ontogeny, adaptive cells are edited to remove auto-reactive specificities ("central tolerance"), but additional peripheral tolerance fail-safe mechanisms exist. For example, only in a context sufficiently suggestive of productive and host-damaging infection will HAMPs, molecules featuring PAMPs/DAMPs, alarmins, and antigens produce a significant immune response. This is one reason that effective live *Mycobacterium bovis* BCG vaccination requires *productive* infection.

During initial pathogen encounter, the first host elements to come into contact with potential pathogens are mechanical and soluble barrier defences, followed by surface-adjacent innate and memory sentinel leukocytes (which also interact with soluble barrier defences). It is these local cells which respond immediately to invasion or damage in a manner appropriate to the inciting cause, and which first recruit and activate additional innate cells from the circulation (also shaping their function to the inciting cause), and then – via secondary lymphoid organs or locally – activate the adaptive system, whose effectors only become available for recruitment/utility after a delay, and which provides memory for a more rapid response to subsequent encounter of the same pathogen. Bi-directional

cross-talk continues between almost every cell type, both within and between the innate and adaptive cell compartments (as well as with non-immune cells).

Thus, innate ($\pm$ memory) responses precede and inform adaptive responses, until both act in concert, bi-directionally fine-tuning each other commensurate with the remaining type and degree of danger. Leukocytes continue to monitor the remaining level of danger, and down-modulate their own inflammatory function – as well as the inflammatory function of other cells – as the danger passes. Once pathogen and tissue debris are cleared, no driving force maintains inflammation, and a multitude of mechanisms facilitates resolution of inflammation and healing, thereby restoring tissue homeostasis (fig. 1).

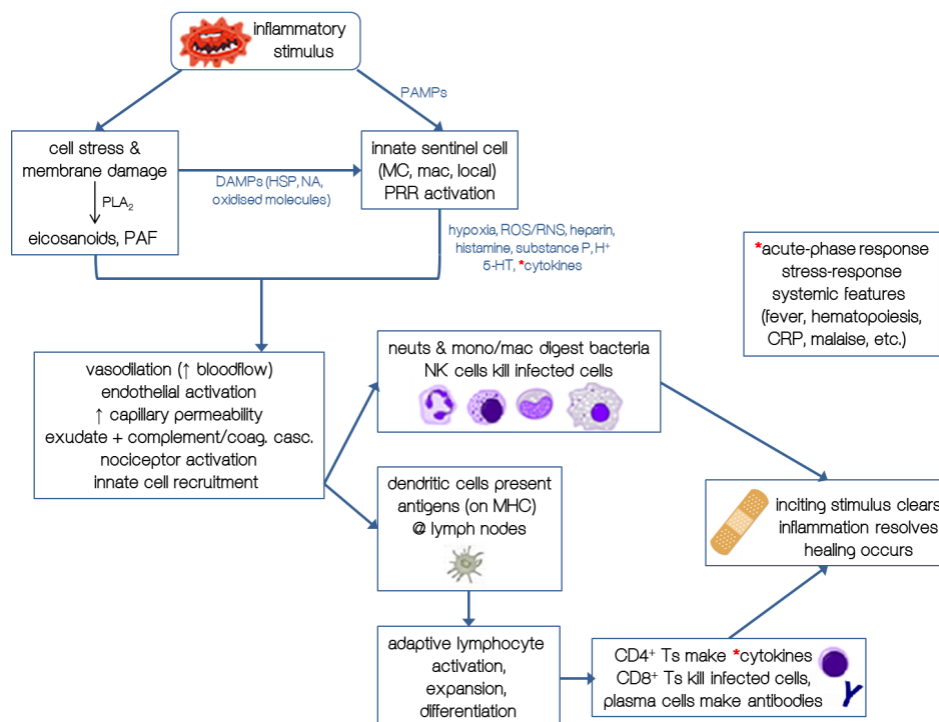


Figure 1 Simplified Integrated Inflammatory Response. **PLA₂** phospholipase A₂, **PAF** platelet-activating factor, **HSP** heat-shock proteins, **NA** nucleic acids, **MC** mast cells, **mac** macrophages, **PRR** pattern recognition receptors, **ROS/RNS** reactive oxygen/nitrogen species, **5-HT** 5-hydroxytryptamine, **CRP** C-reactive protein, **coag. casc.** coagulation cascade, **NK** natural killer, **MHC** major histocompatibility complex.

The unique combination of stimulated receptors and initially-responding cells (as well as the local tissue microenvironment) to a large degree shapes the function of recruited innate and adaptive cells, tailoring each immune response to the inciting stimulus (although some components of the response are common between different stimuli). Ideally, only the biological program most appropriate to combating that stimulus is executed. This, combined with pathogen tissue tropism and pathogen/immune host-damaging mechanisms, underlies the predictable clinical and cellular/biochemical patterns distinguishing different diseases. Certain mechanisms, signs, and symptoms are common to most inflammatory processes (Table 1).

Table 1 Cardinal Manifestations and Mechanisms of Acute Inflammation

Cardinal Manifestation	Proximal Mechanism	Distal Mechanism	Teleological Purpose
rubor (redness)	$\uparrow$ local blood-flow	endothelial activation	provide immune cells and metabolic factors from circulation
calor (heat)		vascular dilation	
tumor (edema)	vascular interstitial exudate	endothelial activation $\uparrow$ vascular permeability	prevent further injury
dolor (pain)	nociceptive signalling	lower nociceptor activation threshold	

Fluor (secretion, to aid in pathogen expulsion) ^[2] and hypercoagulability (to limit blood loss and facilitate healing) also typically occur during inflammation, but are not always clinically evident.

The few innate cytokines possessing a long enough half-life to act systemically (IL-1, IL-6, TNF α) also produce common (nonspecific) manifestations of inflammation, such as altering the hypothalamic set-point to produce fever, and inducing: anorexia/malaise, the hepatic acute-phase response (APR), and the hypothalamic-pituitary-adrenal (HPA) axis stress response ^[3] (cortisol and epinephrine act at a slight delay to regulate inflammation). Cytokines mediating these effects also significantly alter the HPT (thyroid) and HPG (gonadal) endocrine axes ^[4] ^[5]. This is one example of the exquisite inter-dependency of complex adaptive biosystems (*vide infra*): the immune system is bi-directionally linked with other distributed, dynamic biosystems, including the endocrine system, the nervous system, carbon-energy metabolism, and commensal microflora, among others.

Some pathogens (e.g. influenza and the human immunodeficiency virus (HIV)) exhibit a rapid-evolution phenomenon termed antigenic 'drift' (de-selection of immunogenic antigens over time due to host immune pressure), rendering immune memory only partially effective or ineffective. Rapid-enough drift forces continual mounting of a new primary immune response to ever-changing antigens. It is difficult for the immune system to "get a handle on" such pathogens. Other successful pathogens such as *Mycobacterium tuberculosis* (Mtb) naturally express different antigens at different times during infection (antigenic 'shift') as Mtb undergoes metabolic changes (often in response to host pressures), producing a similar problem. At any stage of Mtb infection, only certain antigens are rapidly-increasing/abundant enough to be immunogenic, and these antigens are often not crucial microbial functional nodes. Many pathogens – especially HIV and Mtb – significantly evade and modulate host immune responses at nearly every step of the executing pathogen-tailored biological programme (*vide infra* Section 7.1.3), interfering with pathogen clearance and normal resolution of inflammation.

In the great majority of cases, however, host immunity (transient inflammation with a predictable trajectory) effectively eliminates the pathogen. Indeed, the ideal immune response is acute and well-controlled: as part of peripheral tolerance, the immune system encodes *within activation* multiple delayed resolution mechanisms. Unnecessary, aberrant, or chronic inflammation is - by definition - pathological (including no longer following a predictable trajectory), and can significantly damage the host. For example, chronic inflammation leads to repeated attempts at resolution and healing on a background of ongoing inflammation, an improper execution of two opposing biological programs which leads to pathology (e.g. fibrosis, amyloid deposition) and loss of function.

7.1.2. General Principles Impeding Disentanglement of Cause and Effect Within the Immune System

The immune system is a dispersed, complex, adaptive (dynamic) biosystem. Components interact bi-directionally with each other and with components of other complex adaptive biosystems.

Within the immune system, multiple different cell types produce common effector molecules (complicating inferences of source), individual soluble effectors have pleiotropic (often overlapping or convergent) functions (complicating inferences of effector), and functions are often altered depending on contextual factors such as biological/intracellular compartment, post-translational processing stage, availability of co-factors/interaction partners/functional targets, and presence/absence of specific decoys/antagonists (complicating inferences of function). For example, many different leukocyte types produce IL-6, which can act on multiple different cell types in pleiotropic ways, and shares certain functions with other cytokines (e.g. IL-1 α , IL-1 β , IL-6, TNF α , IL-8, and MIP-1 α and β are all endogenous pyrogens [6]). Some cytokines (e.g. TNF α) can act via multiple different receptors/signalling cascades to produce differential effects [7], and effects of others (e.g. TGF β) are altered depending on where on the inflammation-resolution continuum the microenvironment currently is.

Immune components also operate in a highly anatomically-compartmentalised manner, further significantly complicating interpretation of findings. Circulating leukocytes may be engaged in steady-state surveillance, may be en route to infiltrating residential/lymphoid tissues or an inflamed site, or may be post-efferocytic and en route to disposal/recycling. Similarly, cells within lymphoid organs may be

nascent, resident, traversing, may have homed there after activation, or may be in the progress of egress after activation. Investigating the cells in any single compartment cannot provide sufficient information about the other compartments. For example, increased frequency of a circulating cell type could result from any of the following: enhanced marrow production/release, redistribution from various possible resident tissues/secondary lymphoid organs (SLO), half-life prolongation, or down-regulated elimination/recycling. Similarly, decreased frequency of a circulating cell type could result from decreased production, redistribution to various possible resident tissues/SLO, or various mechanisms of depletion.

Similarly, protein (e.g. cytokine) abundance may increase/decrease due to any combination of the following: transcription rates, mRNA stability/degradation rates, translation rates, protein stability/degradation rates, and protein secretion/exocytosis (e.g. neutrophil degranulation) or shedding from the cell surface. A corollary is that detection of increased/decreased protein levels does not necessarily reflect protein expression induction/suppression. Especially when receptor-ligand interactions or secretion of intracellular proteins is involved, it can be difficult to interpret the direction of change of gene product expression. For example, higher protein abundance is often interpreted as indicating an increase in requirement for/activity of the parent functional module. However, to regulate cell sensitivity (like a rheostat), level of the corresponding cell surface receptor often increases when ligand levels are too low (and vice versa). A surface protein (e.g. receptor) may also undergo enhanced expression (through fusion of pre-existing intracellular reserve vesicles with the plasma membrane), or may be internalised and recycled back to the surface. In both instances, there may be no differences in overall cellular protein level, even though cellular sensitivity to the receptor ligand has changed. Furthermore, if only intracellular (and not circulatory/supernatant) proteins have been measured, it is very difficult to infer whether altered levels of secretory proteins reflect differences in expression or secretion: in the case of secretion/exocytosis or shedding, an observed decrease in intracellular protein levels may actually be due to increased production and loss. It is perhaps also more difficult to clearly interpret down-regulation rather than up-regulation of a protein, especially if a baseline (e.g. healthy volunteer/pre-perturbation) state dataset is not available for comparison. For example, immune responses often simultaneously activate and oppose activation of a functional module (for regulatory purposes), making it difficult to determine whether inhibitory responses are dominant or merely a consequence of activation.

Each leukocyte type can also assume a continuum of different phenotypes dependent on the compartmental/microenvironmental context within which it is operating. Both systemic and local microenvironment cues (which are themselves complex and difficult to study) play a large role in the behaviour of a cell type within a particular compartment, and the signals of interest are usually strongest in the compartment within which pathology is localised, becoming weaker the further away from the site of pathology tissues are sampled (nonetheless, systemic disorders such as septicaemia and TB-IRIS do produce strong signals both locally and systemically). It is very difficult to determine the functional/biological significance of any quantitative cell alterations without also examining qualitative phenotypic characteristics of those cells (e.g. maturity/activation or inflammatory/tolerogenic-regulatory state).

Despite all these factors complicating inference of cause and effect, immune function in healthy individuals nevertheless conforms to certain overarching paradigms which enable us to make meaningful predictions about the behaviour of its components, as well as the entire integrated system. However, pathogen- and pharmaceutical drug-mediated modulation of the immune system from equilibrium adds additional, sometimes impenetrable, layers to the existing complexity (especially when each individual modulant represents an incompletely-understood entity). The immunological context of TB-HIV co-treatment is particularly complex, given the highly-variable timing and intervals of TB and HIV acquisition and infection relative to each other, the opposing and synergistic immunomodulatory effects of these pathogens, and the selective removal of these effects by ATT and ARV therapy (which themselves independently impact immune function). In this context, abnormal new and heterogeneous conditions (and perhaps even equilibria) likely arise, under which it becomes very difficult to predict how the immune system will behave in response to different perturbations.

7.1.3. Immune Responses to & Modulation by Mtb & HIV Infection

7.1.3.1. Host Responses to Mtb

Due to ethical and practical constraints, *in vitro* and animal (zebrafish, mouse, guinea pig, rabbit, non-human primate) studies of immune responses to Mtb outnumber human studies, with the latter often limited to *ex vivo* or *post-mortem* approaches. No model exactly recapitulates human pathology, and each model shares only some elements of pathology with the others, complicating comparability and integration. Further contributing to study heterogeneity is the use of different primary/immortalised cells, mycobacteria other than Mtb, and infection of animals via various routes (not only aerosol). Thus, many questions regarding Mtb-evoked immune responses (and correlates of protection/pathology) remain unanswered.

Little is known regarding early events after Mtb inhalation [8]. Despite no direct evidence, it is believed that alveolar macrophages (AM) are the first point of contact with inhaled Mtb [9] [10] [11]. Indeed, Mtb-containing respiratory droplet nuclei traversing the respiratory tract without deposition is *possible*: notwithstanding electrostatic interactions, nuclei/bacilli are ~40 000x smaller than tracheal diameter, and ~200x smaller than even alveolar diameter. However, airway length and branching may result in deposition and engagement of airway mucosal immunity *prior to alveolar access*. This would influence immune/infection kinetics (perhaps in a prophylactic manner): in rabbits, large respiratory droplets containing many bacilli deposit tracheally, but produce little infection [12]. Murine aerosol Mtb exposure can indeed produce lymph node (LN) infection *from the airways* [13], but this may be a function of significantly smaller airway diameter, as macaque aerosol Mtb exposure produces LN infection *from the lungs* [14].

The Mtb capsule and host-derived droplet nucleus coat, likely modified by host humoral factors (e.g. airway hydrolases, soluble PRR), significantly impact early host-pathogen interaction, for example neutralising Mtb or determining which PRR combination is engaged [15]. *Receptor identity significantly impacts Mtb intracellular fate and downstream immune function*. That ~50% of exposed individuals (~20% in hyperendemic regions) remain Mtb-uninfected (including failing to develop T-cell memory, as inferred from proxies such as the tuberculin skin test (TST) and interferon-gamma release assay (IGRA)) and that 90% of individuals successfully control Mtb infection (latency) strongly suggests that some inhaled Mtb encounter pre-alveolar mucosal immunity in a way that impacts exposure outcome, and/or that some individuals possess pre-existing natural anti-Mtb immunity (also likely mucosal) [16] [17] [18]. Respiratory tract mucosal defences are tailored to airborne pathogens. Apart from mechanical modalities (expulsive reflexes, branching, the mucociliary escalator, and inter-epithelial tight junctions), the upper and lower airways exhibit unique palettes of humoral and cellular defences (Table 2).

Table 2 Selected Humoral and Cellular Airway Defences

	Airways	Alveoli
humoral factors	mucins AMP soluble PRR (e.g. ficolin-3) complement natural/inducible antibodies	ficolins (e.g. ficolin-3) collectins (e.g. SPA-D, MBL) pentraxins (e.g. CRP/PTX1, induced PTX3) complement (e.g. C2-5, factor B) antibodies
cells	mucosal epithelia mast cells innate lymphocytes MAIT GEM cells macrophages DC natural/long-lived plasma cells adaptive memory lymphocytes	alveolar epithelia innate lymphocytes MAIT AM DC

Innate lymphocytes and mucosal-associated invariant T-cells (MAIT) are CD8⁺ cytotoxic cells enriched in the airways [19] [20]. They respond rapidly to Mtb or Mtb-infected MR1⁺ airway epithelia to become early sources of IFN γ , TNF α , and granulysin (though they can also be activated TCR-independently by IL-12 and IL-18 or by IL-17 and IL-22) [20]. Germline-encoded mycosyl (GEM) cells are CD4⁺ T-cells with semi-invariant TCR [21]. Natural and inducible airway antibodies include IgM, abundant IgA, airway-specialised IgD, and IgG [15]. Alveolar antibodies include IgM and IgA, but are predominantly of the IgG type. While alveolar macrophages (AM) are the most abundant alveolar phagocyte, dendritic cells (DC) are also present, and alveolar epithelia make up ~99% of alveolar surface area [9] [22] [15] [18].

Cellular receptors that interact with Mtb include: surface complement receptors (CR, recognising both free and opsonised Mtb), surface receptors for the constant region of immunoglobulins (FcR), surface toll-like receptors (TLR, including -2 and -4), surface C-type lectin receptors (CLR, e.g. DC-SIGN), intracellular TLR (including -8 and -9), intracellular NOD-like receptors (NLR, e.g. NOD1 and -2),

intracellular cGAS-STING, and intracellular inflammasome sensors (AIM2, NLRP3, and possibly NLRC4) (Tables 3 and 4).

Early detection of Mtb by surface TLR is thought to initiate the immune response, via MyD88-NFkB and MAPK-AP-1 inducing expression of IL-1 β , IL-18, IL-6, low-level TNF α , IL-8, IL-12, IL-15, IL-23, IL-10, various chemokines (e.g. MCP-1), and NO, among other effector molecules [23] [24]. These act in autocrine and paracrine manners, helping to activate and stimulate other local cells, recruit circulating leukocytes, and - for the systemically-acting cytokines - exert distal effects (e.g. induction of acute phase reactants – including fibrinogen, CRP, LBP, haptoglobin, and annexin-1 - and hypothalamic effects). Signalling (independently of MyD88) by TLR-4 also induces expression of type I IFN. Only at a slight delay after Mtb uptake - once Mtb or its products (e.g. nucleic acids (NA)) access the cytosol – do *intracellular* PRR become activated. Many of these (e.g. TLR-9, cGAS-STING) also contribute significantly to type I IFN production [25]. Inflammasomes (e.g. NLRP3, AIM2) sense Mtb nucleic acids (including RNA, via DHX33, for NLRP3) - as well as NLRP3 likely being activated by the many other mechanisms outlined in Table 3 (including phagocytosis-mediated decreases in intracellular K⁺, altered ATP levels, and cytosolic cathepsins) – to contribute to IL-1 β and IL-18 maturation, and possibly pyroptosis [25] [26]. Myeloid TREM-1 may amplify TLR- and NLR-initiated signalling [27]. Potential impacts of soluble PRR are discussed below.

Table 3 Ligands & Signalling Cascades of Cell-associated PRR [28] [29] [30] [31] [32]

Class	PRR	Cell Compartments	Ligands	Signalling Pathways	Functional Outcomes
TLR	1/2	surface	Gram+ PDG	MyD88-NFkB MAPK-AP-1	inflammatory cytokine production
	3	early endosome	viral dsRNA siRNA host RNA	TRIF-IRF3 [33]	IFN production
	4	surface	Gram- LPS palmitate	MyD88 [34] [35] [36]-NFkB MAPK-AP-1 TRIF-IRF3 [33]	inflammatory cytokine & IFN production
	5	surface	flagellin	MyD88-NFkB MAPK-AP-1	inflammatory cytokine production
	2-6	surface	acylated lipopeptides		
	7	ER $\rightarrow$ late endosome	viral ssRNA bacterial RNA	MyD88-NFkB MAPK-AP-1 MyD88-IRF7	inflammatory cytokine & IFN production
	8	ER $\rightarrow$ late endosome	viral RNA bacterial RNA	MyD88-NFkB MAPK-AP-1	inflammatory cytokine production
	9	ER $\rightarrow$ late endosome	bacterial CpG DNA viral CpG DNA <i>P. falciparum</i> crystalline hemozoin	MyD88-NFkB MAPK-AP-1 MyD88-IRF7	inflammatory cytokine & IFN production
	10	endosome	HIV gp41 viral dsRNA with TLR2 recognizes bacterial ligands	MyD88-NFkB MAPK-AP-1	IL-8, inflammatory cytokine & IFN production
inflammasome sensors (NLR, ALR)	NLRP1	nucleus cytosol	bacterial muramyl dipeptide	NLRP3/NLRC4/AIM2-CASP1 matures IL-1 β , IL-18, and gasD or LPS/NLRP1-CASP4/5 mature gasD & activate NLRP3/AIM2-CASP1 (FasL induces myeloid release of NLRC4/NLRP3/CASP1-independent, ASC/CASP8-dependent mature IL-1 β and IL-18, & extracellular pro-proteins can be activated by myeloid proteases)	gasD pores release IL-1 α , IL-1 β , IL-18, HMGB1, S100A8/9, etc. sustained gasD activation produces lytic death (pyroptosis)
	NLRP3	cytosol $\rightarrow$ mitochondria-associated ER (in association with cardiolipid, MAVS, Mfn2, oxmtDNA, etc.)	ATP-P2X7R-K ⁺ flux cytosolic cathepsin bacterial/host NA (e.g. mtDNA) ROS urate cholesterol crystals osmotic stress deubiquitination beta-amyloid ECM degradation products		
	NLRC4	intracellular	flagellin host mtDNA, ROS		
	AIM2	cytoplasm	dsDNA host HMGB1-DNA, RAGE		
RLR	RIG-I (DDX58)	cytoplasm	viral ssRNA, dsRNA	MAVS-IRF3/7 RIP2-NFkB	type I IFN & inflammatory cytokine production
	MDA5 (IFIH1)			no signalling; regulates RIG-I & MDA5	decreased RIG-I & MDA5 activity
	LGP2 (DHX58)				
CLR	dectins	surface	fungal β -glucans, mycobacterial mannose (glycolipids also bind dectin-2)	Syk/CARD9-NFkB	inflammatory cytokine production
	mincle			activates Raf-1 signalosome	context-dependent enhanced/suppressed inflammation
	DCAR				
	MR				
n/a	TREM-1	surface	?platelets ?extracellular actin ?PGLYRP1	via DAP12 amplifies TLR and NLR MyD88-mediated signalling (e.g. during sepsis)	enhanced inflammation

Ligation of cell-associated PRR triggers signalling cascades that induce stimulus/cell/microenvironment/receptor-specific appropriate functions. Although simultaneous triggering of multiple PRR-activated cascades (distinct and overlapping) is normal during immune responses (either co-operating or counter-regulating each other), polymicrobial inflammation (e.g. during HIV-induced microbial translocation) can overwhelm or pervert normal cascade cross-talk. Co-receptors assisting PRR include scavenger receptors (e.g. CD14, CD36) and other PRR (e.g. FcγR, NAIP5). Apart from TLR-7/9 and inflammasome sensors, additional cytoplasmic nucleic acid sensors include non-RLR helicases (DDXs), PKR, OAS, and cGAS (signalling via STING). In co-operation with IFI16, STING also forms an *intranuclear* inflammasome. Additional potentially cell-activating receptors not discussed in this table include cytokine/chemokine receptors, integrin receptors, and CR and FcR (with entry via CR versus FcR often circumventing versus potentiating, respectively, intracellular killing of Mtb). IFN-stimulated genes (ISG) include many of the above, including inflammasome sensors, RLR, DDXs, OAS, cGAS, IFIs, etc. (and RLR can induce inflammasome assembly, as can glycolipid CLR ligands – via induction of mtROS production and K⁺ efflux^[37]). Purified human neutrophils express relatively high levels of IL-33, NLRC4, ASC, AIM2, and CASP1 proteins, with LPS additionally inducing IL-1α and NLRC4 transcription [38]. However, neutrophil NLRP3 protein levels are below the limit of detection [38]. **PDG** peptidoglycan. **LPS** lipopolysaccharide. **NAs** nucleic acids.

Table 4 Early Soluble and Cell-associated PRR-mediated Mtb Recognition

Mtb Ligands	Soluble/Cell-associated PRR	Functional Outcomes
antibody complex	FcR	intracellular killing
complement complex: C1q, C3b, C4b iC3b	CR1 CR3 (also recognises Ag85C) esp. CR4	activates inflammation bypasses microbicidal, inflammation
LAM, manLAM, lipomannan, 19kDa lipoprotein, TDM, PIM	TLR-2 (also recognises 38kDa lipoprotein) TLR-4 (also recognises Mtb heat-shock proteins, 38kDa glycoprotein, Rv1068)	IL-1β, IL-6, (TNFα), IL-12, ROS/RNS same + type I IFN
LAM (PDG-like), manLAM	MR DC-SIGN	anti-microbial, anti-inflammatory decreased antigen presentation
?	Dectin-1	via Syk-CARD9-NFκB induces IL-6, TNFα, IL-12
manLAM	Dectin-2	via FcγR-Syk-CARD9 activates leukocytes
TDM	Mincle	via FcγR-Syk-CARD9 activates leukocytes for inflammatory cytokine & NO production
TDM	CLEC5F8	phagocytosis, pro-inflammatory cytokine production, DC maturation, T-cell priming, and respiratory burst
PIM	DCAR	?
Mtb nucleic acids in endosomes/phagolysosomes &/or accessing cytosol via ESX-1 secretion system	TLR-8 TLR-9 NOD2 NLRP3 AIM2 STING	?resolution type I IFN, inflammation IL-1β, IL-6, TNFα, IL-12p40, RANTES IL-1β, IL-18 same type I IFN

Mtb TLR ligands include LAM, ManLAM, 19kDa lipoprotein, lipomannan, TDM, PIM, N-glycolylmuramyl dipeptide (part of PDG), membrane-associated lipoproteins (e.g. 19kDa lipoprotein, LprA/Rv1270c, LprG/Rv1411c, and LpqH/Rv3763), and CpG DNA [39] [40] [24]. Acting on innate phagocytes, TNFα activates vitamin D, and, together, TNFα and vitamin D receptor (VDR) ligation cause cytosolic cathepsin release. Overall, TLR-2 signalling favours Mtb killing (via phagolysosome fusion, production of ROS/RNS and vitamin D-dependent cathelicidin, microbicidal apoptosis, and inflammatory cytokine production) [25]. Both TLR2 and 4 are over-expressed during Mtb infection, and are required *in vitro* to initiate viable infection [41] [24]. Ligation of TLR-2/4/9 and NOD2 all contribute to induction of autophagy [25]. Inflammasome component (e.g. NLRP3, CARD8) gain-of-function mutations limit intra-macrophage Mtb growth [42], and the PYCARD/ASC adaptor (but neither NLRP3 nor CASP1) is implicated in granuloma formation [43]. In terms of co-receptors, CD14 (which, for example, binds Mtb chaperonin 60.1) assists TLR-4 and -9, and CD36 (which, for example, binds ManLAM and lipomannan) and MARCO assist TLR-2 [25]. Certain Mtb products suppress inflammation, phagolysosome maturation, and antigen presentation (e.g. ManLAM – via MR – suppresses IL-12 in favour of IL-10, IL-1Ra, and type II IL-1R, while also activating anti-inflammatory PPARγ and inhibiting phagolysosome maturation [25]). It is known that impaired lysosomal digestion of Mtb and its products hinders macrophage migration, favouring secondary necrosis *in situ* and interfering with microbicidal and antigen clearance [44]. **LAM** lipoarabinomannan. **ManLAM** mannosylated LAM. **TDM** trehalose dimycolate. **PIM** phosphatidylinositol mannoside.

Little is known regarding modulation of Mtb-host interaction by *soluble* PRR. However, ficolin-2 is protective, ficolin-3 (including in co-operation with MBL) enhances phagocytosis, and SPA/D polymorphisms are associated with increased susceptibility [45] [46] [47]. Within alveoli (and perhaps the airways), Mtb activates the classical complement pathway via C1q, and complement-opsonised Mtb is taken up via CR1 (binds C1q, C3b, C4b), CR3 (CD11b; binds iC3b), and especially CR4 (binds iC3b) *in vivo* (though *in vitro* human monocyte/macrophage CR3 accounts for up to 80% of Mtb uptake [48]). Indeed, human AM express higher levels of CR4 than either CR1 or CR3 [49] [50]. Both CR3 and CR4 are also capable of taking up non-opsonised microbial ligands [50]. In circulation, Mtb also activates the alternative complement pathway via MBL, and degree of complement activation correlates with active TB severity [16]. In the airways, IgA (especially including anti-α-crystallin) may confer passive protection, and serum anti-HBHA IgM excludes Mtb from alveolar epithelia [16].

Consistent with the physiological functions of CRP and complement, Mtb opsonisation by these favours more immunologically-silent uptake. Uptake via CR3 or CR4 (the major routes for AM) circumvents cellular microbicidal [51] [25]. For example, entry via CR3 suppresses the respiratory burst and endosome maturation [52]. Virulent Mtb may selectively bind CR3 [53], and CR1 ligation may contribute to TB immunopathogenesis. Of the humoral opsonins, only IgG (via FcγR) most successfully induces microbicidal, antigen presentation, and induction of a Th1 response [54]. Indeed, uptake of PTX3-opsonised bacteria via FcγRIII also induces inflammatory cytokines. Genetic PTX3 variants alter Mtb infection susceptibility and later plasma PTX3 levels correlate with TB severity [55] [56] [57]. Nevertheless, Mtb infection induces FcγR expression (including intracellular TRIM21), and whole blood myeloid FcγRIA (high-affinity, binding mainly IgG1 and -3) and FcγRI signalling transcriptomic signatures

identify active TB [58] [59] [60]. Thus, unlike CR ligation, FcR ligation (depending on antibody isotype, subtype, and the cellular balance of activating/inhibitory FcR) enhances anti-Mtb responses. Regarding cells of the airways, Mtb can directly infect bronchial epithelia [20] which present Mtb antigens via: MHC-related MR1 (presenting vitamin B-derived metabolites) to MAIT [19], CD1a-c (presenting lipids, including glycolipids) to $\gamma\delta$ T-cells, CD1b (presenting glucose monomycolate) to GEM cells [61] [62], and CD1d to NKT cells. Findings suggest that airway mast cells may degranulate in response to Mtb [63], and that bronchial epithelia are highly-responsive to cytokines (e.g. IL-1 β and type I IFN) produced by Mtb-infected myeloid cells [18] (major airway leukocytes [11]). In the alveoli, epithelia, AM, DC, and pulmonary innate lymphocytes (NK, NKT, and $\gamma\delta$ T-cells, as well as MAIT) can all become activated by opsonised/complexed/free Mtb/products. It is not known whether Mtb infects type I pneumocytes, but *in vitro* it infects and replicates in human type II pneumocytes [9] (when activated, these produce various pro-inflammatory cytokines, MMPs, antimicrobial peptides, hydrolases, complement, and surfactant, specifically conditioning macrophages in ways that enhance intracellular inhibition of Mtb growth and replication [64]), and Mtb DNA is detectable in these cells *post-mortem* [15]. Alveolar epithelia, too, are able to present Mtb antigens (MAIT may target infected epithelia [64]), and may represent a replication niche: "early Mtb invasion of alveolar epithelia [is] essential for the establishment of infection", and Mtb virulence correlates with the ability to induce alveolar epithelial necrosis (e.g. of type II pneumocytes, via ManLAM and PIMs) [47]. During active human TB, airway MAIT frequency increases, and mice lacking MR1 or MAIT exhibit decreased pulmonary BCG control [20].

Mtb-evoked cytokine and cellular networks are complex. For example, there is early activation of DC (which produce high IL-23 levels) and local innate lymphocytes, which produce IFN γ (all), TNF α (all), granzymes (δ 2, MAIT), granulysin (NK, δ 2, MAIT), perforin (NK), IL-17 (esp. $\gamma\delta$ T), IL-22 (esp. NK), and G-CSF plus GM-CSF (both from NK and only the latter from NKT cells) [65] [66] [67]. Both IL-1 β and IL-18 from myeloid cells (especially in the presence of IL-12 and IL-15) enhance innate lymphocyte/MAIT IFN γ production, while IL-18 (especially in the presence of IL-12 and/or IL-15) also supports innate and adaptive T-cell cytotoxicity, augments TNF α and GM-CSF production, and favours IL-17 and IL-22 production (IL-23 also favours production of the latter two) [20] [68] [47] [26]. Via induction of GM-CSF, IL-17 enhances neutrophil provision, but IL-17 also enhances $\gamma\delta$ T-cell function and promotes Mtb-stimulated DC secretion of IL-12, favouring subsequent Th1-type responses once adaptive T-cells become available (also including production of IFN γ and TNF α , as well as cytotoxicity). GM-CSF-polarised macrophages produce high levels of IL-23, but only produce IL-12 on exposure to IFN γ , which conversely dampens DC IL-23 production [69]. Stromal-derived IL-7 enhances MAIT sensitivity to lower levels of Mtb ligands [70], boosting their cytotoxicity [20] [68]. Mtb also elicits IL-21, which supports Th1 expansion [71].

Early during infection, Mtb accesses the alveolar interstitium (free or within macrophages/DC) [9]. In guinea pigs, *free* Mtb reach the LN within days [47]. Epithelial necrosis, or Mtb transcytosis through epithelia and associated basement membranes may be mechanisms of early interstitial access [47]. Proliferating bacteria within alveoli, AM, and the interstitium induce IL-8-mediated pulmonary neutrophil influx [10] (indeed, neutrophilia – with higher levels of immature neutrophils/G-MDSC – and a neutrophil-derived IFN-inducible gene signature distinguishes active from latent TB, with degree of neutrophilia associated with TB severity and – likely MMP-8-mediated – pulmonary damage [64] [72]; neutrophils may also be required for memory induction [67]). These recruit and activate additional neutrophils, monocytes, and DC from circulation [15] [67] [73] (e.g. uptake of infected apoptotic granule-containing neutrophils by infected macrophages directly enhances microbicidal activity [15]). As mentioned, pre-existing IL-23 elicits neutrophil IL-17 and IL-22 [74] [75] [67].

Approximately two weeks post-exposure, DC – via *the lymphatic system* – arrive at the mediastinal LN for antigen presentation to lymphocytes (indeed, macrophages can also carry pulmonary Mtb to the draining LN [76], and B-cells also recognise soluble and non-peptide antigens passing through/presented here). Mtb/antigens *in circulation* will also activate *splenic* adaptive lymphocytes. Lymphocyte activation is slow, conditioned by the local LN microenvironment: IL-2 drives clonal expansion of activated CD4⁺ T-cells, while DC-derived IL-12 (along with IL-15, IL-18, and type I IFN) favours Th1 polarisation [1] and CD8⁺ T-cell cytotoxicity. Surprisingly, IL-17, too, supports Th1 polarisation [77] [67]. As alluded to regarding antigenic shift, at different times during natural infection, T-cells are

presented with differing immunodominant Mtb antigens (e.g. ESAT-6, CFP10, Rv2031c, Rv2654c, Rv1038c, etc.) and epitopes, resulting in dynamic antigen-specific T-cell responses over time [78]. Antibodies are indeed protective against intracellular (as well as extracellular) pathogens (e.g. via neutralisation or FcR-mediated phagocytosis), but the roles of B-cells and antibodies in the anti-Mtb immune response are historically poorly studied [16].

Approximately six weeks post-exposure, activated adaptive lymphocyte effectors eventually start producing antibodies and infiltrating the infected site, where functional polarity of local myeloid cells and lymphocytes influences the cytokine milieu to condition incoming T-cells. For example, IL-12 and IL-18 continue to favour Th1 polarisation, the major circulating $\gamma\delta$ T-cell subset (V γ 9V δ 2 a.k.a. δ 2) via enhancement of antigen-presenting cell [APC] functionality supports Mtb-specific CD4⁺ $\alpha\beta$ T-cell function [79], IL-2 from activated T-cells in concert with IL-27 from macrophages and DC oppose Th17 polarisation [80], and NK cells produce cytokines further supporting CD8⁺ T-cell cytotoxicity [15]. Th1-polarised CD4⁺ T-cell effectors sustain high levels of IFN γ (which induce epithelial IL-22R), and Th17 also likely produce IL-22 for enhanced epithelial defence [75] [47] [67]. Mtb antigens may also directly stimulate Th production of IL-22 [75] [67]. High IFN γ levels support macrophage M1 polarisation and full activation (increased production of IL-18, TNF α , NOS2, iNOS, NO, vitamin D-dependent cathelicidin, and induction of microbicidal autophagy) [81] [82] [83] [84] [85] [86] [87] [88] [40], as well as inducing epithelial NOS2-dependent NO production [89] [90] and a non-haematopoietic indoleamine oxidase (IDO)-dependent protective response [20]. Along with IFN γ , IL-1 β and TNF α induce iNOS and thus NO production by macrophages [91]. Excessive NO production resulting from mycobacterial infection likely leads to protein thiol nitrosylation as well as (via peroxynitrite, formed by reaction of NO with superoxide) nitration of tyrosine to form nitrotyrosine [91]. Both iNOS and NO correlate with Mtb bacteristasis and bactericidal activity, human NOS2A polymorphisms alter TB susceptibility, and thiol nitrosylation inhibits NLRP3 inflammasome assembly to decrease immunopathology [91] [92]. However, human anti-Mtb NO roles remain uncertain [93] [94] [91]. Together, IFN γ and TNF α synergise to favour maturation of and antigen presentation by APC, while the former also induces expression of various T-recruiting chemokines (e.g. CCL2, CCL5/RANTES, CXCL10/IP-10 [47]).

Like innate T-cells, CD8⁺ T-cell effectors produce IFN γ and TNF α [40], and – via cytotoxic molecules – target both free Mtb and infected cells to contribute to Mtb control [95] [96]. CD8⁺ T-cells, polarised towards Tc1, are cytotoxic (via granzysin) to infected cells and Mtb, and also contribute IFN γ and TNF α [40]. IL-18, for example, favours CD8⁺ T-cell cytotoxicity [26]. Due to its favouring of cytotoxicity by innate and adaptive lymphocytes, IL-18 can contribute to host tissue damage, but is naturally opposed by IL-18BP [26]. The humoral response is prominent during active TB, but the protective/deleterious roles of B-cells and antibodies are still more complex than those of T-cells. Active TB exhibits raised circulating mycobacteria-specific plasmablast and plasma cell frequencies [16]. About 90% of TB patients also exhibit raised serum anti-mycobacterial Ig titres at presentation, with higher titres associated with smear-positive disease [16]. However, immunoglobulin-targeted antigens vary widely between individuals: of ten Mtb culture filtrate proteins, none was universally recognised by serum from active TB patients (in latency, however, high titres of anti-CFP-10 and -ESAT-6 antibodies are frequently observed) [16]. Plasma cell-derived immunoglobulin feeds back and alters Mtb interaction with FcR; the correct IgG subtypes likely enhance intracellular microbicidal activity. Although natural Mtb infection induces anti-Mtb antibodies (whose titres decline on ATT as active disease progressively resolves), passive transfer of these into other patients leads to inconsistent results [16], and B-cell depletion only rarely leads to reactivation (with no obviously discernible pattern accounting for differential responses). However, at least in mice, MAb directed against certain mycobacterial antigens are protective [97].

7.1.3.2. *Mtb Infection Chronicity & Sequelae*

The above humoral and cellular mechanisms repeatedly play out over time, but Mtb has evolved mechanisms to oppose virtually every step of anti-Mtb immune responses (TLR-2-MyD88 signalling [98], inflammasome activation [99], phagocytosis, DC maturation (DC-SIGN-mediated interference), phagolysosome maturation [100], production of IL-12 and ROS/RNS, antigen presentation, Th1 induction

(favouring instead IDO-mediated T_{reg} induction and higher kynurenine levels), apoptosis (favouring instead prolonged survival and necrosis), cytotoxicity, autophagy, etc. (it also exploits intracellular PRR axes such as IFI16-STING-TBK1-IRF3, cGAS-STING-NFκB/IRF3, RIG-I/MDA5-MAVS- NFκB/IRF3, and PKR-eIF2A-NFκB/IRF3 to enhance type I IFN production and interfere with antimycobacterial immunity) [15] [15] [101] [102] [103]). Thus, once an Mtb infection is established, it is difficult to clear and continues to act as a chronic inflammatory stimulus.

Within the alveolar interstitium, phagocytes aggregate around an infected APC, recruiting sequential waves of innate and adaptive cells. Chronic monocyte/macrophage activation by T-cell IFNγ induces formation of multi-nucleated giant cells, while Mtb mycolic acids (MA) induce formation of lipid-laden foamy macrophages, which are poorly microbicidal, and allow Mtb to switch from carbohydrate to fatty acid metabolism. The granuloma core thus consists of infected monocytes/macrophages and neutrophils, surrounded by elongated chronically-activated macrophages (which have undergone epithelioid differentiation), multinucleated (Langhans') giant cells, and foamy macrophages (fig. 2) [1] [104]. Large CD4⁺ T-cell aggregates (and a few scattered CD8⁺ T-cells) surround and penetrate the macrophage area [1] [104]. The outer surface exhibits prominent ectopic B-cell follicles, whose CXCL13-dependent formation requires IL-23 (possibly via IL-23 stimulating IL-17 and IL-22 production, which induces CXCL13 expression by primary lung fibroblasts) [105].

Despite the preference for Th1 maintenance, persistent IL-1β signalling (perhaps via enhanced IL-6 production) favours Th17 polarisation and is required for IL-17 secretion by Th17 [26]. Indeed, TGFβ (from epithelia and – during prolonged inflammation - myeloid cells and induced T_{reg}) in the presence of IL-6 favours Th17 polarisation (further supported by IL-21 and IL-23 [80]) and – via effects on fibroblasts – can induce fibrosis [106]. Since IL-17 enhances neutrophil recruitment and activation, this may contribute to pathological neutrophilia and infiltration. Ongoing inflammation would produce tissue-damaging mediators, including proteases and TNFα, while attempted resolution/healing (including TGFβ production in the presence of IL-6) on a background of ongoing inflammation would contribute to immunopathology (including Th skewing and fibrosis). For example, advanced Mtb/HIV infections both favour M2/Th2 polarisation [107] [108] (perhaps due to both pathogens signalling via DC-SIGN) and increased IL-10 production (both perhaps enhanced by platelets [109] and the latter perhaps enhanced by type I IFN), both of which oppose IFNγ production, and neither of which favours intracellular pathogen clearance. While TNFα is required to prevent reactivation of latent infection, suggestions that it is thus crucial for granuloma “maintenance” are probably spurious: it likely directly maintains bacilli in a non-replicating state (perhaps through modulation of host glucose and lipid metabolism, or phagocyte function), independently of granuloma architecture.

There is no consensus regarding whether granuloma cells are sessile, or can migrate dynamically into and out of the granuloma, but it is intuitive that recruited cells continuing to display the same chemokine receptor set will be retained (as long as receptor ligands are locally produced) until they die, are cleared through efferocytosis, and are replaced by new incoming cells. Additional cells which may be present include DC, innate lymphocytes [110], fibroblasts, etc. [104]. A fibrous or calcified capsule may encompass older granulomas. A single lung exhibits many heterogeneous granulomas, because the composition of each granuloma evolves independently over time. In macaques, most (82%) of granulomas derive from a single Mtb bacillus: rarely are *different* Mtb clones found in the same granuloma [111]. Most LN, on the other hand, drain large lung areas containing multiple granulomas, and exhibit multiple Mtb clones [111].

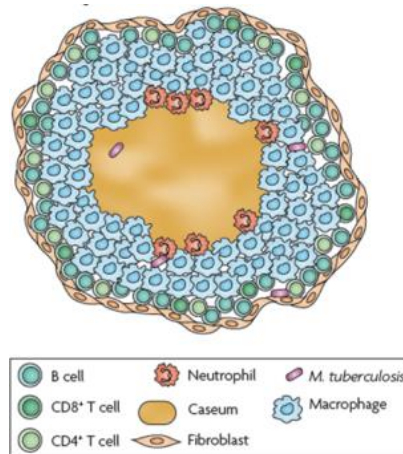


Figure 2 Simplified Granuloma Architecture [104]

As long as myeloid phagocyte activation and function remains appropriate/homeostatic, granulomas represent a hypoxic, oxidative, low pH, and proteolytic microenvironment; via hypoxia and NO triggering the Mtb (e.g. DosR-mediated) stress response and leading to reduced replication, capsule thickening, and a switch from carbohydrate to fatty acid metabolism [112]. Such a phenotype, however, also favours Mtb persistence, and likely resistance/non-responsiveness to ATT. Over time, different granulomas proceed to different outcomes, including:

- **Healing by fibrosis ± calcification**, likely favoured by effective, homeostatic immunity often achieving sterility. However, >20% of such granuloma scars still exhibit viable bacilli. In macaques, nascent granulomas exhibit decreasing bacterial numbers, with an increase in the number of sterilised granulomas (up to 100% in the lungs of some animals) over time (even in monkeys who go on to develop active TB, suggesting a loss of control over Mtb in other parts of the lung or body, though this appears to be immune qualitative rather than Mtb quantitative) [113].
- **Central caseous necrosis**, likely due to somewhat over-active immune responses to a higher bacillary load or more necrotic Mtb strain. Slow, progressive apoptosis and necrosis of infected macrophages and neutrophils occurs under the influence of T-cell cytokines [1]. Caseum contains leukocyte debris and free Mtb (viable and dead), and is nonvascular, such that hypoxia likely opposes Mtb replication and extracellular growth.
- **Liquefaction of the necrotic core**, likely due to host mechanisms which lead to massive synchronous phagocyte death that overwhelms clearance mechanisms. Phagocyte-derived hydrolytic enzymes digest caseum, leading to liquefaction ± cavitation as contents erode out of the granuloma and into neighbouring airways and/or vasculature (granulomas can also occur within SLO, and liquefaction of LN granulomas can result in erosion and draining through the skin). During human active TB (especially in immunocompetent hosts in which liquefaction and airway erosion occurs more frequently), Mtb is coughed up to start the transmission cycle anew. Liquefaction can occur in response to viable or non-viable Mtb, arguing against involvement of pathogen replication-based mechanisms (e.g. quorum-sensing). While Mtb up-regulates many phagocyte proteases, host cytokines (e.g. TNFα) and enzymes are probably key, especially since high tuberculin reactivity (likely reflecting aggressive immune responses) is observed in experimental animals which exhibit liquefaction [112]. Peripheral fibrosis with central liquefaction may reflect a cytokine gradient, e.g. high TNFα and collagenase (induced, for example, by LAM) with low IFNγ centrally, compared to high peripheral TGFβ activity. Or it may reflect a hypoxic gradient which is less intense at the periphery: increasing granuloma size or progressive caseum outgrowing of the blood supply may lead to hypoxically-triggered HIF-1α programmes (also implicated in abscess formation). Liquefied caseum is a good growth medium for Mtb, while toxic fatty acids kill macrophages [114], and peripheral lymphocytes can no longer influence the core (also reminiscent of abscesses).

A single macaque exhibits both stable and propagating granulomas: some granulomas seed new granulomas in the lung and LN [111]. The determinants underlying propagation are incompletely understood, though granulomas with a higher bacillary load are more likely to propagate [111], and earliness of granuloma formation/age of granuloma does not appear to play a role.

7.1.3.3. The Anti-Mtb Recall Response

Primary and secondary inflammatory responses differ in a number of important ways. The understanding of secondary responses to Mtb is even less complete than that of primary responses [115], largely because of the difficulty in studying immune memory in a chronic infection setting. However, a comprehensive paradigm of the recall response is required for rational vaccine design, and recall responses (to Mtb products encountered prior to ATT initiation) may contribute to TB-IRIS. Existing knowledge derives from vaccine and diagnostic research. Though inflammation is marked by intricate inter-dependencies between innate and adaptive cells, the protective nature of the recall – as for the primary – response has predictably been attributed to IFN γ -producing CD4 $^{+}$ T-cells [115], which have thus been the major focus of vaccination and diagnostic strategies [116] (none of which have been particularly successful). Clearly, other leukocyte types *must* be involved in the recall response to Mtb.

Both BCG vaccination and Mtb infection induce T_{mem} formation [117]. While BCG vaccination augments cellular responses to Mtb (due to a subset of shared antigens), protection against aerosol pulmonary infection is variable (0-89%, likely due to prior environmental mycobacterial exposure) and temporary (10-20 years), although it does protect against the most severe forms of childhood infection [118]. Because the routes of natural infection (respiratory mucosa) and vaccination (parenteral) are different, and the immune systems of these locations differ, this may also impact quality of the recall response [119]. Murine vaccination, too, induces protective Mtb-specific T_{mem}, facilitating IL-12 production and more rapid pulmonary CD4 $^{+}$ T-cell IFN γ production in response to Mtb, but only after a delay of 15 days [118] [117] (still shorter than the original six-week interval, in agreement with Chapter 1 inferences regarding TB-IRIS likely being due to a recall response). CD4 $^{+}$ T_{mem} require both TLR engagement by Mtb antigens and TCR ligation to achieve proliferation and cytokine production [39]. Naturally-infected human TB patients (but not TST-negative controls) exhibit BAL fluid recall responses to bronchoscopic PPD (Mtb-specific), also characterised by lymphocytosis (including CD4 $^{+}$ T-cells and cells capable of an antigen-specific IFN γ response) associated with IFN γ -inducible T-recruiting chemokines (IP-10 and MIG, but not MIP-1 α or RANTES) [120]. This suggests that early IFN γ production by *pulmonary-resident* cells recruits antigen-specific Th1-like cells [120].

In mice, an Mtb-specific Th17-derived IL-17 response (associated with expression of IFN γ -inducible IP-9/CXCL11, IP-10/CXCL10, and MIG/CXCL9 – all of which bind CXCR3 to induce Akt signalling for T-cell chemotaxis, as well as monocyte chemotaxis for IP-10) *precedes* and is essential for the accelerated CD4 $^{+}$ T_{mem} IFN γ recall response (and suppression of Mtb replication) [118] [121]. While primary responses are characterised by Th1 activation preceding both Th2 and Th17 activation when stimulating *ex vivo* PBMC [122], *in vivo* recall responses may be characterised by a faster memory Th17 (relative to Th1) response. It is notable that Th17 can be activated by IL-1 β and IL-18 independently of TCR activation, producing IL-17 and IL-22 in response to IL-23, and producing IFN γ in response to IL-12 [123]. In the case of Mtb re-exposure, both IL-12 and IL-21 are dispensable for the Th17 recall response [121], and Th17 are negatively regulated by IFN γ [118]. Initial IL-17 and IFN γ nonetheless likely derive from innate lymphocytes (*vide infra*). This situation is reminiscent of the working model for induction of organ-specific autoimmunity, during which the first wave of infiltrating cells are IL-23-expanded Th17 that subsequently orchestrate Th1 recruitment [80] [124]. Indeed, Th17 are emerging as major effectors of vaccine-induced protection [121], and the mechanisms may include similar IL-17-induced functions as during a primary immune response (neutrophils are required for *induction* of memory), or perhaps Th17-mediated IgA induction.

Emerging data suggest that B-cells modulate T-cell anti-Mtb memory responses: *ex vivo*, splenic B-cells from vaccinated mice maximise CD4⁺ T_{mem} activation and IFN γ responses against re-challenge (surprisingly, PD-L1 contributes to the IFN γ recall response, but in a PD-1-independent manner) [116]. This effect is particularly evident in the interaction between B-cells and high IFN γ -producing CD4⁺ T_{mem}, which, in turn, trigger and amplify B-cell activation [116]. B-cell expression of CD185/CXCR5 (a chemokine receptor involved in homing to splenic and Peyer's patch - but not LN - follicles) is required for co-localisation of T_h17 near B-cell follicle macrophages (and associated Mtb control) [121]. Intestinal mucosa T_h17 are abundant and IL-17 expression correlates with IgA levels: T_h17 may act as precursors of T_h that induce class-switching to IgA within Peyer's patches [119]. Although less is known about the role of T_h17 in airway mucosal induction of IgA, the prevalence of pulmonary T_h17 is also high during airway infections, and IL-17 does increase plgR expression to promote IgA transcytosis and pulmonary B-cell recruitment [119].

Indeed subcutaneous priming with a T_h17-inducing non-Mtb antigen/adjuvant combination, followed by aerosol boosting, leads to recruitment of IgA⁺ B-cells and GPR29/CCR6⁺ (MIP-3 α -, CCL20-, and β -defensin-responsive memory) T_h17 to the lung parenchyma, and sustained high levels of pulmonary IgA production, both coincident with GC formation in draining LN and dependent on T_h17-derived IL-17 [119]. Recruited CCR6⁺ T_h17 become pulmonary residents that persist for at least 10 weeks (the duration of the study) and probably longer [119]. Ligation of CCR6 by CCL20 is important at mucosal surfaces, including for recruitment of DC (immature), T_{mem}, T_h17, T_{reg}, and B-cells [125]. Primary human type II pneumocytes, DC, neutrophils, and B-cells – including B_{mem} (but not AM) - constitutively express CCL20, with expression of both CCL20 and CCR6 up-regulated during inflammation (e.g. due to TLR stimulation by LPS) [126] [125]. CCR6-CCL20 interaction mediates T_h17 entry into Peyer's patches (and other intestinal MALT sites), and B-cell localisation, differentiation, and isotype-switching in Peyer's patches. It may also be essential for B_{mem} recall responses.

Though some researchers find that CD8⁺ T-cells do not participate in Mtb recall responses, induction of CD8⁺ T_{mem} during Mtb infection is likely, because mycobacterial-specific IFN γ -secreting and cytolytic CD8⁺ T-cells are culturable from the circulation of healthy TST-positive individuals [115]. In Mtb-infected mice which were cured and rested prior to aerosol re-challenge, pulmonary CD4⁺ and CD8⁺ T_{mem} were detectable as early as one week post-challenge [115]. Activation marker was expressed on $\geq 30\%$ of these pulmonary T-cells (compared to $< 6\%$ in naïve mice), and *ex vivo* re-stimulation produced IFN γ (which did not occur in naïve mice) [115]. After Mtb infection, CD8⁺ T-cells from both naïve and memory mice were cytotoxic to Mtb-infected macrophages *ex vivo* [115]. However, Carpenter et al. (2016) find that while vaccination with the immunodominant antigen TB10.4 (aka. EsxH) induces a robust specific CD8⁺ T_{mem} response, subsequent challenge with Mtb induces only *transient* expansion of EsxH-specific CD8⁺ T-cells, and CD8⁺ T-cells from a new primary response outnumbers those from the memory response [127]. This is perhaps not entirely surprising, given that the overall number of naïve T-cells greatly outstrips that of specific T_{mem}, although T_{mem} will have the affinity advantage.

Other pulmonary cytotoxic leukocytes - the innate lymphocytes - are also involved in the Mtb recall response. Memory-like NK cells from human TB pleural fluid potently produce IFN γ in response to BCG [128]. BCG, Mtb antigens, IL-15, and IL-12 all enhance levels of IL-22 produced by TB pleural fluid NK cells (IL-17 is not produced by these cells in response to mycobacterial antigens) [128]. The IL-22-producing memory-like antigen-specific NK cell subset (CD45RO⁺) is distinct from that producing IFN γ , and their IL-22 production may participate in Mtb recall responses [128] (although CD4⁺ T_{mem} may also be a significant source of IL-22), including NK cell recruitment [129]. BCG-reactive memory-like NK cells are induced during infant vaccination, with *in vitro* IFN γ expression to BCG stimulation in an IL-12- and IL-18-dependent manner [130]. High frequencies of IFN γ -expressing BCG-reactive NK and NKT-cells exist at baseline in BCG-vaccinated (TST-positive, healthy) individuals, and subsequent BCG boosting facilitates significant durability of memory NK and NKT cells, whose levels "remain elevated up to one year after" boosting [130]. Such re-vaccination also transiently boosts BCG-specific CD4⁺ and CD8⁺ T-cells, as well as $\gamma\delta$ T-cells, all producing type 1 cytokines [130].

Non-human primate $\delta 2$ exhibit rapid recall expansion after Mtb infection of BCG-vaccinated animals, correlating with protection against fatal TB [131]. In a macaque BCG model, the $\delta 2$ subset expands rapidly during primary exposure and on secondary challenge [132]. Furthermore, in BCG-vaccinated (but not naïve) macaques, treatment with IL-17 or IL-22 and especially IL-23 leads to expansion of HMBPP-stimulated $\delta 2$ [132]. Expansion of such cells to HMBPP in the presence of IL-23 appears to be dependent on APC contact [132]. A memory immune response of antigen-specific $\delta 2$ is also seen in human studies [131]: human $\gamma\delta$ T-cells from BCG-vaccinated individuals expand on restimulation with mycobacterial antigens, displaying a memory-like phenotype [133]. Rapid $\delta 2$ recall in vaccinated humans correlates with protection against TB [131]. The Th17 family of cytokines may contribute to recall responses of antigen-specific $\gamma\delta$ T-cells after BCG vaccination/Mtb infection [132].

Whichever cell types are involved in anti-mycobacterial recall responses, BCG-mediated protection wanes as TB becomes chronic, coincident with a reduction in the number of IL-2-secreting CD4⁺ T-cells, T-cell terminal differentiation (increased number of KLRG1⁺ terminally-differentiated T-cells), progressively worse function of Mtb-specific human CD4⁺ T-cells, and increasing mycobacterial load [117]. Immunoregulatory signalling through KLRG1 (or PD-1/2 and PD-1L/2L, as well as the activity of T_{reg}) likely impact "long-term maintenance of equilibrium required to support chronic infection" [117]. Protection can be enhanced by BCG boosting combined with an Ag85B-ESAT6-adjuvant fusion protein, and declining T-cell functionality is partially attenuated by ATT [117].

Thus, a picture of the dynamic Mtb recall response and its protective correlates begins to emerge, featuring the following events. Early local responses by expanding innate lymphocyte residents probably provides early IFN γ (and perhaps IL-22), while early PRR-mediated responses by resident innate phagocytes (including production of IL-6 and TGF β) precede and are required for a transient memory Th17 response. This likely induces transient neutrophil recruitment and activation, as well as B-cell recruitment and IgA secretion, which precede and are required for the Th1/IFN γ response that replaces the early Th17 response (as well as for optimal cross-priming of CD8⁺ T_{mem}), with recruitment of T_{mem} to the site of disease at least partially via IL-22-induced β -defensin. It must be remembered that memory lymphocytes can be activated independently of TCR/BCR ligation (i.e. via cytokines or TLR stimulation). However, TLR-2 is dispensable for Mtb recall responses (and thus likely for the innate phase assisting the recall response): the response of TLR-2 KO mice following secondary Mtb exposure resembles that of WT mice regarding degree of resistance to infection, bacterial burden, frequency and phenotype of recruited IFN γ ⁺ T-cells, and quality of the granulomatous response [134]. Apparent protective correlates may thus include Mtb antigen-specific Th1 and Th17, IL-17, and IgA production.

7.1.3.4. Heterogeneity of Immune Responses to Mtb Complicates Understanding

The nature of comprehensive protective or insufficient anti-Mtb immune responses (both primary and secondary) remains unclear [103]. Almost every immune component (humoral and cellular) involved in the anti-Mtb response can correlate with both host protection and damage, often depending on timing, level, anatomical compartment, and immune context.

For example, most innate leukocytes are protective during early infection, but can become maladaptive during chronic infection. Furthermore, the primary and recall responses fluctuate over the course of Mtb infection, in part because Mtb adapts to host conditions, altering its gene expression profile and thus the palette of antigens to which the immune system is exposed [135]. Certain antigens (e.g. Rv2031, Rv2659, and Rv1284) are expressed during relative quiescence, others (e.g. Ag85B, Rv0203, and Rv1196) are expressed during the early replicative stage, and still others (e.g. ESAT-6, TB10.4, CFP10, Rv3872, Rv3614) are constitutive [135]. It is incompletely known which antigens are immunodominant for T-cell and humoral responses, and indeed which to target for optimum bactericidal activity.

Failure to limit Mtb replication - and to regulate consequent immunity - leads to clinical disease, with signs and symptoms as direct consequences of host inflammatory responses [1]. The quality/strength of response against certain combinations of antigens may correlate with clinical symptom emergence. Inter-individual (and even intra-individual [136]) heterogeneity (fig. 3) makes it difficult to identify such trends, but certain events do predict subsequent development of active TB, including a significant

increase in whole blood IFN γ in response to CFP10 [135]. A longitudinal whole blood transcriptome and plasma proteome study of LTBI adolescents demonstrates that progression to active TB is predicted by elevated interferon signalling and complement cascade events up to 18 months prior, with more temporally proximate alterations in “myeloid inflammation, lymphoid, monocyte, and neutrophil gene modules” [73].

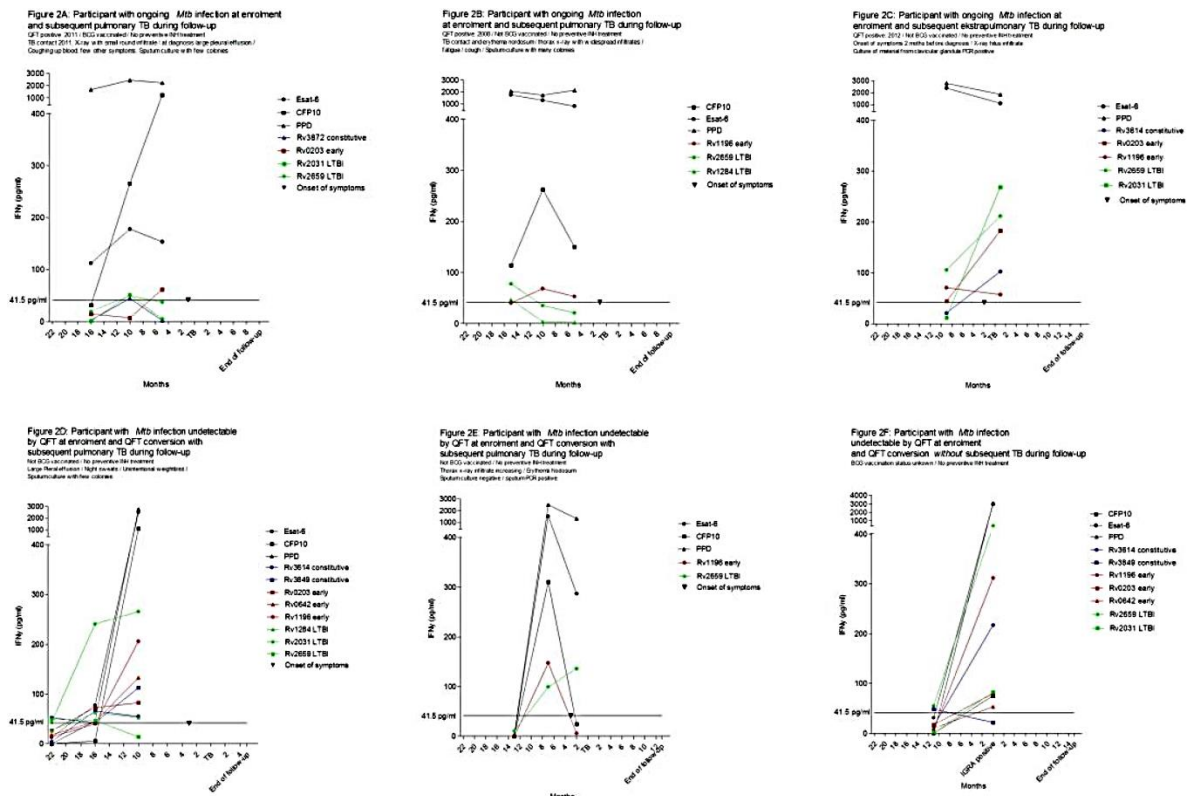


Figure 3: Differential Dynamic Inter-individual Immune Responses to Mtb Antigens after Natural Infection over a One-year Period [135]. 2A-C represent latently infected (by Quantiferon) participants developing active TB during follow-up. 2D-E represent apparently uninfected (by Quantiferon) participants becoming infected (Quantiferon conversion) and developing active TB during follow-up. 2F represents an apparently uninfected (by Quantiferon) participant becoming infected (Quantiferon conversion) but not developing active TB during follow-up. Black squares represent CFP-10 response measurements.

As alluded to, the diverse clinical states observable during human Mtb infection reflect underlying cellular and molecular immune heterogeneity [136]. Site of infection may differ, and there can be a continuum of inflammatory responses, from sub-clinical through local and systemic to dysregulated excessive inflammation [73]. Overall, a complex network of innate and adaptive immune mechanisms interacts with Mtb and its soluble/shed mediators, and contributes to host resistance and damage. “Infection with Mtb involves participation of >90 known antigens and various virulence factors, and results from interaction between the pathogen” and multiple host immune cells and mediators [24]. Looked at from an ‘omics perspective, systemic inflammation is detectable in peripheral circulation as increased levels of “cytokines, chemokines, acute phase proteins, and other inflammatory mediators” [73]. Indeed, a common molecular signature is shared by active TB patients (and is, in some respects, remarkably similar to that exhibited during TB-IRIS). The blood profile is characterised by up-regulated transcription of inflammatory genes, including type I and II IFN-inducible genes (including in circulating neutrophils and which correlates with extent of radiographic pulmonary disease [136]), genes “associated with myeloid cell inflammation and TREM1 signalling”, and FcR/complement pathway members, along with down-regulated transcription of genes encoding B- and T-cell function (perhaps due to the reduction in circulating T-cells observed during active TB) [103] [73]. Interestingly, “numerous

mechanisms of [antiviral] type I IFN impede protective Mtb immunity" [103]. Additional studies have investigated gene expression profiles longitudinally from TB diagnosis through ATT. A 320-transcript signature present after two weeks of ATT was significantly decreased by six months, with similar gene network alterations occurring during treatment: >1200 genes' (including C1q) expression became down-regulated within only one week of ATT, followed by a more gradual increase in humoral response genes [136].

Finally, anti-Mtb immune responses (and responses to ATT) are significantly influenced by various host, pathogen, and environmental factors, including Mtb strain and genotype, route of infection, site of disease [136], host genotype and epigenome (influenced by environment), prior BCG vaccination, host commensal microbiota, co-morbidities (e.g. helminth co-infection [137]) and their treatments, immunocompromise (e.g. poor nutrition, diabetes, malignancy, pharmacological suppression, HIV infection, etc.), stage of infection, and granuloma state [16] [136]. Particular host and Mtb genotypes combine to increase the likelihood of active TB and/or enhance disease severity [138]. In children under the age of five, and during MDR-TB [139], the more Th2-polarised response may favour EPTB [40]. As an example of the influence of site of disease, a high incidence of FoxP3⁺ T_{reg} is observed in EPTB [140]. As alluded to above, IL-10 levels also tend to increase during active TB [141].

The best known risk factor for active TB is immunodeficiency [138]. As mentioned in Chapter 1, HIV, especially, alters immunity in ways that significantly increase risk. Epidemiologically, TB-HIV symbiosis has driven the international spread of both epidemics [142]. Each enhances susceptibility to, progression of, and mortality resulting from the other [143]. Enhanced susceptibility to TB emerges as early as the time of HIV sero-conversion [144].

7.1.3.5. Mechanisms of Mtb-HIV Synergy

While Mtb has been co-evolving with humans for thousands of years, HIV only crossed the species barrier from non-human primates sometime in the 1900s (with AIDS first being recognised as a clinical entity in the 1980s), resulting in less mature bi-directional adaptation in the latter context.

Both pathogens are altered by host humoral factors prior to acquisition across different epithelial surfaces, leading to interactions with heterogeneous initial cell surface PRR, and thus heterogeneous intracellular fates and downstream immune responses. Bacterial-viral co-infection is not uncommon. For example, prevalent viral respiratory tract infections (e.g. influenza) predispose individuals to secondary bacterial infections (e.g. *Streptococcus pneumoniae*) [145] [146] [147] via direct pathogen interactions and/or viral impact on host microbiota and immunity [147] [148]. In a similar manner, both Mtb and HIV significantly modulate host immunity, exhibiting extreme host-detrimental synergy [149]. The scope of known *in vivo* interactions is too broad to comprehensively cover here, but we provide an overview in Table 5.

Table 5 Selected Examples of Pathogenic Synergy Between Heterogeneous Pathogens [150] [151] [152] [153] [154] [155] [156] [157] [158] [159] [71] [143] [160] [15] [161] [162] [163] [164] [165] [166] [149] [167] [168] [143] [138] [16] [169] [158] [155] [162] [166] [151] [150] [152] [149] [154] [160] [45] [71] [158] [161] [170]

	Mtb	HIV
organism type	bacterium	retrovirus
life cycle	facultatively intracellular, antigenic shift	obligately intracellular, antigenic drift
infection site	respiratory mucosa/alveoli	genital/rectal mucosa
tropism	lungs, SLO	GALT, inflamed tissue, SLO
infectious course	chronic, incl. 'dormancy'	chronic, incl. 'latency'
targets	epithelia, monocytes/macrophages, DC	epithelia, monocytes/macrophages, DC, CD4 ⁺ T-cells
implicated PRR	CR (classic: C1q, C3; lectin: MBL), FcR, TLR (2, 4, 9), CLR (DC-SIGN), NLR (NOD1, 2), cGAS-STING, inflammasomes (AIM2, NLRP3), ISG (IFI16)	CR (classic: C1q, C3; lectin: MBL), TLR (2, 3, 4, 7/8, 9), CLR (DC-SIGN), RLR (RIG-I), cGAS-STING, inflammasomes (NLRP3), ISG (IFI16, DDX3, PQBP-1)
immune response	anti-intracellular pathogen (Th1-dominant) phagolysosomal/autophagic/apoptotic destruction by phagocytes, cytotoxic destruction of infected cells	antiviral response (early IFN α , sustained IFN γ) ISG-mediated viral restriction, cytotoxic destruction of infected cells
immune correlates	protective: IL-1 β , IL-18, TNF α , IL-12/IFN γ axis, STAT1, poly-functional (IL-2 ⁺ IFN γ ⁺ TNF α ⁺ IL-17 ⁺) Th1 possibly: early IL-17, iNOS/NO, HIF-1 α , cytotoxic lymphocytes	susceptibility in elite controllers/long-term non-progressors influenced by: HLA polymorphisms, variant chemokines/receptors (CCR5, CCR2, CX3CR1, CCL3L1, CCL5/RANTES, CXCR1, etc.), antiviral ISG (IRF-1, APOBEC3G, TRIM5a, MX2, etc.) possibly also: CR2, C4BPA, TLR-3, DC-SIGN, TIM-3, ERAP-2, defensins, CYP7B1, etc.

correlates of advanced disease	type I IFN-dominant response, ↓ lymphocyte functionality, possible skewing towards Th2, higher IL-10	↓ CD4 ⁺ T-cell counts and functionality, possible skewing towards Th2, higher IL-10
mechanisms driving progression of the other pathogen	↑ target cell recruitment ↑ target cell HIV co-receptor expression and entry ↑ target cell activation and HIV transcription, replication ↑ viral evasion of host immunity (e.g. type I IFN)	depletion, chronic activation, dysfunction of most leukocyte types ↑ target cell Mtb uptake receptors ↓ microbicidal, efferocytic receptors (e.g. FcR, CD36) ↓ DC-induced NK IFN γ production and cytotoxicity ↓ antigen presentation to innate lymphocytes (e.g. NKT cells) and adaptive lymphocytes ↓ innate and adaptive mucosal lymphocyte IL-17 and IL-22 production ↓ CD4 ⁺ T-cells, esp. mucosal Treg and Th17, esp. Mtb/HIV-specific T _{mem} ↓ DC IL-23 impairs induction of Th17, ectopic B-cell follicle formation ↓ T-cell help limits BCR/Ig repertoire ↓ protective anti-Mtb Ig subtype ↓ B _{mem}
synergy	chronic cell hyperactivation, ↑ Mtb and HIV entry co-receptors (e.g. CD4, CCR5, CXCR4) infiltration by immature neutrophils/G-MDSC ↑ TLR-2 and TLR-4, increasing HIV replication and type IFN, respectively ↓ TLR responsiveness via DC-SIGN-Raf-1 signalling, thus ↓ appropriate cell activation induce MMPs and same cytokines, e.g. IFN α , IL-1 β , IL-18, IL-6, IL-8, TNF α , IL-12, IL-15, IL-22, IFN γ , IL-10 innate lymphocytes: ↓ cytokine expression, cytotoxicity, cross-talk with myeloid cells (e.g. NK-DC, δ 2-monocyte) myeloid cells: altered polarisation and ↓ chemotaxis, phagocytosis, respiratory burst, iNOS (via type I IFN), autophagy, microbicidal, antigen presentation, apoptosis (favouring necrosis) thus ↓ antigen clearance induce myeloid IDO-mediated ↑ kynurenine and ↓ tryptophan ↓ macrophage, NK cell, T-cell intracellular glutathione (e.g. via ↑ TNF α , ROS) ↑ lymphocyte death and inhibitory receptors (e.g. TRAIL, PD-1) and their ligands (e.g. TNF, myeloid cell PD-L1); type I IFN also up-regulates death receptor (TRAIL, DR5, Fas) and FasL expression ↓ IL-2 suppresses T-cell expansion induce IL-10 ⁺ TGF β ⁺ T _{reg} and Th2, and oppose Th1 TCR repertoire depletion/restriction ↑ CD8 ⁺ T-cell frequency, but ↓ cytotoxicity B-cell depletion (↓ T-cell help), ↓ mucosal IgA chronic inappropriate immune activation (incl. via dysbiosis, translocation) damaged SLO architecture, inhibiting lymphocyte activation and memory generation	

In some ways, Mtb and HIV also impact host immunity differentially. For example, the former favours monocytois, neutrophilia, and NK cell expansion, while HIV favours the opposite [171] [172] [173] [174] [155]. However, co-infection has been observed by different studies to produce both higher and lower monocyte counts [175] [176].

Mtb and HIV infection also have *indirect* impacts on each other, e.g. via disturbance of mucosal microbiome composition. Interestingly, *intestinal* commensals impact *pulmonary* CD4⁺ T-cell polarisation: the gut-lung axis [177] [178]. Reciprocally, pulmonary inflammation impacts circulating and gastrointestinal (GIT) microbes [179]. Thus, pulmonary inflammation alters systemic host-microbiome interactions, leading to translocation. It is unclear whether HIV directly induces alveolar epithelial changes leading to local translocation, but this remains a possibility. Airway dysbiosis may contribute pulmonary TB pathophysiology: both TB and ATT profoundly impact the human lung microbiome [178]. In fact, Mtb infection alters both respiratory and intestinal microbiota, but no quantitative consensus regarding diversity exists, and it is not known which changes, if any, correlate with prognosis [178]. Given that similar levels of intestinal fatty acid-binding protein (I-FABP, a proxy of translocation) are measurable during HIV-monoinfection and TB-HIV co-infection, intestinal translocation may contribute less to the intense chronic immune activation observed during co-infection than does Mtb-induced inflammation [180] at local sites of infection.

HIV-associated immune impairment even alters the physiology (and thus drug susceptibility, transmissibility, etc.) of Mtb itself. Mtb naturally adapts to withstand immune responses and diverse tissue microenvironments with variable pH, nutrient and oxygen availability, and levels of ROS/RNS [162]. Conditions (e.g. hypoxic or high NO) which limit Mtb aerobic respiration induce the DosR regulon, whose 48 gene products enhance Mtb survival and virulence, including via altering adaptive immunity to enhance T-cell-mediated granuloma formation [162]. Sputum gene expression profiling reveals that during co-infection with HIV, the DosR regulon is less induced (although of course this may be due to differential Mtb numbers in sputum), with lower host expression of IFN γ and higher host expression of IL-10 [162]. Lower DosR expression suggests that Mtb are experiencing less stress during HIV co-infection. It may be skewing by HIV of immunity towards alternative activation which produces the hypothesised “less hypoxic/lower NO [granuloma] conditions” [162].

Much is made of so-called “T-cell exhaustion” during chronic progressive HIV infection. This term refers to up-regulation of inhibitory T-cell surface receptors - such as PD-1, Tim-3, and CTLA-4 (on HIV- and Mtb-specific T-cells) – during HIV infection and progression to active TB [143] [181]. Given that resolution

is programmed into initiation of inflammation, lymphocyte activation itself inevitably triggers increased inhibitory receptor expression to *dampen* T-cell function when cognate ligands (expressed by myeloid cell types during the resolution phase) become available. Indeed, during the chronic stage of HIV infection, PD-1L is up-regulated on myeloid cells, likely reflecting attempts at resolution on a background of ongoing inflammation (due to non-clearance of inflammatory stimuli). However, so-called “exhausted” T-cells can be *re-activated* via immune checkpoint blockade (e.g. during anti-cancer therapy): clearly these cells are still capable of responding (exhaustion is not a terminal immunometabolic state or phenotype); rather, their function is being suppressed through normal protective mechanisms (whose effect during abnormal chronic inflammation becomes maladaptive). “Exhaustion”, therefore, is a misleading term for chronic expression of inhibitory receptors (indicating T-cell activation), not to be confused with true anergy (adaptive cell non-responsiveness to particular antigens).

The later post-infection ARV therapy is initiated, the less complete the degree of immune recovery that is possible. Significantly incompletely-reversible sequelae of HIV infection include disturbed SLO architecture, altered TCR repertoire diversity (including loss of certain Mtb antigen-targeting T-cell specificities [182]), altered mucosal δ 2 proportions and functions, diminished mucosal Th17 counts and functions (perhaps due to continued IL-23 signalling inhibition) [183], immune hyperactivation, and persistent low-level type I IFN signalling .

Thus, HIV alters many elements of the anti-Mtb immune response (even impacting adaptive gene expression of Mtb itself), and Mtb reciprocally alters many anti-HIV immune responses, altogether resulting in the unusually powerful observed Mtb-HIV pathogenic synergy. Infection with HIV alters the clinicopathological features of TB [1] in the following major ways:

Clinically, certain TB features (e.g. radiological) can be less pronounced [142] [162], although extrapulmonary and disseminated (severe) TB are more common. Presentation is often atypical [162] [184], with more frequent hilar lymphadenopathy, diffuse pulmonary involvement, and altered anatomical distribution [142] [184]. Existing granulomas are less prone to liquefaction and cavitation, and therefore less often erode into airways to produce smear-positivity or haemoptysis [142]. HIV often leads to reactivation of latent TB, the presentation of which may differ from that of primary TB (*if* apical/posterior upper or superior lower lobe lesions - where O₂ tension is higher – are more frequent, this may lend support to the hypothesis that hypoxia contributes to liquefaction). HIV-coinfected individuals also display less robust TST responses. In addition to the difficulty in diagnosing TB on a background of HIV, co-infection also increases the risk of ATT resistance, despite good adherence to treatment [142].

Histologically, fewer/smaller granulomas are present, and exhibit significantly disorganised architecture with poorer demarcation [149] [162]. In animal models, poorly-delineated granulomas may be anhypoxic, a finding which may extend to humans [162] and which would support the hypothesis that hypoxia contributes to liquefaction.

Altogether, although HIV suppression of host immunity leads to a higher Mtb burden and increased likelihood of dissemination, it also partially ameliorates TB *immunopathology* [1].

7.2. Response to, Off-target Effects of, & Immunopharmacology of ATT & ARVs

7.2.1. Anti-tuberculous Therapy

Successful ATT reduces the Mtb load (also making available a wider epitope variety, although Mtb antigens are eventually cleared from the system), thus normalising various immune and endocrine parameters [185]. For example, ATT decreases (normalises) IL-1 β [186], IL-6, CRP, IFN γ , TGF β , [185], IP-10, VEGF [187], and CD4⁺ T-cell (not including T_{reg}) PD-1 expression levels [188]. ATT also helps normalise increased cortisol, estradiol, prolactin growth hormone, thyroid hormone/T4, ghrelin, and dopamine, and decreased epinephrine, DHEA, testosterone, and leptin levels [189] [190] [191] [192] [193].

Successful ATT also reduces host amylin, increases host estradiol, T3, and leptin, and initially increases (then decreases) adiponectin [141] [186]. Hormone-associated pathways impacted during effective ATT include “inflammasome”, “cytokine-mediated communication between immune cells”, “VEGF ligand-receptor interactions”, and “signalling of the myeloid cell receptor, TREM1” [141]. Furthermore, successful ATT effectively lifts the immune sanctions imposed by *Mtb*'s immunomodulatory mechanisms.

However, ATT also has direct effects on the host microbiome. It is active against various microbes, and may impact mycobacterial intestinal commensals (e.g. *M. avium* subspecies *paratuberculosis*, implicated in Crohn's disease pathogenesis [178]). However, relative to other antibiotic regimens, ATT-induced *Clostridium difficile* diarrhea is uncommon, suggesting that first-line ATT impacts the intestinal microbiota to a lesser extent than other antibiotics [178]. Nevertheless, ATT (especially rifampicin), like ARV therapy, leads to murine gut dysbiosis (with multiple affected taxa known to be associated with altered immune function) which persists for at least three months after cessation of therapy [194].

Furthermore, many antimicrobial agents directly modulate immunity. Rifampicin apparently suppresses humoral and cellular immunity *in vitro* and *in vivo* [195]. Publications report conflicting direct effects of ATT on innate phagocyte function [196]. Rifampicin inhibits IL-1 β and TNF α secretion, while enhancing IL-6 and IL-10 secretion by stimulated human monocytes [197]. It also enhances PGE₂ production by granulocytes [198], and is beneficial against the inflammation of rheumatoid arthritis [199]. INH initiates a lupus-like syndrome in certain individuals (albeit very rarely) [200], induces low-level *ex vivo* T-cell expansion at low doses (despite no intrinsic mitogenic properties), and suppresses T-cell expansion at high doses (not mediated by its cytotoxic effects) [200] [201]. In the presence of PZA, stimulation of human monocyte TLR-4 with LPS reveals a concentration-dependent inhibitory effect on secretion of IL-1 β , IL-6, TNF α , and MCP-1 (the only mediators investigated), whereas stimulation with TLR-2/6 and TLR-1/2 agonists reveal concentration-dependent inhibitory effects only for IL-1 β and MCP-1, respectively [202]. Little information is available regarding the immunomodulatory effects of ethambutol.

Given that other body systems impact the immune system and vice versa, other off-target effects of ATT should not be discounted. Adverse drug reactions to ATT are common, and include hypersensitivity and hematologic disturbances (rifampicin and INH), and hyperuricemia and arthralgia (PZA and ethambutol). Rifampicin also induces cytochrome P450 (CYP3A4 [203] and CYP3A5 [204]), INH also causes neuropathy (the reason for prophylactic co-prescription of vitamin B6), PZA (also the most hepatotoxic drug in the regimen) can cause hypersensitivity, and ethambutol can cause optic neuritis.

7.2.2. Antiretroviral Therapy

Many studies investigate immune dynamics following ARV initiation, but the full extent of immunological impact remains unknown [205]. Similarly to ATT, ARV agents impact host immunity both indirectly (via effects on VL, lifting of HIV-induced immunomodulation, and off-target effects on various body systems) and directly (via off-target immunomodulatory effects).

Regarding beneficial effects related to viral suppression, there is gradual restoration of pathogen-specific immune responses, including CD4⁺ T-cell reconstitution [206]. An impressive >90% reduction in VL occurs within the first two weeks of ARV therapy [207]. The CD4 count increase occurs in 2 phases [207]. An initial rapid increase during the initial weeks' VL drop extends over the first two to three months (mainly due to redistribution of sequestered activated CD45⁺RO⁺ T_{mem} from lymphoid tissue, and reduced apoptotic cell death [207]). The slower second phase of CD4 expansion occurs over the subsequent months to years (≥ 3 -4yrs if ARV therapy was initiated in advanced HIV [207]), mainly due to reconstituting thymic function expanding CD45⁺RA⁺ naïve cells to produce a sustained CD4 count increase. During chronic infection, LN T_h expand, concomitant with germinal centre hyperplasia and hypergammaglobulinemia [208]. *Single-cell transcription factor* profiling of circulating HIV-Gag-specific CD4⁺ T-cells during ARV therapy demonstrates early (transient) potent Th1 effector responses, followed by increases in T_{reg} and T_h-like memory (T_{cm}) cells (T_h egress from lymphoid tissue germinal centres may thus account for delayed circulating T_{cm} increases) [208]. Although the circulating CD4 count increases during ARV therapy, lymphoid CD4⁺ T-cell populations (e.g. GALT, LN) remain low [209]. Thus, while ARV

therapy selectively normalises some disturbed immune indices (e.g. CD4 count, some T-cell activation markers), innate and adaptive immunity remain incompletely restored, and other indices become further perverted [205] [209] [210].

As mentioned, the length (and thus stage) of HIV infection at which ARV therapy is initiated to a large extent determines the degree of possible immune recovery. The degree of immune activation is also negatively associated with immune reconstitution: some patients do not even achieve satisfactory recovery of circulating CD4 count [211] [205]. Incomplete immune reconstitution and persistent hyperactivation despite ARV therapy continue to be a challenge [212]. For example, even effective ARV therapy only partially normalises microbial translocation [213], and residual T-cell and especially innate activation (attributed largely to monocytes/macrophages [205]) persists [209]. This is associated with an increased risk (relative to HIV-uninfected individuals) for clinical conditions associated with chronic inflammation (e.g. cardiac disease, clotting disorders, pulmonary hypertension, emphysema, and stroke) [209], and for mortality from non-AIDS causes [205].

Various genetic and environmental factors (e.g. composition of commensal microbiota, diet, substance use, opportunistic co-infections, pharmacological agents, etc.) also impact the rate and degree of immune normalisation during ARV therapy [214] [215]. TB is one such co-infection, and the quality of its impact may also be influenced by the timing of TB relative to HIV acquisition, as well as the timing of ATT initiation relative to TB acquisition and stage of infection. Thus, a complex state of immune abnormality and imbalance persists, making it difficult to predict immune system behaviour according to the principles that govern immune trends in a healthy individual.

In terms of direct immunomodulatory effects independent of viral suppression, efavirenz is best-studied. It is associated with endothelial dysfunction and increased: LDL, F2 isoprostanes (prostaglandin-like molecules formed by free radical-mediated peroxidation of arachidonate; markers of lipid peroxidation due to oxidative stress [216]), and CRP [217] (not observed with ARVs such as maraviroc [218]). *Ex vivo*, efavirenz induces acute direct mitochondrial toxicity, increases superoxide anion production, and enhances vascular permeability by reducing junction protein levels [217]. *In vitro* and in animal models, efavirenz also enhances neutrophil and monocyte endothelial adhesion by augmenting expression of CD11b and c, as well as CD18 (components of Mac-1 and gp150,95) [217].

Tenofovir has diverse immunomodulatory properties *in vitro*. Findings regarding anti- versus pro-inflammatory effects on macrophages, lymphocytes, and murine hepatocytes are in opposition [219]. Complicating matters further (but likely explaining the disparity), *ex vivo* tenofovir effects are *cell-specific*, including – like other reverse transcriptase inhibitors – modulating serum and cervico-vaginal lavage cytokine and chemokine levels, and peripheral leukocyte responses [220]. This includes eliciting IL-8 from CD4⁺ T-cells, and IL-8 and MIP-3a from blood-derived macrophages [220]. When exposed to tenofovir *ex vivo*, female reproductive tract CD4⁺ T-cells do increase production of IL-8 (endometrium, ectocervix), but also of TNF α (cervix, ectocervix) [220]. Endometrial CD14⁺ cells increase production of IL-8, TNF α , and MIP-3a [220]. *Ex vivo*, tenofovir enhances IL-8 and TNF α secretion by epithelia from endometrium and ectocervix (but not cervix), and reduces IL-8 secretion by fibroblasts from endometrium and cervix (while ectocervix fibroblasts increase IL-8 secretion) [220]. In HIV-negative individuals, combination tenofovir/emtricitabine reduces CD8⁺ T-cell CD38/HLA-DR co-expression and circulating sCD27 (a correlate of immune activation shed by activated CD4⁺ T-cells) [219]. Little information is available regarding the direct immunomodulatory effects of emtricitabine in isolation.

In terms of adverse drug effects, NRTIs are linked to mitochondrial toxicity and resulting metabolic and cardiovascular disturbances (e.g. lactic acidosis, hyperlipidemia), as well as (in almost a third of patients) peripheral neuropathy [212]. Mitochondrial DNA polymerase- γ inhibition leads to mitochondrial dysfunction, anaerobic respiration, lactic acidosis, oxidative damage, and clinical symptoms [221]. At least clinically speaking, NRTI-mediated mitotoxicity only manifests after several months, although this does not preclude earlier sub-clinical molecular and cellular events. The therapeutic and (at least partially) toxic effects of NRTIs depend on intracellular triphosphate nucleotide analogue formation [221]. At least *ex vivo*, female reproductive tract epithelia and CD4⁺ T-cells respond to tenofovir by decreasing nucleotidase-mediated dephosphorylation of nucleotides, but

circulating macrophages and CD4+ T-cells increase nucleotidase activity in response to the same stimulus [220]. Together, intracellular emtricitabine (NsRTI) and tenofovir (NtRTI) decrease dATP and dCTP production by 11% and 14%, altering the intracellular dNTP pool [219].

Both S.A.'s first-line NRTIs (at the time of the study) concentrate in monocytes, producing four-fold higher levels compared to T-cells [219]. A tenofovir/emtricitabine- (rather than abacavir/lamivudine-) based regimen is significantly positively associated with risk of subclinical atherosclerosis progression [222]. Tenofovir is also commonly associated with nausea and vomiting, headache, fatigue, heartburn, diarrhea, dyslipidaemia, hyperphosphataemia, and hypocalcaemia [223] (associated with reduced bone mineral density) [223] [221]. More rarely and more seriously, proximal renal tubulopathy causes excessive renal phosphate and calcium loss, and 1-hydroxylation defects of vitamin D [223]. Although the *in vitro* capacity of tenofovir and emtricitabine to inhibit polymerase- γ is lower than that of other NRTIs, tenofovir-induced proximal tubule damage is due mitochondrial toxicity arising from drug over-accumulation (due to uptake by organic anion transporter-1 and -3: OAT1 and 3) [221]. Tenofovir may also lead to reduced urate levels, however; urate correlates with systemic inflammation, including CRP, TNFa, and IL-6 [219].

NNRTIs are associated with neuropsychiatric disturbance, hypersensitivity, liver toxicity, and lipid abnormalities [212]. Efavirenz, especially, has a non-ideal toxicity profile, mainly manifesting as neuropsychiatric abnormalities (dizziness, insomnia, disturbed dreams, and depression) [224]. Longer-term effects include loss of bone mineral density, likely mediated by renal toxicity alteration of phosphate and calcium metabolism [225] .

7.3. Complexity & Elucidating TB-IRIS Pathogenesis

The proof that order can arise from disorder [226] underpins Complexity Theory. In the case of biology, simple physical laws are the basic programming from which "all the extraordinary richness ... shown by living things" arises [227]: living systems self-assemble and function via successive spontaneous biochemical events [228]. Although individual physical laws are deterministic, living systems represent complex adaptive (responsive, dynamic) systems exhibiting non-deterministic behaviours (Table 6). A living organism represents a hierarchical complex system with highly-interacting functional modules: every conceivable layer is connected to every other, even if indirectly, which means that every system causally impacts every other system in various ways. Each functional layer on its own, too, represents a complex adaptive system that is more than the sum of its parts, and exhibits "utterly new ... properties" not predictable from the characteristics of their individual parts [228].

Table 6 Properties of Complex Adaptive Systems

Initial	Consequent
self-assembly/autopoiesis	openness/unboundedness
numerosity/multiplicity of components	functional modularity (overlapping)
rich bi-directional interactions	hierarchicality (every level is also a complex adaptive system)
self-regulation (*stochasticity, feedback)	dynamic equilibrium robust to perturbation (though key nodes/edges do exist)
entropy-driven decay of regulatory capacity	emergence

Hierarchical levels interact bi-directionally with each other, forming a continuum of inter-dependencies.

*Stochasticity: randomly determined, with a distribution of outcomes along a continuum (not just binary).

Complexity Theory is highly relevant to biological systems, and "has revolutionised our understanding, prediction", and control of the behaviour of systems such as the brain [229]. System evolution and current system state are influenced by external and internal conditions, and are dependent on the system's preceding (causal) history [230]. Even though complex biosystems (such as the immune system) may be conceptualised as enormous if/then algorithms, it is very difficult (but likely not impossible) to trace the path of causality through such a system; to predict how any particular perturbation or perturbations will propagate through the system and which functional outcome(s) will result; to disentangle cause and effect (as well as disentangling association versus causal relationships).

For example, mitochondrial/ER stress may be a *cause* or a *consequence* of dysregulated intracellular trafficking.

Such systems thus pose challenges in analysis (simultaneously studying the trajectories of all components and interactions of the system over time), representation (each current method has advantages and shortcomings; fig. 4), deep conceptual understanding and simulation (following the causal flow of information propagating through the system), and manipulation (e.g. optimisation to prevent/repair decay/malfunction).

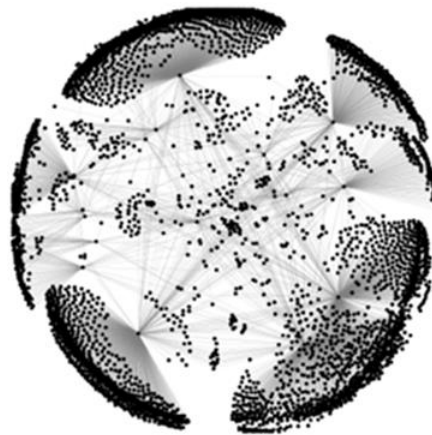


Figure 4: Network Diagram Representing Nodes and Edges of a Complex System. Although such an approach partially represents components, interactions, and functional groups, it fails to represent compartments and dynamic behaviour (among other important properties).

Complex systems are highly relevant to biomedicine, because complex biosystems (with high inter-individual variability and significant noise) underlie complex diseases (not determined by a single gene product, but in which multiple genetic and environmental factors interact to produce the pathological phenotype) (fig. 5).

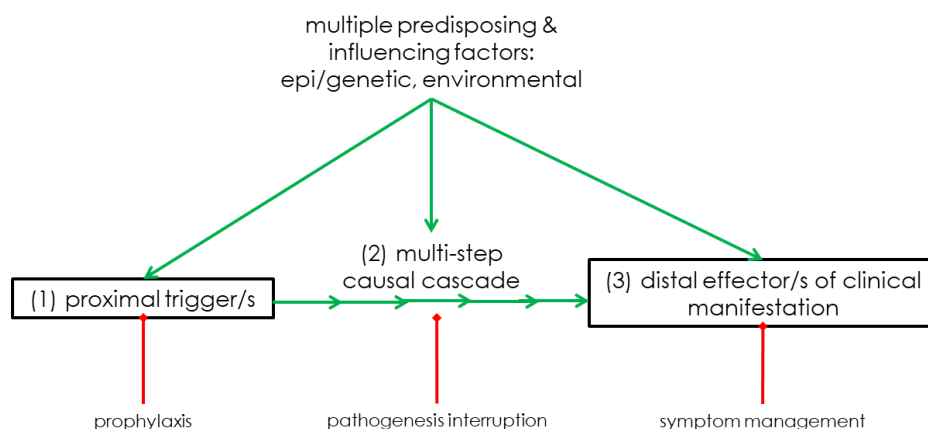


Figure 5: General Chain of Complex Disorder Pathogenesis. Potential points of intervention are indicated in red.

The classical reductionist, isolationist research paradigm produces understanding only at the single-component level, in a manner not necessarily reflective of its *in vivo* function. At best, this provides a single pixel of a larger image, and, at worst, is misleading (observations made at the single-component level are often not borne out by the holistic system, because isolated components behave differently

when they are not embedded within their native web of interactions). We thus find ourselves close to the limits of resolution of classical Cartesian reductionist approaches. In many cases, normal physiology remains incompletely understood, identification of preclinical disease is not a priority (despite prevention being preferable to cure), the pathogenesis of many (especially complex or multi-system) diseases remains unknown, and there is only a modicum of recognition that a single disease manifests as many different phenotypes (as well as that therapeutic strategies have differential impact) across individuals (i.e. population approaches have been preferred to personalised approaches, also for economic reasons) [231].

The preceding information makes clear that complex and dynamic biological systems such as the immune system (and their roles in complex diseases) cannot be satisfactorily studied under a reductionist or isolationist paradigm [232]. Going forward, only a deep conceptual understanding of complex adaptive biosystems can drive the next wave of innovation in healthcare (optimisation, risk stratification, prevention, diagnostics, prognostics, treatment, and treatment monitoring). Conceptually, we are shifting away from the reductionist paradigm, towards a more holistic idea: that all health and disease phenotypes reflect the interactions of complex adaptive biological subsystems, both with each other and with their environment (fig. 6).

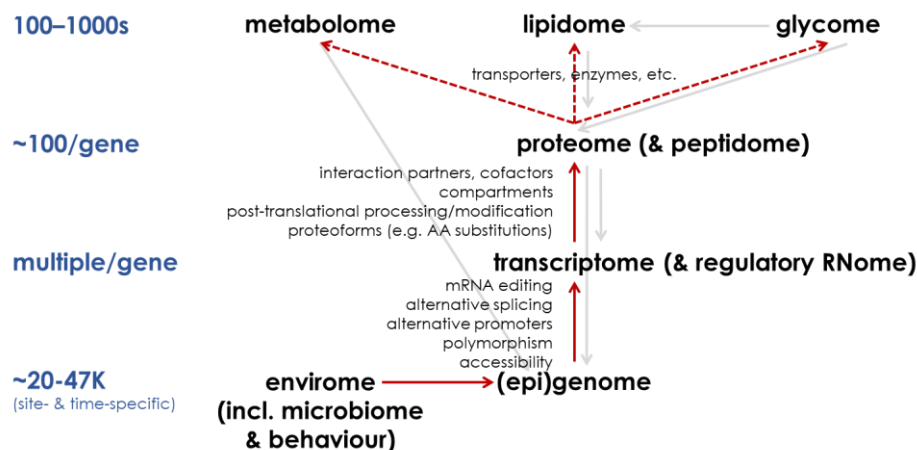


Figure 6: Selected Interactions of Complex Adaptive Biological Subsystems. Disease-causing endogenous/exogenous factors alter protein expression in multiple physiological pathways, leading to pathological cellular behaviour and the disease phenotype [233]. Integration of multi-omic data is termed trans-omics.

This shift in scientific philosophy, coupled with revolutionary progress in molecular biology analytic technology and computational approaches/capacity is beginning to facilitate true attempts to understand biomedicine at the systems level [234]: to create holistic models which more accurately represent and predict the behaviour of entire complex biological systems (although this is still – for now – a somewhat prohibitive undertaking). Meaningful interrogation of complex immunological disorders requires methods capable of investigating not only multiple components in multiple compartments simultaneously, but also their alterations over time. This is the purview of systems biology.

Latest-generation proteomic techniques, in particular, are ideally suited to studying complex adaptive biosystems such as the immune system, and thus to understanding complex disorders such as TB-IRIS. Since proteins are the major molecules executing cellular (and indeed inter-cellular) functions, they are most proximally responsible for cells' functional states. Indeed, most existing effective drug targets are proteins. Furthermore, the proteome is many orders of magnitude larger than what can be predicted from the genome or even transcriptome (fig. 6), due for example to alternative splicing events, post-translational processing, and post-translational modification, all of which can significantly alter protein

function. Biological complexity is more a function of this plastic continuum than of absolute genetic content [232].

As discussed in Chapter 3, one major limiting factor is that our knowledge of existing functional biological networks, as well as the sampling of molecules during 'omics experiments, is incomplete. Large numbers of nodes and edges within the functional modules of interest may be missing, and many irrelevant ones may be present [232]. Lu et al. (2007) demonstrated that peripheral rather than highly-connected genes are the most disturbed during pathology [235], and Goh et al. (2007) demonstrated that most disease-related genes are actually non-essential, and are typically not central hubs within functional molecules (i.e. are not good therapeutic targets) [236]. However, even partial maps (i.e. a relatively random selection of nodes and an incomplete list of edges) "provide a useful hypothetical description of protein-protein interactions", and we may still model biological systems despite a degree of incompleteness [232]. Even modelling only a subset of complex biosystems data facilitates innovations in health optimisation and disease risk stratification, prevention, diag/prognostics, treatment, and monitoring.

Finally, although current techniques are unable to make sufficient use of high-quality 'omics data to comprehensively model all the information and events within a complex system, the future promises the use of deep learning approaches (perhaps in combination with quantum computing) for improved understanding, representation, and *in silico* simulation of complex adaptive biosystems.

7.4. Hypotheses Arising from First Principles: Integrated Baseline and Pathogenesis-associated Literature

Paradoxical TB-IRIS has been classically conceptualised as dysregulated immune recovery causing an overzealous inflammatory response to residual Mtb antigens. We are able to derive a great many specific and plausible hypotheses regarding TB-IRIS predisposition and pathogenesis from first principles (Table 7; all references are laid out in Chapter 1), but many questions regarding the pathogenic chain and potential interventions remain unanswered (*vide infra*).

Table 7 Literature-derived Hypotheses Regarding TB-IRIS Predisposition & Pathogenesis/Manifestation

Factor	Predisposition	Pathogenesis & Manifestation
Mtb/HIV co-infection	advanced	partially-treated
VL (& likely type I IFN)	high	falls rapidly
Mtb load	high	?high
immunometabolic state	altered: ?cachexia cholesterol homeostasis eicosanoid cascade (↑ HETE)	↑ Trp conversion to NAD further altered: cholesterol homeostasis eicosanoid cascade (↑ COX-1, 9-HODE)
cellular adaptogen levels (e.g. glutathione)	?depleted	?further depleted
myeloid metabolic (incl. mitochondrial) stress (pro-inflammatory)	likely (incl. Fe load-associated oxidative stress)	likely (incl. Fe load-associated oxidative stress, possibly TNF-induced mROS)
myeloid mobilisation	+ (?ROS, C5a, IL-8)	+ (?ROS, C5a, IL-8, G-CSF)
myeloid activation	+	++
inflammasome activation	+	++
TLR activation (& IL-1R/IL-18R/TLR-MyD88-NFκB signalling)	likely impaired	++ (?due to lifting of Mtb/HIV/type I IFN suppression)
TREM-1 enhancement of MyD88 signalling	?	+
elevated cytokine profile	IL-1β, IL-18, IL-8, IFNγ, IL-27 (likely high IFNα) (equivalent TNFα) suggests host stress- rather than pathogen- induced inflammation	*IL-1β, *IL-18, *IL-6, *TNFα, IL-8, IFNγ, *IL-10 (increasing IL-12 & MCP-1) (equivalent IFNα) suggests pathogen-induced inflammation ± host stress
myeloid cytokine involvement	increases risk (likely indirect)	involved in pathogenesis & manifestation (directly)
ATT/myeloid activation & *MMP-induced matrix turnover-enhanced provision of Mtb Ag	+	++

myeloid cell death	likely primed for necrosis > apoptosis	likely co-ordinated necrosis (pyroptosis, ferroptosis, necroptosis) releasing host inflammatory stress molecules, host tissue-damaging molecules, & granuloma-sequestered Mtb/antigens, mimicking new infection
microbicidal/clearance of Mtb/antigens	↓ despite myeloid activation (? due to ↓ glutathione, ↓ oxidative burst, ↓ NO, ↓ autophagy, complement- rather than FcR-mediated uptake, etc.)	likely ↓ despite myeloid overactivation (? due to ↓ glutathione, ↓ NO, ↓ autophagy, ↑ IL-10, complement- rather than FcR-mediated uptake, etc.); thus inflammatory stimulus persists
restraint of inflammation	risk of ↓ restraint (e.g. classical complement activation, IL-12-induced Th1 polarisation)	↓ restraint (e.g. classical complement activation, IL-12-induced Th1 polarisation, decline in type I IFN, decline in autophagy stimulus)
coagulability	+	+
innate cytotoxic cell activity	↑ (δ2, NK – with degranulation)	↑ (δ2, NK – without degranulation, *granzyme B possibly from neutrophils)
adaptive lymphocyte activity	↓ CD4 ~ / ↑ CD8 ↓ anti-PGL-Tb1 antibody titre (other antibodies equivalent)	CD4 mobilisation & reactivation (Mtb-specific polyfunctional T _{em}) simultaneous Th1, Th2, Th17, & T _{reg} polarisation (T _{reg} ineffective) ~ / reactivated CD8 ↓ anti-PGL-Tb1 antibody titre (↑ post-IRIS) (other antibodies equivalent)

*corticosteroid-suppressed mediators

Thus, as mentioned, epidemiological and clinical observations strongly suggest that more advanced TB/HIV-coinfection is what predisposes to the TB-IRIS phenomenon (and could also account for highly-variable incidence rates between studies). More profound and less reversible consequent immunometabolic equilibrium disturbances may represent a state specifically primed for TB-IRIS, i.e. predisposed to dysregulated immune and metabolic function (including induction of host cell metabolic stress) on therapeutic lifting of dual pathogen-induced immune embargoes (and possibly requiring longer treated intervals to re-approach homeostasis).

Baseline metabolic disturbances may include, for example: dyslipidemia (e.g. type I IFN-inhibited cholesterol and steroid hormone production, also impacted by the altered eicosanoid cascade balance), glucose intolerance, cachexia, glutathione depletion (increased vulnerability to oxidative stress), cellular iron loading (with resultant oxidative stress), susceptibility to oxidative/mitochondrial/ER stress (e.g. an inflammatory state of high energy demand, or decreased capacity for stress-compensatory autophagy), and thus overall a metabolic state primed for necrosis (necroptosis, ferroptosis, and/or necroptosis) rather than apoptosis of stressed cells.

Baseline immune disturbances may include, for example: increased type I IFN ± Th2-type ± IL-10/TGFβ signalling, inhibition of inflammasome/IL-1 activity and TLR signalling via MyD88 e.g. by VL-elicited type I IFN (but inflammation-induced increased expression of components, including enhancing TREM-1 and other intracellular NA-sensing PRR), higher Mtb-induced IL-27 (with T-lymphocyte sensitisation to IL-12) ± IL-32/TLSP levels, abnormally-activated myeloid cells (exhibiting e.g. higher complement production/decreased opposition of complement activity, lower NO production (perhaps due to higher type I IFN levels inhibiting iNOS), possibly elevated IL-10 production, and defective microbicidal/antigen clearance/antigen presentation – perhaps also due to lower myeloid-activating IFN_γ levels, with consequent higher granuloma-sequestered Mtb/antigen loads), more advanced SLO disruption, mucosal Th17 depletion, an altered TCR repertoire (perhaps with relatively more quiescent Mtb-specific T_{cm} escaping HIV-mediated depletion), innate lymphocyte provision of myeloid-activating IFN_γ, and thus overall an immunological state primed for exaggerated, abnormal rather than well-regulated, normal myeloid/lymphoid responses. For example, more advanced HIV (i.e. more pronounced T-cell depletion) likely produces the higher-trending baseline levels of IL-7 in patients who go on to develop TB-IRIS, and IL-7 may (e.g. via acting as a rheostat increasing T-cell sensitivity to even low levels of Mtb products) create ideal conditions for reactivation of Mtb-specific polyfunctional T_{mem}. Given the apparent improper activation of myeloid cells (i.e. their inability to efficiently kill intracellular Mtb or clear antigens) in these patients, it is likely that their granulomas harbour not only dead but also live Mtb (despite partial ATT, since granulomas are akin to abscesses, with poor rifampicin and INH penetration resulting in sub-therapeutic granuloma concentrations [237]; even host-activated PZA will be less effective in an immunologically-inactive granuloma).

Overall, literature supports a relatively-unrestrained further increase in myeloid (including complement) activation and myeloid necrosis within granulomas at the time of TB-IRIS, with simultaneously increased innate lymphocyte activation, induction of incompatible adaptive lymphocyte response programmes, and transition to inflammation chronicity (likely due to prolonged persistence/delayed clearance of inciting stimuli ± failure of immune regulatory mechanisms). As discussed in Chapter 1, corticosteroids likely support myeloid-mediated antigen clearance as well as affecting energy metabolism and inhibiting innate and adaptive lymphocyte responses, but partial prevention and alleviation of TB-IRIS symptoms may be due largely to suppression of downstream mediators (e.g. IL-6, TNFα) directly responsible for clinical manifestation, rather than acting on proximal triggers.

Our hypothesis - most parsimoniously explaining all above observations – is that in more advanced Mtb/HIV-coinfection, viable and non-viable Mtb (and derived antigens) remain partially sequestered within tissues (within myeloid phagocytes such as neutrophils and macrophages, including within granulomas) at sites of TB disease, with ATT stress possibly inducing production of additional antigens by viable Mtb/increased availability of additional antigens as bacilli are destroyed. We propose that Mtb-sequestering myeloid cells are already metabolically stressed (predisposed to inflammatory forms of cell death, e.g. due to depleted glutathione decreasing antioxidant capacity in the context of iron loading-induced oxidative stress) and immunologically primed (predisposed to relatively less restrained, more abnormal immune responses, e.g. due to inhibition of inflammasomes and TLR signalling) at baseline.

Since TB-associated granuloma liquefaction is likely due to sudden co-ordinated myeloid death in affected organs, we further propose that the addition of ARV therapy in this context (either directly inducing mitochondrial stress or indirectly inducing mitochondrial stress via TNFα or lifting of HIV-mediated immune embargoes), rather than producing Mtb clearance, triggers co-ordinated death of tissue-sequestered (including granuloma-resident) myeloid cells (at least in the lungs and LNs, if not necessarily other TB-affected organs), leading to release of pro-inflammatory molecules in a manner mimicking new infection: host stress molecules (e.g. nucleic acids, ATP, HMGB1, alarmins/cytokines, etc.) and previously-sequestered Mtb/antigens (including via MMP-mediated matrix turnover ± granuloma liquefaction and cavitation) in a manner mimicking new infection as well as reactivating whichever Mtb-specific memory lymphocytes – perhaps including innate – remain (recall that the majority of mycobacterial IRD - including TB-IRIS - cases arise during redistribution of memory T-cells from SLO [1]). Indeed, ARV-mediated recovery (especially in patients experiencing excellent virological responses) of granuloma hypoxic conditions and NO production - further stressing Mtb bacilli - as well as antigen presentation may enhance these processes. Granuloma myeloid cell reactivation is supported by the observation of elevated circulating active vitamin D and hypercalcemia during some instances of mycobacterial IRD [1]. Even early publications noted that “LNs involved in mycobacterial IRD may show clinical suppuration, and biopsy specimens of affected tissues may reveal a degree of necrotising inflammation ... unusual in the context of profoundly immunocompromised patients” [1]. Although clinically-apparent suppurative necrosis (e.g. of LNs) only typically appears *after* onset of TB-IRIS, this does not necessarily preclude microscopic (clinically non-apparent) necrosis. Even if overzealous myeloid cell infection/stress-response pathways and co-ordinated death only contribute to (i.e. do not *initiate*) the inflammation of TB-IRIS, they would certainly explain the observed tissue damage as well as contributing towards further-delayed Mtb/antigen clearance. The massive resultant release of autoantigens into aberrant biological compartments in a pro-inflammatory context (i.e. high likelihood of neoepitope-generating modifications) will almost certainly also lead to generation of some autoantibodies or reactivation of pre-existing auto-reactive natural/memory B-cells (provided these mechanisms remain functional).

Although this study has been the first to propose such a host-cell-necrosis-and-content-release-contributing-significantly-to-inflammation hypothesis, a recent publication looking to related inflammatory disorders for insights regarding TB-IRIS pathogenesis has also come to the independent conclusion that immune reconstitution triggers myeloid inflammasome overactivation and lytic death with release of antigenic content, thereby contributing to T-cell activation, higher levels of IFNγ, and “a positive feedback loop of further activation” and cell death, causing hypercytokinemia [238].

However, their hypothesis does not take into account pre-existing factors stressing myeloid cells, nor the contribution of host inflammatory molecules; additionally, since innate lymphoid cells are also a source of IFN γ , hypothesised uncoupling of myeloid activation from T-cell activity is likely incorrect).

Thus, via normal routes, release of host- and pathogen-derived inflammatory molecules potentiates myeloid activation (overzealous due to baseline predispositions), innate cytotoxic lymphocyte activation (though perhaps only partially-effective), and reactivation and expansion of Mtb-specific poly-functional CD4 $^{+}$ T-cells (with induction of multiple incompatible response programmes, including only partially-effective FoxP3 $^{+}$ T $_{reg}$ responses). It is possible that perturbed cholesterol/isoprenoid pathway precursors released during cell death activate $\delta 2$: it is possible that even at baseline there is relative preservation of CD1-mediated versus MHC-mediated antigen presentation (though HIV does also interfere with innate lymphocyte function, ARV therapy restores the $\gamma\delta$ T-cell repertoire and $\delta 2$ IFN γ responses to phosphoantigens in a manner apparently dependent on mycobacterial-specific IFN $^{+}$ Th [131]). A secondary response does not preclude a simultaneous new primary response, and partially-effective FoxP3 $^{+}$ T $_{reg}$ responses do not preclude effective FoxP3 $^{-}$ Tr1 or regulatory myeloid cell responses. Perhaps IRIS arises only in those exhibiting sufficient remaining anti-Mtb memory despite HIV, or in those in which Mtb lipid phosphoantigen-reactive innate T-cells cross-react with high levels of host isoprenoids (it is apparently unknown whether these molecules share mimotopes).

Despite T-cell-mediated myeloid cell activation, defective antigen clearance likely persists (e.g. due to depleted glutathione, CR- rather than FcR-mediated uptake, IL-10 inhibiting uptake and microbicidal, etc.), along with lack of inflammatory restraint/ineffective regulatory mechanisms leading to persistent stimulation and chronic inflammation. Overactivation would place significant additional metabolic (carbon-energy, oxidative, etc.) stress on leukocytes, and this is reflected in the proteomic results below. This study is the first to reveal detailed neutrophil metabolic behaviour prior to and at the time of TB-IRIS. Myeloid-derived cytokines are likely directly responsible for various clinical manifestations of TB-IRIS (fever, malaise, weight loss), as well as elicitation of a hepatic acute-phase response (e.g. producing haptoglobin and pentraxins like CRP), while antigen deposition and lymphocyte expansion in secondary lymphoid organs is likely responsible for lymphadenopathy ($\pm$ hepatosplenomegaly). Activated myeloid cell products also produce hypercoagulability, both at baseline and at the time of TB-IRIS manifestation. Airway inflammation co-exists with myeloid infiltrates, and local tissue damage likely results from a combination of: proteolytic enzymes (released by dying myeloid cells), the effects of complement and TNF α , and the actions of cytotoxic cell types (including neutrophils) and their granule contents. Although cytotoxic cell mediators typically induce apoptotic cell death, complement and TNF α can cause both apoptotic or necrotic cell death, and proteolytic enzymes as well as intracellular infection/stress detection systems can cause necrotic cell death.

Apart from possible adjuvant (e.g. glutathione) depletion, it is possible that higher levels of myeloid metabolic stress in IRIS-predisposed patients are contributed to by achievement of higher circulating/intracellular concentrations of ATT or ARV or their metabolites (recall that tenofovir-induced mitochondrial toxicity is related to OAT1/3-mediated intracellular accumulation), though it is equally possible that metabolic stress may be in response to strong TNF α and/or IFN γ stimulation (or that metabolic stress itself – e.g. via PPAR α -mediated alteration of efavirenz disposition – leads to higher ARV levels, thereby exacerbating metabolic stress). Note that threshold-exceeding levels of TNF α can also cause undirected myeloid motility and impaired antigen clearance, as well as favouring necrosis over apoptosis. Incomplete restoration of immunometabolic homeostasis by ARV therapy (especially in more advanced HIV) may also contribute to TB-IRIS. For example, although ARV therapy suppresses viral replication and decreases VL, it only partially restores LN architecture [239], and persistent immune hyperactivation with low-level “type I IFN signalling” can interfere with normal immune reconstitution [240]. Indeed, various immune rheostat balance discrepancies also noted during active TB (esp. advanced) may be involved in TB-IRIS (e.g. M1/Th1 versus M2/Th2, the IFN γ /TNF α balance, and the type I/type II IFN balance); many of these are discussed below.

Since there is no apparent age-disparity in who develops IRIS, and younger patients will have been more likely to receive BCG vaccination, this is unlikely to be a risk-modulating factor. Similarly, since

there is no apparent gender disparity in who develops IRIS, and females' pDC produce higher levels of type I IFN, pDC-generated type I IFN is unlikely to be a risk-modulating factor.

7.5. Selected Remaining Questions

Thus, although a great many plausible inferences can be drawn from existing literature, additional questions remain.

Which additional factors beyond degree of TB/HIV-coinfection most strongly influence TB-IRIS risk? Is risk mainly genetic-, lifestyle-, infection/strain-, or treatment- (e.g. relative timing of Mtb and HIV acquisition, presence of comorbidities/co-treatments, the specific ARV regimen used, etc.) related, or is it a combination of these factors? Is there a relative defect in steroid production/increased steroid resistance at baseline in TB-IRIS developers? What are the specific cellular/molecular mechanisms predisposing to overzealous subsequent inflammatory responses? What is the relative contribution of host versus viable/non-viable Mtb (or even HIV) products to TB-IRIS pathogenesis? What are the exact identities (e.g. mtDNA, HMGB1, host isoprenoids, specific Mtb antigens that act as TLR-2/TLR-4/CD14 ligands, etc.) of the most proximal triggers of TB-IRIS pathogenesis? Since HIV is known to preferentially deplete memory and Mtb-specific T-cells, how do reactivation responses initiate TB-IRIS (e.g. do only predisposed patients retain the correct specificities of memory lymphocytes)? What are the dynamics of certain insufficiently-studied cytokines (e.g. type I IFN, Th2-type cytokines, IL-10, IL-15, IL-21, IL-23, IL-27, IL-32, TSLP) and chemokines during the course of ARV therapy/TB-IRIS pathogenesis? What are the dynamics of their receptors on different leukocyte types in different biological compartments? Do myeloid cells in fact undergo co-ordinated necrosis *in situ* during ARV therapy? What are the identities of TCR/BCR ligands? Are they a combination of protein, lipid, and carbohydrate antigens? What is the contribution of B-cells and antibodies (e.g. IgE) to TB-IRIS pathogenesis? What is the complete intercellular causal model of TB-IRIS pathogenesis (especially $\gamma\delta$ T-cell, NK cell, NKT cell, and neutrophil interactions)? What are the exact mechanisms by which effective anti-TB-IRIS prophylactic/ameliorative therapy functions? Are we able to identify causally-responsible signalling pathways and more specific novel treatment targets (which can ideally be targeted by lifestyle changes or existing compounds/drugs already approved for other indications)? Are easily-accessible, high-performance risk stratification, prognostic, diagnostic, and therapeutic monitoring biomarkers available? Prognostic markers and "a validated diagnostic test for TB-IRIS ... would be extremely valuable" [238].

By interrogating the neutrophil proteome, this thesis has attempted to answer some of these questions. Below we discuss neutrophil proteomic findings, whether they are consistent with literature-derived hypotheses, and how they may extend literature-derived hypotheses.

7.6. Neutrophil Proteomic Findings: Predisposing & Pathogeneses-associated Immunometabolic Changes

Proteomic changes (detailed in Chapters 4 and 5) suggest class-specific metabolic and immune functional alterations summarised here (Tables 8 and 9). As per Chapters 4 and 5, tables present differences between the class (prior to ARV initiation) and healthy volunteers, as well as how this is altered in each class by two weeks of ARV therapy (see key below each table).

Table 8 Predicted Neutrophil Metabolic Changes from Baseline through ARV Exposure

	CTRL	IRIS
insulin sensitivity	/ $\rightarrow$ over-anabolism corrected	$\uparrow$ $\rightarrow$ over-anabolism corrected
PKA signalling	likely-dominant $\rightarrow$ $\downarrow$	
AKT-mTOR signalling	$\downarrow$ (?inactive AMPK de-represses mTORC1) $\rightarrow$ $\downarrow\downarrow$ (?AMPK represses mTORC1)	$\downarrow$ (?inactive AMPK de-represses mTORC1) $\rightarrow$ $\downarrow\downarrow$ (?farnesylated RHEB activates AMPK & mTORC1)
glycogen flux	$\downarrow$ $\rightarrow$ /	
glycolysis	$\downarrow$ glucose entry, $\uparrow$ post-G3P/DHAP flux $\rightarrow$ $\uparrow$ early glycolysis glucose flux $\downarrow$ glycolytic pyruvate $\rightarrow$ acetyl-CoA ($\uparrow$ Gly)	$\downarrow$ glucose entry, $\uparrow$ post-G3P/DHAP flux $\rightarrow$ $\uparrow$ early glycolysis glucose flux (incl. $\uparrow$ glucose entry), $\uparrow$ glycolytic pyruvate $\rightarrow$ acetyl-CoA ($\downarrow$ Gly)

HIF1a induction	↑ (incl. pyruvate → lactate + NAD) → /	↑ → /
PPP	↓ glucose entry (↓ NADPH), ↑ downstream activity → ↑ early PPP glucose flux (↑ NADPH)	↓ glucose entry, ↑ downstream activity → /
glyoxal stress	↑ → ?↑ (high glycolytic flux persists)	↑↑ → ?↑ (high glycolytic flux persists)
TCA cycle	↑ malate → pyruvate ↓ α-KG + glutamate → succinyl-CoA (α-KG buildup may feed glycolysis/PPP) → α-KG buildup corrected	↑ malate → pyruvate ↑ malate → oxaloacetate (?↑ malate-Asp shuttle transfer of NADH electrons to respiratory chain, consuming Glu, producing Asp + α-KG) ↓ α-KG + Glu → succinyl-CoA (α-KG buildup may feed glycolysis/PPP) ↓ adenylosuccinate → fumarate → α-KG buildup corrected
oxphos	↓ complex I & II ↑ complex V ↑ reliance on pyruvate → lactate → ↓↓ complex I ↑ electron transfer from FADH2 via complex II ↓ complex III (*possible mt depolarisation) ↓ complex V	↓ complex II & III (*possible mt depolarisation) ↑ complex V → ↑ electron transfer from NADH via complex I ↓ complex IV ↑ complex V (*possible attempt to prevent mt depolarisation)
mitochondrial stress & cell death	likely ↑ NRF1-mediated mt biogenesis & antioxidant response ↑ inhibition of apoptosis (but external > internal apoptosis capacity) de-repressed mTORC1 may ↓ mitophagy (↑ mt stress, mtROS, mtDNA damage) → ↓ ER Fe-S cluster binding possible ↑ mt import of respiratory chain Fe parts ↑ mt-site protein degradation ↑ inhibition of mtROS production & mtDNA damage ↑↑ inhibition of apoptosis (external & *CASP8-mediated & mt internal) *absent CASP8 means ↓ TNF/Fas-CASP8-CASP10-CASP3/7-mediated apoptosis	no evidence of NRF1-mediated signalling ↑ inhibition of apoptosis (but external > internal apoptosis capacity) likely ↑ proneness to necrosis de-repressed mTORC1 may ↓ mitophagy (↑ mt stress, mtROS, mtDNA damage) → ↑ folding QC esp. for mt proteins imported under stress ↑ mt ADP uptake ↑ ATP-alternate E-production ↑ capacity for mt membrane permeabilisation ↓ resistance to apoptosis (↑ external granzyme/TNF/Fas-CASP8-*CASP10-CASP3/7 & especially internal mt-BAX-apoptosis) possible ↑ NLRC4-CASP4/5-mediated pyroptosis &/or lipid peroxidation-mediated ferroptosis &/or DNA damage-induced necroptosis *↑ CASP10 expression
oxidative stress (ROS)	↑ (likely exacerbated by ↓ NADPH, GSH, GPx/Trx reductase) → ↑↑ ↑ & ↓ antioxidant responses (e.g. TXNIP-mediated Trx inhibition ↓ Cys-peroxiredoxins for lipid deperoxidation)	↑ (likely exacerbated by ↓ NADPH, GSH, GPx/Trx reductase, esp. in IRIS) → ↑↑ ↑ & ↓ antioxidant responses (e.g. TXNIP-mediated Trx inhibition ↓ Cys-peroxiredoxins for lipid deperoxidation ↓ GPx1 for peroxide detoxification SH3BGRL-mediated glutaredoxin regulation ↑ GPx4 to prevent lipid peroxidation & ferroptosis)
nucleotide metabolism	Δ purine (adenine) metabolism ↑ mt pyrimidine salvage & catabolism for ↑ (NAD-dep.) purine (guanine) synthesis ↑ PRPP + Trp → NAD (NAD also derives from anaerobic glycolysis) ↑ G3P + Glu (?from α-KG buildup) → Ser (not entering mt, not used to generate charged folates required for Met & de novo purine synthesis, suggesting instead use for glycerophospholipid &/or sphingosine synthesis) → ↑ DNA repair but necroptosis if this fails (e.g. due to absent DTXL3) ↓ pyrimidine cannibalisation for purine synthesis (incl. ↓ adenosine/AMP synthesis) ↑↑ PRPP + Trp → NAD ↑ ribose/ribose → xylulose ?→ G3P (rather than nt synthesis) ?→ Ser	Δ purine (adenine) metabolism ↑ mt pyrimidine salvage & catabolism for ↑ (NAD-dep.) purine (guanine) synthesis ↓ ribose-5-P → G3P ↓ α-KG + Lys → Glu ↑ G3P + Glu → Ser (+ ↑ Glu consumption by other processes) → ↑ DNA repair (esp. in IRIS) but necroptosis if this fails (e.g. due to absent DTXL3) ↑ DNA precursor pool size somewhat ↓ pyrimidine cannibalisation for purine synthesis continued ↓ pyrimidine precursors (may exacerbate ARV-induced mtDNA depletion, to which neutrophils may be esp. susceptible) perhaps prioritising purine/ATP synthesis ↑ PRPP + Trp → NAD ↑ nt → ribose/ribose → xylulose ?→ G3P (rather than nt synthesis) ?→ Ser (not used to generate charged folates, suggesting use for glycerophospholipid/sphingosine synthesis)
lipid metabolism	↑ FA synthesis ↑ FAO (incl. NADH & FADH2 to oxphos) → ↑ lipid synthesis (?preferential PPARα-glycerolipid) & ↓ FAO	↑ acetyl-CoA/FA/glycerolipid (from G3P) synthesis ↑ FAO-derived FADH2 to oxphos → ↓ lipid synthesis, ↑ TG/fat-soluble vitamin uptake, & ↑ FAO
integrated CoA, TCA cycle, amino acid, B-vitamin, & NH₄ metabolism	carbon/lipid findings relevant to acetyl-CoA: ↑ glycolytic pyruvate → lactate (not acetyl-CoA) ↓ mt acyl-CoA uptake in exchange for carnitine (favouring carbohydrate rather than FA oxidation) ↑ FA/glycerol levels ↑ FAO (with ↑ NADH and FADH2 to oxphos) ?↑ branched-chain AA catabolism → ↓ glycolytic pyruvate → acetyl-CoA ↑ (likely glycerol-)lipid synthesis ↓ FAO Thus, acetyl-CoA initially derives from FAO/ketogenic AA rather than glycolysis, then apparently from none of these (despite consumption by lipid synthesis). ↓ vitB5 recycling ↓ early vitB5 → CoA but ↑ late CoA synthesis ↓ PLP synthesis ↓ PLP-dep. Glu → Gln ↓ Lys + built-up α-KG → Glu	carbon/lipid findings relevant to acetyl-CoA: unknown glycolytic pyruvate → lactate (HIF1a suggests, but no LDHA dysregulation) ↓ mt FA-CoA uptake in exchange for carnitine ↑ FA/glycerol levels ↑ glycerolipid synthesis from G3P ↑ FAO → ↑ glycolytic pyruvate → acetyl-CoA ↑ TG uptake ↓ lipid synthesis ↑↑ FAO Thus, acetyl-CoA initially derives from FAO rather than glycolysis (↑ OA production may suggest gluconeogenesis) & is consumed by lipid synthesis, then from glycolysis & FAO (with ↓ consumption by lipid synthesis). ↓ PLP synthesis ↓ PLP-dep. Glu → Gln

	↑ G3P + Glu → Ser (↓ entry into mt) ↑ late urea cycle Arg → ornithine + urea ↑ tryptophan + PRPP → NAD → ↑ vitB5 regeneration (due to ↑ CoA catabolism) ?PLP normalisation (adaptation to INH) initial ↓ CoA would impair glycolysis-TCA linking (pyruvate already converts to lactate rather than acetyl-CoA) G3P/serine apparently diverted to glycerolipid synthesis, while α-KG may feed into glycolysis/PPP (requiring TCA replenishment, likely by succinyl-CoA from FAO) both Arg and Trp are consumed (for NH₄ detoxification and NAD synthesis respectively); arginine consumption may impair NO synthesis ARV therapy corrects many of these abnormalities (including B-vitamin), though acetyl-CoA for ↑ lipid synthesis must derive from AA given ↓ in pyruvate production & FAO	↓ Lys + built-up α-KG → Glu ↑ G3P + Glu → Ser ↑ late urea cycle Arg → ornithine + urea → ↑ CoA synthesis ?PLP normalisation ↑ intra-mt portion of malate-Asp shuttle (delivers NADH to oxphos, consumes glutamate, produces aspartate & α-KG) ↑ Asn → Asp + NH₄ (neither combines with Glu to form Gln & NH₄ not fully detoxified by urea cycle, given ↓ mid-cycle arginosuccinate → Arg + fumarate) apparently normal glycolysis-TCA linking via acetyl-CoA; ↑ OA production suggests facilitation by gluconeogenesis (though OA may also participate in the citrate shuttle, transporting mt acetyl-CoA into the cytosol), but FA rather than carbohydrates may be the major energy-producing substrate (though acetyl-CoA & G3P/Ser are simultaneously consumed for lipid synthesis), while α-KG may feed into glycolysis/PPP (with TCA replenished by succinyl-CoA from FAO) arginine consumption may impair NO synthesis ARV therapy corrects many of these abnormalities (including B-vitamin), but further ↑ glycolytic drive for the TCA, & ↑ FAO rates NH₄ production is enhanced while detoxification is impaired (including failure to regenerate arginine); enhanced Asp production may feed the dicarboxylic acid cycle (helps convert Ser-derived pyruvate into glucose for gluconeogenesis); cellular uptake of monosaccharides is enhanced
methionine-SAMe-cysteine-GSH continuum	earlier Mtb/HIV infection → lesser ↓ GSH (literature) ↑ Met → SAMe (ATP/NAD-dep.) likely (due to ROS) ↓ homocysteine → Met likely (due to ROS) ↑ trans-sulfuration to Cys & thus GSH/GPx/Trx reductase (requiring *PLP & *vitB5) or H₂S (requiring GSH) *likely depleted possible homocysteine buildup → ?corrected GSH production (due to ↑ PLP & vitB5) ↑ GSR-mediated GSH reduction (i.e. ↓ homocysteine buildup)	later Mtb/HIV infection → ↓↓ GSH (literature) likely (due to ROS) ↓ homocysteine → Met likely (due to ROS) ↑ trans-sulfuration to Cys & thus GSH/GPx/Trx reductase (requiring *PLP & vitB5) or H₂S (requiring *GSH) *likely depleted likely homocysteine buildup → PLP recovery but ↓ trans-sulfuration to Cys & thus GSH/GPx/Trx reductase or H₂S (i.e. ↑↑ homocysteine buildup) GSH may become especially limiting: all 3 precursors (Gly, Glu, likely Cys) are ↓
H₂S metabolism & cyanide detoxification	attempted ↑ H₂S synthesis (GSH may be limiting): ↓ step 1 (Cys → pyruvate) ↑ step 2 (SAMe-stimulated homocysteine + Cys → Ser) ↑ step 3 (Asp-stimulated Cys + α-KG → 3MCP) ↑ step 4 (GSH/thioredoxin/dihydrolipoic acid-dep. 3MCP → persulfides → H₂S, or sulfate → thiosulfate) ↑ H₂S catabolism (quinone-dep.) to thiosulfate & thus (GSH-dep.) to GSH persulfide (thiosulfate can also be converted back to H₂S/sulphite thiocyanate) ↑ reconversion thiosulfate → sulphite (also detoxifies cyanide to thiosulfate; cyanide detoxification can also consume vitB12) → ↑↑ H₂S synthesis (if Cys & GSH/Trx available) & ?↑ cyanide detoxification	attempt at ↑ H₂S synthesis (GSH likely limiting): ↓ step 1 (Cys → pyruvate) / step 2 ↑ step 3 (Asp-stimulated Cys + α-KG → 3MCP) ↑ step 4 (GSH/thioredoxin/dihydrolipoic acid-dep. 3MCP → persulfides → H₂S, or sulfate → thiosulfate) ↑ H₂S catabolism (quinone-dep.) to thiosulfate & thus (GSH-dep.) to GSH persulfide apparent thiosulfate buildup (absent enzyme which transfers thiol group to GSH, GSH limiting) → ↑↑ H₂S catabolism & ?↑ cyanide detoxification
Fe & xenobiotic metabolism	↑ intracellular Fe sequestration ↑ Fe-S cluster & Fe-S protein synthesis (possibly due to Fe-S binding by ISG RSAD2) ↑ xenobiotic metabolism (incl. AhR signalling) → ↓ xenobiotic conjugation to GSH for elimination (& ↓ in additional xenobiotic responses)	↑ Fe-S cluster & Fe-S protein synthesis (possibly due to Fe-S binding by ISG RSAD2) ↓ heme → biliverdin ↑ xenobiotic metabolism (incl. AhR signalling) → ↑ intracellular Fe sequestration ↓ xenobiotic conjugation to GSH for elimination & ↓ acetaldehyde breakdown/vitA activation by ALDH (but otherwise ongoing xenobiotic responses e.g. to prevent lipoprotein oxidation & for ↑ heme breakdown/AhR activation)
isoprenoid/sterol metabolism	FA/glycerol/mevalonate/IPP buildup ↓ protein prenylation & cholesterol/steroid hormone/Q10 synthesis (with attempted ↑ steroidogenesis/↓ steroid hormone degradation) → likely ↓ SREBP-induced sterol synthesis de-repressed FPPS relieving buildup & facilitating prenylation & thus RHEB AMPK/mTORC1 activation	FA/glycerol/mevalonate/IPP buildup ↓ protein prenylation & cholesterol/steroid hormone/Q10 synthesis ↑ HDL export ↓ steroidogenesis → ↓ sterol synthesis de-repressed FPPS relieving buildup & facilitating prenylation & thus RHEB AMPK/mTORC1 activation (RHEB becomes elevated only in the IRIS group)
amino acid & protein homeostasis	altered protein expression & ER architecture/Ca²⁺/glycosylation, including: ↓ folding QC ↑ chaperone activity ↑ UPS flux	altered protein expression & ER architecture/Ca²⁺/glycosylation, including: ↓ folding QC ↑ chaperone activity (esp. in the IRIS group) ↑ UPS flux.

	de-repressed mTORC1 may ↓ autophagy possible compensatory ↑ auto/mitophagy attempts → ↓ ER nascent protein processing ↓ folding QC (TMX4 up-regulation is likely a response to redox rather than folding conditions) ↓ glycosylation (together suggesting ↓ co-translational translocation or ↑ ER dysfunction; additional findings suggest de-repressed protein translation) ↓ protein insertion into ER membrane (↔ reversal of ER lumen expansion as lipid biosynthesis and protein-folding requirements decline) ↑↑ chaperones ↑ ubiquitination (& neddylation) of selected targets for UPS-mediated degradation ↓ SUMOylation of selected targets ↓ UPS-mediated degradation of misfolded/fusion proteins (incl. ↓ immunoproteasome activity) likely ↑ secretory protein export from ER (& targeting of abnormally-folded proteins to ERAD), retaining capacity for UPR (incl. ERAD) although apparently no misfolded protein stress likely-continued AMPK inactivity despite activation attempts, though mTORC1 may be inhibited (e.g. via TXNIP) esp. in the CTRL group (with decreased likelihood of activation attempts), which may de-repress auto/mitophagy	↑ repair/degradation of damaged proteins ↓ translocation of misfolded/fusion ER proteins for UPS-mediated degradation de-repressed mTORC1 may ↓ autophagy indications of ↓ auto/mitophagy → ↓ parts of ER nascent protein processing ↓ parts of glycosylation (but overall ↑ processing, folding QC, and glycosylation of ER-entering nascent proteins) ↓ protein insertion into ER membrane ↑↑ chaperones (esp. for proteins entering mt under stress) ↑ ubiquitination (& neddylation) of selected targets for UPS-mediated degradation ↑ SUMOylation of selected targets ↑ UPS-mediated degradation of misfolded/fusion proteins ↑ glycoprotein (& glycolipid) transport (possibly incl. retrograde transport of aberrant cargo) between the ER and Golgi likely ↓ UPR capacity (with possible ↑ ERAD branch) likely-continued AMPK inactivity despite activation attempts, though mTORC1 may be inhibited (e.g. via TXNIP, though activation attempts by farnesylated RHEB are likely); may partially de-repress auto/mitophagy

Note that PKA signalling (which would favour FAO) is dominant at baseline (at which time FAO is indeed up-regulated), but decreases on ARV therapy, and that AKT-mTOR signalling (which would favour lipid synthesis) is apparently decreased throughout (this does not preclude other mTOR-activating pathways). Glycolysis (which would be enhanced by mTORC1 or HIF-1α) transitions from depressed early- and enhanced late-pathway to recovered early-pathway. Indeed, pro-anabolic mTORC1 would also enhance pyrimidine, lipid, and protein synthesis. HIF-1α activation typically occurs in response to inflammation and hypoxia, induces anaerobic glycolysis, and alters oxygen and iron homeostasis. DNA synthesis typically requires vitamins B9 and B12 (folate; charged folates are also required for Met synthesis). Nucleotides can be degraded into precursors PRPP/ribose/ribulose, which can also be used to generate NAD or G3P. Ser can be synthesised from Gly (likely elevated in the CTRL but decreased in the IRIS group), and Ser metabolites (ethanolamine, choline) can enter charged folate, glycerophospholipid/sphingosine, or glucose synthesis (gluconeogenesis, via entering the Asp-dependent dicarboxylic acid shuttle as pyruvate). The TCA cycle product α-KG can be siphoned off and used for Glu (multi-purpose precursor) synthesis. Succinyl-CoA also derives from TCA cycle α-KG, as well as from FAO. Vitamin B5 is required both for the antioxidant response and for CoA production. CoA is a cofactor (acyl carrier) in metabolic reactions, facilitating the linkage of glycolysis to the TCA cycle, as well as activating FA for metabolic reactions. Acetyl-CoA derives from mitochondrial pyruvate, from Phe/Tyr/Leu/Lys/Trp, and from FAO. It enters the cytosol via the citrate shuttle (acetyl-CoA + OA → citrate, which is transported to the cytosol to recombine with CoA, using ATP → acetyl-CoA + OA). Acetyl-CoA feeds into both the TCA cycle and into lipid synthesis (both antioxidant responses and FA synthesis require NADPH, the majority of which is normally supplied by the PPP. Acetyl-CoA is also exchanged for carnitine, facilitating entry of the former into mitochondria. CoA, carnitine, and creatine are related to sulphur amino acids. CoA and carnitine assist in muscle ATP production (NADH and FADH2 provision of electrons to the respiratory chain) and in long-chain FA import into the mitochondrion for FAO (only when carnitine levels are high; when they are low the favoured major energy substrate becomes carbohydrate). Creatine also contributes to muscle ATP production. During fasting, amino acids can be used for gluconeogenesis. During repletion, solely ketogenic amino acids (i.e. producing acetyl-CoA/acetate) can enter FA/TAG synthesis or are metabolised as fuel (but cannot contribute to glucose production). Of the ketogenic amino acids (Phe, Leu, Ile, Lys, Trp) only Phe, Ile, & Trp are also gluconeogenic. Amino acids producing KG, succinyl-CoA, fumarate, or OA can directly supplement the TCA cycle, allowing withdrawal of OA as the major gluconeogenic substrate. The ER participates in more than Ca²⁺ homeostasis and protein folding, modification, and trafficking, with additional key roles in lipid/cholesterol synthesis and CYP450-mediated xenobiotic detoxification. Endogenous cyanide is produced by microbicidal leukocytes and muscarinic cholinergic signalling, and cyanide detoxification can consume vitamin B12. Key: ↑ increased, ↓ decreased, → change in response to ARV therapy, / unknown status, orange class-specific changes suggested by data, red class-opposite changes suggested by data, mt mitochondrion/mitochondrial, ER endoplasmic reticulum, ROS reactive oxygen species, nt nucleotide, FA fatty acid, TG triglyceride, TAG triacylglycerol, FAO fatty acid oxidation, PPP pentose phosphate pathway, TCA tricarboxylic acid cycle, oxphos oxidative phosphorylation, vit vitamin, PLP active form of vitB6, GSH reduced glutathione, AA amino acid, Fe-S iron-sulphur, AhR aryl hydrocarbon receptor, UPS ubiquitin-proteasome system, UPR unfolded protein response, ERAD ER-associated protein degradation. All other abbreviations constitute accepted gene, protein, amino acid, or metabolic intermediate/reducing equivalent nomenclature.

Table 9 Predicted Neutrophil Immune Activity Change from Baseline through ARV Exposure

	CTRL	IRIS
inflammatory signalling capacity (provided SHP1/2 restraint lifted)	↑ → /	↑ → /
regulatory receptor expression	↑ → ↑	/
activation	/ → ↑	/
recruitment	/	↓ → ↑
chemotaxis	↓ →	↓ → ↑
adhesion	↓ (preserved SELL rolling, ↓ selectin-activated integrin) → ↓ (SELL rolling & integrin)	↓ (non-integrin & profound integrin) → ↑ (transient rolling)
extravasation	↓ → ↓	↓ → /
migration through ECM	/	/ → ↑
degranulation	↓ → /	↓ → ↓
phagocytosis	↓ (esp. complement-opsonised) → ↑ (efferocytosis)	↓ (esp. complement-opsonised) → ↑ (esp. complement- & IgG-opsonised)
respiratory burst	↓ → /	↓ → ↓
microbicidal substances	/	↓ → /
cytotoxicity	/	/ → ↓
Fe sequestration	↑ → /	/ → ↑
complement inhibition	↑ → ↑ (C1qBP inhibits classical pathway)	↓ → ↑ (CD55 inhibits cascade amplification)
Th1/cytotoxic/Th17 induction	↓ → ↑ (NK, Th1, & esp. CD8 ⁺ T-cells) but ↓ IL-1α, IL-6, TNFα, IL-10, TGFβ (ARV)	↓ → ↑ (esp. NK, but also Th1 & CD8 ⁺ T-cells) with ↑ TNFα, IFNγ (ARV)
Th2/Treg induction	↑ → / but ↑ IL-4/13 (ARV)	↑ → / but ↑ TGFβ (ARV)
eicosanoid cascade	↓ arachidonate provision, LT (incl. LTB4) synthesis, LTB4R → ↓ LT & ALOX15-mediated HETE/HPETE synthesis (preservation of PAF)	↓ arachidonate provision, LT (incl. LTB4) synthesis → ↓ LT (incl. LTC4), ALOX15-mediated HETE/HPETE, & COX-2-mediated prostanoid synthesis (↓ protective PGE2)

		(? PAF inactivation)
coagulation	/	/ → ↑ (PLAUR, plasmin)

Key: ↑ increased, ↓ decreased, → change in response to ARV therapy, / unknown status, orange class-specific changes suggested by data, red class-opposite changes suggested by data

7.6.1. Immunometabolism: The Inextricable Links between Metabolic & Immune Function

Metabolism provides energy and molecular building blocks for cellular function (while degrading/recycling/removing waste products). It necessarily also modulates cell signalling (e.g. certain metabolites are also signalling molecules) [241]. Unlike during the leukocyte resting state, activation increases energy and biomolecule requirements; metabolic reprogramming must occur to meet these needs (otherwise, abnormal/ineffective immune responses result) [241].

Myeloid activation induces significant reprogramming, including preferred use of glucose as the substrate for ATP generation (though lipids and amino acids may also be used). For example, LPS-stimulated TLR-4 triggers increased reliance on glycolysis rather than oxphos, even in aerobic environments [241]. In this context, glycolytic pyruvate is channelled towards lactate production (less efficient, but more rapid ATP generation) rather than driving the TCA cycle and oxphos (the electron transport chain also becomes remodelled to increase production of microbicidal mtROS) [241]. Rapid ATP provision may be required to support, for example, increased protein, fatty acid, and nucleic acid synthesis [241]. Simultaneously, flux through the PPP is elevated in order to generate NADPH and ribulose-5-phosphate for maintenance of redox homeostasis [241]. Thus, LPS-mediated myeloid activation favours switching from oxphos to increased glucose uptake, glycolysis, lactate production, PPP flux, and suppressed TCA function [242].

Resulting TCA cycle alterations cause a build-up of succinate - which stabilises transcription factor HIF-1α [241]. In a normoxic context, inhibitor HIF1AN prevents HIF-1α-mediated induction of hypoxic response genes (HIF-1α also modulates the activity of NFκB and other proteins) [243]. However, as mentioned in Table 8, both hypoxia and inflammation (including via oxLDL) can activate HIF-1α [244]. The resulting transcriptional programme involves over 40 hypoxic response genes, including those involved in energy metabolism (metabolic adaptation to hypoxia) and apoptosis [243]. HIF-1α up-regulates glycolysis (in macrophages increasing transcription of GLUT1, HK2, PFKFB3, and PDK1) for ATP generation (incl. anaerobic) - which extends neutrophil lifespan (including inhibiting spontaneous apoptosis) - and antimicrobial peptide expression and NETosis [241] [244]. However, it suppresses the respiratory burst (and thus ROS-dependent bactericidal activity), while preserving chemotaxis and phagocytosis [244]. HIF-1α activation induces pro-IL-1β transcription, while myeloid glycolytic rate alterations apparently do not affect IL-6 and TNFα secretion [241]. However, upregulation of several specific glycolytic enzymes (e.g. PKM2) in macrophages produces a pro-inflammatory phenotype by (via STAT3) enhancing IL-1β and IL-6 production [245]. Very few studies have investigated the metabolic changes occurring concomitantly with neutrophil activation, but it is known that Mtb infection-induced hypoxia - via HIF-1α - enhances neutrophil MMP-8 and -9 secretion [246]. While inflammatory microenvironments are often hypoxic, the neutrophils in this study are derived from circulation, rather than from the inflammatory site, but HIF-1α may still be induced e.g. via increased circulating lactate levels or inflammatory mediators.

Mechanistically, resting cells in a nutrient-replete environment exhibit homeostatic anabolic turnover signalling. By virtue of hyperglycemia being pro-inflammatory, insulin has anti-inflammatory (and anabolic) effects [247]. Similarly anabolic PKA signalling is anti-inflammatory. On the other hand, activated myeloid cells signal via pro-inflammatory PI3K-AKT-mTOR (the latter being "a key orchestrator of metabolism in response to nutrient availability and energy requirement") to channel glucose into enhanced glycolysis (antagonized by anti-inflammatory AMPK, which preferentially enhances oxphos) [241]. Apart from a falling ATP:AMP ratio, AMPK can also be activated by oxidative stress [248]. At least in mice, inhibition of AMPK boosts glycolysis while suppressing FAO (which also leads to decreased expression of retinoic acid synthesis-regulating enzyme ALDH2, thereby potentially interfering with FoxP3⁺ T_{reg} induction) [241]. Interestingly, mTOR can induce production of NO, which also poisons the respiratory chain (but of course type I IFN oppose iNOS activation). It is worth noting that Mtb products

induce cellular membrane and mitochondrial damage, and that efavirenz induces iNOS and NO expression and thus may induce mitochondrial toxicity (including affecting respiratory complexes, especially complex I) [249] via this mechanism as VL (the stimulus for type I IFN production) decreases; however, Mtb products may maintain type I IFN production in the context of TB-IRIS.

Leukocyte activation alters not only glucose but also cholesterol and lipid metabolism. Cholesterol-containing plasma membrane regions are crucial for signal transduction and phagocytosis, cholesterol incorporation into membranes is needed to enlarge intracellular organelles, and isoprenoid intermediates of cholesterol synthesis are required for prenylation of metabolism- and inflammation-involved signalling molecules (e.g. proteins) [241]. Cholesterol homeostasis (e.g. via rate-limiting HMG-CoA reductase expression and activity) is stringently regulated by transcription factors including liver X-receptor (LXR) and sterol regulatory element-binding proteins (SREBPs) [241]. Dimerization of LXR (activated by intracellular oxysterol accumulation or arachidonate) with retinoid X receptor (RXR) is required for its transcription factor function [250] [251]. While RXR can function as a homodimer, it frequently heterodimerises with other nuclear receptors which act as dietary component and xenobiotic sensors, such as LXR, peroxisome proliferator-activated receptor (PPAR) for fatty acids and their metabolites, vitamin D receptor (VDR) for active vitamin D, retinoic acid receptor (RAR) for all-trans retinoic acid (ATRA), Farnesoid X receptor (FXR) for bile acids, and constitutive androstane receptor (CAR) and pregnane X receptor (PXR) for xenobiotics [250] [251]. PXR is one of the master regulators of xenobiotic detoxification, including enzyme (e.g. CYP3A4) expression [252]; PXR and CAR (also involved in bilirubin and thyroid hormone metabolism) – both also induce CYP3A5 expression [204]. In some heterodimerisation cases (e.g. with thyroid hormone receptor or VDR, depending on cell type) RXR does not require activation by its own ligands, and in others (e.g. RAR) its own ligands only enhance activation; indeed, the RXR ligand is unclear and may be any or a combination of fatty acids (e.g. DHA), retinoids (possibly including dihydroretinoids), and other carotenoid metabolites [251].

Activated LXR favours (e.g. via inducing ABCA1 and ApoE) cholesterol efflux and reverse transport while opposing cholesterol accumulation; it also regulates lipogenesis (via inducing SREBP1c – though it has the opposite effect on other SREBPs – and CREB, which directly stimulate fatty acid synthase-mediated fatty acid synthesis) and enhances glucose tolerance (decreasing hepatic gluconeogenesis while increasing GLUT4-mediated peripheral glucose uptake) [241] [250] [251]. Indeed, fungal β -glucan-stimulated human monocytes up-regulate cholesterol synthetic enzymes (opposable by fluvastatin-mediated HMG-CoA reductase inhibition), and LPS-induced TCA cycle citrate production also “drives fatty acid biosynthesis ... for expansion of the ER and Golgi to support cytokine [and other secreted protein] release” [241]. Elevated citrate levels may also favour acetyl-CoA citrate shuttling, transporting mt-generated acetyl-CoA into the cytosol.

Simultaneously, LXR inhibits expression of inflammatory NF κ B and AP-1 target genes [241]. In β -glucan-pre-exposed mice, atorvastatin decreases LPS-induced plasma IL-1 β [241]. In the context of atherosclerosis, macrophage uptake of modified LDL produces foam cells: conversion of desmosterol to cholesterol is inhibited and desmosterol accumulates, activating LXR, suppressing SREBP1/2 processing, and resulting in a less inflammatory phenotype (although foamy macrophages can still be inflammatory within atherosclerotic plaques) [241]. Conversely, when cellular sterol (oxysterol, desmosterol, cholesterol) concentrations fall (no longer stimulating LXR), ER-sequestered SREBPs are freed; SREBP1 favours LDL uptake and cholesterol and fatty acid synthesis – membrane enlargement and prenylation intermediates are both important during inflammation (indeed, NF κ B induces SREBP1a expression, enhancing both lipogenesis and inflammasome activation) [241]. It is worth noting that – at least in endothelia – HIV opposes reverse cholesterol transport via altered endothelial caveolin-1 dynamics [253].

While enhanced glycolytic rates appear to be a general (non-specific) leukocyte response to activation (e.g. to help support pro-inflammatory cytokine production) by many types of TLR agonist/pathogen, but additional complex metabolic signatures are also induced in some cases (such as simultaneous enhancement of both glycolysis and oxphos, e.g. when myeloid cells are stimulated using *Escherichia coli*) [241]. Inflammatory activation either suppresses or enhances oxphos in a

context-dependent manner (e.g. acute versus chronic inflammation). The nature of activation-induced complex metabolic signatures is cell type-, microbe-, and microenvironment-specific, leading to unique signatures associated with different conditions (e.g. acute versus chronic inflammation, the presence of certain cytokines, the extracellular presence of certain metabolites, etc.) [241]. For example, TLR-2 versus TLR-4 stimulation drive completely different metabolic responses [to support] different [cellular] functions" [241].

Although the effect of cytokines (especially in combination) on metabolic function has not been as well-studied as the effect of TLR stimulation, some trends are emerging. Type I IFN typically promote glycolysis, while either diminishing oxphos (with decreased oxygen consumption being dependent on HIF-1 α induction) or boosting FAO-driven oxphos (where the FA for oxidation derive from *de novo* FASN-mediated synthesis from pyruvate) [254]. Both LPS and the Th1-type cytokine IFN γ polarize macrophages towards a pro-inflammatory M1 phenotype, unsurprisingly accompanied by: enhanced glycolysis (including HIF-1 α -dependent increased expression of the fatty acid synthase complex FASN), accumulation of succinate (driving mtROS generation) and citrate (while increasing ACLY-mediated conversion of citrate into acetyl-CoA and OA to favour both FA synthesis and ROS/NO production), and enhanced oxphos (via mTORC1 inhibition) despite inhibiting SIRT1 expression (and thus mt biogenesis) [241] [254]. The Th2-type cytokine(s) IL-4 (and IL-13) polarise(s) macrophages towards an M2 (alternately-activated) phenotype in a PPAR γ -dependent manner; such macrophages "rely on oxphos" and exhibit PPAR γ -driven fatty acid oxidation (FAO) of triacylglycerols, taken up into lysosomes by scavenger receptors [241]. Alternately-activated myeloid phenotypes (via PPAR γ) favour TAG uptake and FAO-driven oxphos. Lysosomes also process oxLDL, apoptotic cells, and autophagy-derived cargo; oxLDL "induces profound" lysosomal dysfunction, including via formation of NLRP3-activating cholesterol crystals [241]. As mentioned in Tables 4 and 8, mycobacterial infection (e.g. via ManLam-MR interactions) potently increases macrophage PPAR γ expression, which favours lipid droplet accumulation and decreases mycobactericidal activity [246]. Logic dictates that decreased clearance of ManLam (e.g. due to myeloid lysosomal dysfunction) would then especially favour PPAR γ expression on myeloid lysis. Direct metabolic effects of IL-6 remain unknown, but it up-regulates the macrophage IL-4 receptor [241]. Macrophages exposed to anti-inflammatory IL-10 undergo DDIT4-mediated mTOR suppression, with resultant mitophagy inhibiting inflammasome-dependent IL-1 β maturation [241].

Interestingly, TCA cycle-derived α -KG has many cellular functions relevant to stressed cell metabolism. For example, it can (via malate) be converted to pyruvate to enhance NADPH production (glutaminolysis) [254], can act as an energy source while inducing mTOR signalling (thereby preventing muscle catabolism) [255], as a Gln precursor can additionally be useful in preventing muscle wasting during states of increased protein catabolism (e.g. infection) [256], and as a Gln homologue can enhance myeloid function (e.g. phagocytosis, ROS production, cytotoxicity, reduced glutathione (GSH) synthesis, and cytokine production) [256]. However, it also inhibits NF κ B and (possibly as a consequence) induces PXR-mediated xenobiotic detoxification [257]. Like other TCA cycle intermediates, α -KG exhibits antioxidant properties by directly reacting with hydrogen peroxide to form succinate [258] (including during ammonium- or aldehyde-induced oxidative stress), helps restore antioxidant levels and antioxidant enzyme activity, and "can prevent [free radical-mediated] damage to mtDNA [256]. It also antagonises cyanogens (and thus generation of cyanide, which would otherwise induce ROS and inhibit antioxidant enzymes, as well as impairing mitochondrial oxphos) [256]. Interestingly, α -KG also regulates prolyl hydroxylases (and thus HIF-1 α as well as collagen synthesis; decreased α -KG or Fe²⁺ levels allow cytosolic HIF-1 α to accumulate [256]) as well as (via chromatin epigenetics) regulating expression of various genes [256].

Just like carbohydrate and lipid metabolism, amino acid metabolism, too, is altered by myeloid activation, and Arg metabolism is a key pathway regulating leukocyte function (changes in Arg concentration also alter cellular metabolism) [259]. Arg is a precursor to urea, NO, ornithine, citrulline, Glu, Pro, creatine, etc. [260]. T-cell activation requires Arg, ornithine, and citrulline, and Arg supplementation enhances T-cell and NK cell activity and promotes pro-inflammatory cytokine production [259]. Normally, Arg is taken up across the plasma membrane, after which it can be acted on by four possible enzymes: NOS (to produce citrulline and NO), arginase (ARG1; to produce ornithine

and urea), Gly aminotransferase, and/or Arg decarboxylase [259]. Product NO (able to diffuse through membranes) can react with superoxide to form peroxynitrite (which can nitrosylate thiols) or become nitrate (which can nitrate Tyr) [259]. Thus, overall Arg metabolism depends on NOS (constitutive and inducible) and ARG1 [259]. During inflammation, both iNOS and COX-2 are induced, and the presence of NO induces COX-2- and mPGES1-mediated PGE₂ production (mPGES1 tyrosine nitration enhances its activity) [259]. PGE₂ has both pro- and anti-inflammatory roles (e.g. suppressing type I IFN antitumor activity and eliciting MDSC and T_{reg}) [259]. Urea cycle enzyme ARG1 is a marker of the M2 macrophage phenotype, whose expression can also be induced by hypoxia and lactate [241]. Indeed, at least tumour-associated macrophages (TAMs) exhibit higher expression of all urea cycle enzymes, as well as nitrogen metabolising transaminase Gpt and glutamine synthetase [241]. Too, "Arg-derived metabolites, Cys/cystine, and Trp metabolism are important mediators of the immunosuppressive activity of MDSCs", whose high-level NO generation may also contribute [241].

Although innate myeloid cells do not exhibit classical memory, metabolic reprogramming (including epigenetic changes) results in "trained immunity": more rapid and pronounced responses after prior insults [241]. For example, in LPS-induced tolerance, "NAD⁺-dependent activation of class III histone deacetylases (Sirtuins) [inhibits] gene transcription", and in activated monocytes, certain TCA cycle intermediates (e.g. succinate, fumarate) directly antagonise histone and DNA demethylases, leading to epigenetic alteration [241]. Regarding BCG vaccination, glycolytic enhancement is a key element of trained immunity [241].

Regarding metabolic changes during more *chronic* inflammation, cachexia is a wasting syndrome – involving weight loss, muscle atrophy, fatigue, weakness, and loss of appetite – associated with chronic disorders (e.g. TB, COPD, diabetes, cancer) and ageing [261]. The best-investigated candidate mediator is TNF α , which activates E3 ubiquitin ligases (and thus UPS-mediated muscle degradation) [261]. Other candidate mediators and/or mechanisms include IL-6 (anti-IL-6 antibodies suppress non-muscle wasting aspects of human cachexia), TGF β family members myostatin and activin A (which signal via ACTRIIB, whose blockade prevents murine cachexia), growth hormone resistance (with elevated growth hormone and lower IGF-1 levels), insulin resistance (with elevated glucose and insulin levels), and angiotensin II (which can lead to insulin resistance through suppression of GLUT4 translocation) of the renin-angiotensin-aldosterone (RAA) system [261].

As indicated in Chapters 4 and 5, proteomic results suggest a possible dysregulation of angiotensin II levels or sensitivity (as well as alterations in TNF α production and signalling, supported by literature) in the context of TB-IRIS. Cachexia often co-occurs with elevated circulating angiotensin II, exogenous administration of angiotensin II to rodents produces muscle wasting, and ACE inhibitors can ameliorate weight loss, muscle wasting, and insulin insensitivity [261]. Angiotensin II enhances circulating levels of IL-6, TNF α , and glucocorticoids (all of which impact muscle metabolism); alters IGF-1 signalling; enhances UPS-mediated muscle degradation; decreases hypothalamic levels of neuropeptide Y and orexin (decreasing appetite); alters brain levels of Agouti-related protein, POMC, TRH, and CRH; and – via activating phosphatase PP2Ca – inhibits AMPK activity (thereby depleting ATP) [Yoshida 2015]. Via this latter mechanism, angiotensin II also likely induces mitochondrial dysfunction: PP2Ca knockdown in angiotensin II-treated animals restores mitochondrial function and ameliorates muscle wasting, and the AMPK agonist AICAR restores ATP levels, ameliorates muscle wasting, and blocks angiotensin II-induced E3 ubiquitin ligase expression (possibly via Akt-mediated inhibition of FoxO1) [Yoshida 2015]. The RAA system-mediated mechanisms of cachexia are incompletely understood, however (e.g. angiotensins act via various receptor subtypes, AT1R blockade induces a compensatory increase in angiotensin II, and ACE inhibitors concomitantly increase angiotensin I), and clinical trials incorporating blockade of angiotensin II receptors have not yet been successful in treating cachexia [Yoshida 2015].

Angiotensin II also contributes to muscle atrophy via inducing ROS generation (via both NADPH oxidase and mitochondria; this effect is opposed by antioxidants) and Ca²⁺ elevation (thereby activating Ca²⁺-dependent proteases such as calpain), and activating E3 ubiquitin ligases and caspase 3 (thereby also stimulating 20S proteasome activity) [Yoshida 2015]. Furthermore, during various chronic diseases associated with cachexia, metabolic dysregulation is noted (in the form of down-regulation of the latter part of glycolysis, of ATP generation, and of glucose utilisation by muscle) and may contribute mechanistically [261].

7.6.2. Links: PPAR/RXR, LXR/RXR, FAs, cholesterol, lipid mediators and phagocytic inflammation

Key lipid metabolism regulators are the three-member PPAR family of ligand-de-repressible TFs [262]. Activated PPARs dimerise with RXR to bind PPAR response elements (PPREs) in the promoters of target genes [262]. Each PPAR member exhibits a unique tissue distribution, and enhances expression of distinct genesets: PPAR α (mainly in hepatic and brown adipose tissues) is a major positive FA oxidation regulator and is involved in peroxisome proliferation, PPAR δ is more ubiquitous and has similar functions, and PPAR γ (mainly in adipose tissue) is necessary for adipogenesis [262]. The effects of PPAR δ and PPAR γ on peroxisome proliferation are less well-studied, though a high-fat diet or cold exposure appear to - via PPAR γ activation - increase peroxisome proliferation, and transcriptional co-activator PGC-1 α enhances cold-induced peroxisomal biogenesis independently of PPAR α , suggesting the involvement of PPAR γ or PPAR δ [262].

The pro-resolving effects of spontaneously-apoptotic neutrophils are associated with PPAR and LXR, which also play an important role in efferocytosis. For example, apoptotic cell uptake activates PPAR γ (which attenuates macrophage ROS production), PPAR induce expression of efferocytic lipid receptor CD36 (efferocytosis following tissue injury prevents interference of excessive inflammation with tissue repair), and LXR is required for steady-state apoptotic neutrophil clearance (which activates LXR, thereby repressing the IL-23/IL-17/G-CSF axis and granulopoiesis). Deficiencies in PPAR γ/δ , LXR α/β , or RXR α (which dimerises with LXR) cause apoptotic cell clearance defects, and makes animals resistant to the pro-resolving effects of spontaneously-apoptotic cells, by preventing inhibition of macrophage IL-1 β , TNF α , and IL-12 production, and preventing induction of macrophage IL-10 and TGF β synthesis [263] [264]. Macrophage CD36 - via uptake of spontaneously apoptotic cells - inhibits TLR (e.g. TLR-2) signalling and LPS-mediated NF κ B activation and favour IL-10 production; CD36 is also implicated in atherosclerotic foamy macrophage development as well as (in animal models) establishment and progression of TB (perhaps because Mtb can utilise host lipids as a carbon source) [265]. Without CD36, however, alveolar apoptotic cell clearance becomes delayed, potentiating production of neutrophil-recruiting chemokines (e.g. KC) and inflammation (although lower levels of Th2 cytokines and fibrosis, as well as decreased macrophage ARG1 expression, are also observed) [266]. Interestingly, Th2-type cytokines IL-4 and IL-13 (via STAT6) elicit higher PPAR γ expression, and M2-polarised macrophages exhibit increased expression/activity of PPAR γ and PPAR δ [267]. Additionally, IL-4 modulates 15-LOX activity to increase production of 13-HODE and 15-HETE, which are potential PPAR γ ligands [267]. Furthermore, pioglitazone (a PPAR γ agonist) ameliorates murine monocytosis and neutrophilia, improving infection outcomes. Finally, CD36 is also the receptor by which type II pneumocytes take up fatty acids for constitutive surfactant synthesis (comprising mainly phosphocholine lipids and ~10% SPA through D) [265]. Surfactant/SPA-opsonised phagocytosis probably plays a role during early Mtb infection [268], and SPA - which resembles C1q - also regulates complement [50] and helps maintain only pulmonary macrophage and neutrophil CR3 and CR4 expression [269]. SPA also boosts MR expression, while inhibiting TLR-2 expression, and may - like CRP - support of more immunologically silent infection/debris clearance, thereby modulating TB risk. Indeed, human SPA and SPD polymorphisms are associated with increased Mtb susceptibility [64].

Hyperlipidemia interferes with efferocytosis, favouring secondary necrosis [270]. In addition, cholesterol accumulation (e.g. due to deficiency of transporters ABCA1/G1 which mediate efflux to HDL) in myeloid cells activates the NLRP3 inflammasome (and thus IL-1 β as well as IL-18 maturation), significantly boosting neutrophil accumulation and NETosis [271].

7.6.3. Immunological Counter-regulatory Rheostats: Metabolism, IFN, IL-1/PGE2, IL-12/IFN γ /NO, and IL-17

7.6.3.1. Type I and II IFNs

Both Mtb and HIV infection lead to type I and II IFN responses. These cytokines signal through different receptors to induce expression of differential and overlapping ISG, often with antiviral functions [272] (including antiviral metabolic reprogramming [242]).

Type I IFNs (IFN α subtypes, IFN β , and “several other poorly-defined gene products”) signal via a heterodimeric receptor (comprising IFNAR1 and 2 subunits) and transcription factors STAT1 and 2 (although IFNAR1-dominant IFN β signalling favours AKT rather than JAK–STAT activation) to regulate expression of >300 IFN-stimulated genes (ISGs), many of which have documented antiviral function [273]. Such ISG do indeed restrict viral replication and inter-cellular transmission, but also regulate innate and adaptive immunity, for example: enhancing NK antiviral activity, altering the response to TLR ligands such as LPS [146], enhancing macrophage and neutrophil microbicidal activity (including towards *M. avium*) and macrophage-mediated antibody-dependent cytotoxicity, favouring monocyte differentiation into DC, down-regulating macrophage IFN γ receptor expression (thereby decreasing macrophage MHC-II expression and apoptosis), favouring Th1 polarisation, opposing macrophage-mediated Th2 recruitment, enhancing CD8⁺ T-cell antiviral activity, opposing activated/memory T-cell apoptosis, inhibiting T_{reg}, and altering antibody production [272] [273] [274].

ISG induced by type I IFN also significantly impact metabolism (esp. lipid and cholesterol metabolism). Emerging evidence indicates that type I IFN especially impact energy and lipid and cholesterol metabolism, all “essential for viral replication” [242]. As mentioned above, type I IFNs have been observed to increase glucose uptake and glycolysis [242]. Increased mTOR activation and mtROS production (as occurs during an activation-induced switch away from myeloid oxphos) can both contribute to dysregulated type I IFN production, and in turn type I IFN can support a switch away from oxphos (although in some murine leukocytes it can also enhance oxphos [242]). At least in mice, type I IFN are also able to increase oxidation of fatty acids produced by FASN from glycolysis-derived pyruvate, involving enhancement of PPAR α activity [242]. Furthermore, type I IFN can inhibit sterol synthesis (a strongly antiviral action) and up-regulate cholesterol-25 hydroxylase (which converts cholesterol to 25-hydroxycholesterol, inhibiting viral entry and growth) [242].

Type II IFN (IFN γ) promotes antiviral and antibacterial innate immunity (including ISG overlap with type I IFNs), including: inhibiting the M-CSF receptor [254], enhancing antigen presentation and IL-12 expression (favouring Th1 but opposing Th17 polarisation) [272], helping T-cells condition phagocytes for enhanced intracellular killing (e.g. via enhancing phagocytosis, ROS production, iNOS-mediated NO production, the latter which also inhibits viral replication and increases bloodflow), and contributing to T-cell recruitment (via ISG). Metabolism is altered by IFN γ , too, in part through up-regulated expression of LPS receptor TLR-4 and its co-receptor CD14 [242]. Additionally, IFN γ partly inhibits mTORC1 (decreasing global mRNA translation – including for viral proliferation – while favouring translation of antiviral protein mRNAs, and inducing autophagy) and induces IDO (as do type I IFN, to a lesser extent), depleting intracellular Trp (potentially sufficiently to produce mTORC1 suppression – *vide infra*) to produce immunosuppressive kynurenine [242]. It also inhibits expression of NAD-dependent SIRT1, thereby inhibiting expression of several key metabolic genes [242].

Acutely, both type I and II IFNs have beneficial effects in combating infection, but chronic production can cause abnormal and/or suppressed immune function [272]. For example, chronic TLR-9 activation of murine pDC leads to type I IFN-dependent lymphoid tissue morphology alterations similar to those observed in HIV (including temporary increases in LN and spleen cellularity, followed by local depletion of activated T-cells) [181]. Chronic type I IFN stimulation also induces a tolerogenic DC phenotype and up-regulates DC and macrophage PD-L1 and IL-10 expression [272]. Type I IFN signalling blockade in HIV-infected humanised mice during the chronic stage “diminished HIV-driven immune activation, decreased T-cell exhaustion marker expression, restored HIV-specific CD8⁺ T-cell function, and ... decreased viral replication”, and combining such treatment with ARV therapy further boosts viral suppression and shrinks the pool of HIV reservoirs [240] [275]. IFN γ , even acutely, opposes Th2 polarisation, and – chronically – upregulates PD-L1 expression (including on neutrophils) and differentiation of MDSC (in conjunction with GM-CSF, for example) [272]. As mentioned above, both type I and II IFN induce expression of IDO, another immunosuppressive mechanism. Indeed, in response to HIV, myeloid cells (especially pDC) produce large amounts of type I IFN and IDO, and these in fact synergise to induce T_{reg} [276] (which also secrete TGF β), explaining the association between type I IFN and fibrosis [277] (and yet, massive pDC production of IFN α during acute HIV is transient, disappearing

during transition to chronic infection transition [278], though type I IFN production does continue, despite HIV-mediated opposing mechanisms).

As mentioned in Chapter 4, TGF β and AMPK counter-regulate each other: instead of AMPK-induced oxphos, autophagy, and facilitation of (mTORC1-opposing) type I IFN production, TGF β would instead favour gluconeogenesis and mTORC1 activation (with concomitant biomolecule synthesis and opposition of autophagy).

7.6.3.2. Effects of Type I and II IFNs during Mtb Infection: Type I IFNs Inhibit IL-1/PGE₂ and IL-12/Th1/IFN γ /NO/autophagy Antimycobacterial Responses

Regarding IFN effects on the immune response to Mtb, type I IFN effects during bacterial (rather than viral) infection are less predictable, with somewhat capricious (bacterium- and timing-dependent) functions [273] [279]. Cytokines such as IL-1 β , IL-12, IFN γ , and TNF α , are correlates of protection against active TB, largely due to the IL-12/IFN γ axis: early APC-derived IL-12 favours Th1 polarisation and IFN γ production, contributing to full phagocyte intracellular microbicidal activation, including TNF α production and its autocrine effects [273]. Both "self-healing" *M. leprae* and latent Mtb infections are associated with protective Th1-type IFN γ -driven immunity, while chronic and disseminated leprosy exhibit insufficient IFN γ with a dominant type I IFN response, and many blood transcriptomic studies suggest a pathogenic role for type I IFN during Mtb infection: as mentioned above, active TB is associated with a dominant type I IFN-inducible signature (which "correlates with the extent of lung radiographic disease") [279]. Unsurprisingly, this signature originates from neutrophils and monocytes rather than from T-cells [58] [280], and ISG (e.g. STAT1/2, IFITs, GBPs, MXs, OASs, IRFs, etc.) overexpression apparently precedes progression to active disease [73] [281]. Although this early signature also occurs in up to 25% of LTBI patients, these may be at the highest risk of progression to active TB [58]. At least during *M. leprae* infection, significant type I IFN (and ISG, e.g. CCL2, IL-10, OASL) production are associated with simultaneous suppression of IFN γ signalling and autophagy (required for mycobacterial clearance), as well as altered metabolism (enhanced glucose uptake, PPP-mediated NADPH generation, and decreased oxphos) [282].

However, the effects of type I IFN on Mtb infection outcomes appear to be timing- and dose-dependent. For example, while early/low/tonic-level type I IFN signalling may prime protective antimycobacterial immunity, later/high type I IFN levels are detrimental [273] [103]. Regarding protective effects: human patients receiving BCG for bladder cancer, recombinant IFN α enhances IL-12 and IFN γ responses [274], multiple case reports indicate that active TB patients unresponsive to conventional treatment and/or with recurrent diseases improve after co-administration of ATT and IFN α , and younger patients with mycobacterial infection and complete/partial IFN γ receptor signalling deficiencies also derive therapeutic benefit from combined ATT and IFN α administration [273] (which likely suggests partial compensation by the shared ISG induced by both type I and II IFN). Regarding detrimental effects: hepatitis patients undergoing antiviral IFN α therapy experience TB reactivation, a rare IFNAR1 polymorphism is "associated with increased resistance to TB" (though it is unclear whether this represents a lowered susceptibility to infection or to progression), and an inherited ISG15 deficiency leading to enhanced type I IFN responses increases susceptibility to mycobacterial infection [273]. In mice, factors enhancing type I IFN responses (e.g. nasal administration of a TLR-3 ligand, coinfection with influenza A virus, deletion of MAP3K8 which negatively regulates TLR-mediated type I IFN induction, and non-functional Usp18) lead, variously, to increased TB pulmonary pathology, decreased host resistance, impaired Mtb clearance, increased bacterial loads, and decreased survival [273]. More virulent Mtb strains are known to induce more pronounced pulmonary IFN α expression, and resultant IFNAR-mediated suppression of Th1-type immune responses and enhancement of anti-inflammatory responses increases host mortality [279].

It appears to be the type I/type II IFN balance that determines whether the immune response is protective against or drives progression of Mtb [103]. Indeed, murine model-based studies suggest that it is only on a background of IFN γ signalling that type I IFN exert a pathological effect (suppressing IFN γ antimycobacterial activity) during Mtb infection; indeed, in the absence of IFN γ signalling, type I IFN enhances TNF α production, opposing ARG1 expression and favouring a more protective M1 (rather

than M2) macrophage phenotype [273]. It is possible that partially-treated TB in the context of TB/HIV-coinfection leads to a dominant antiviral type I (rather than a protective anti-Mtb type II) IFN response, or more persistent type I concomitantly with type II signalling. Negative type I IFN impact on mycobacterial infection containment occurs via IL-10-dependent and -independent mechanisms (Table 10).

Table 10 Mechanisms by Which Type I IFN Support and Oppose Mtb Immunity [273] [103] [279] [274]

Immune Factor	Tonic/Early/Transient	High/Late/Chronic
IL-1 α	?	inhibits production
IL-1 β	?	inhibits production, and thus production of IL-1-elicited PGE ₂
IL-12	facilitates, synergising to induce NK cell production	inhibits production
CD4 ⁺ T-cell IL-12R β -chain	increases expression, enhancing IL-12 signalling and IFN γ responses	?
TNF α	facilitates	inhibits production
myeloid IFN γ receptor (i.e. sensitivity)	?	inhibits expression (thus also inhibiting induction of target genes, e.g. iNOS, IL-12/23 p40, intracellular killing, etc.)
IL-10 (anti-inflammatory)	favours production, thereby regulating pro-inflammatory responses	enhances production, suppressing IL-12 production and via STAT3 suppressing production of IL-6, TNF α , IFN γ (and resultant MIP-1 β , and iNOS/NO)
IL-27 (anti-inflammatory)	?	enhances production (<i>M. leprae</i>), which is anti-inflammatory
myeloid cells	enhance DC maturation recruitment, differentiation, survival of protective cells	delays normal neutrophil apoptosis favours accumulation of infection-permissive cells, and necrotic death

Type I IFN thus enhance/synergise with or inhibit/oppose Th1-type immunity [273] [274]. Mechanisms by which type I IFN may oppose anti-Mtb immunity include elicitation of IL-10 (known to increase susceptibility to Mtb) and possibly IL-27 expression, via these and other mechanisms suppressing production of IL-1 (and IL-1-elicited PGE₂) as well as of Th1-type cytokines (TNF, IL-12, IFN γ) and myeloid iNOS expression, inhibiting autophagy, delaying/inhibiting normal neutrophil apoptosis, and promoting "accumulation of permissive myeloid cells at the site of infection" [273] [103] [283] [103].

7.6.3.3. IL-1/PGE₂ Inhibit Type I IFN Responses and Alter IL-17 Responses

Both IL-1 and IL-1-induced PGE₂ limit production and pro-mycobacterial effects of type I IFN production [103] [103]. Providing exogenous PGE₂ (or increasing its production via ALOX5 blockade, provided COX enzymes are functional) during Mtb infection reverses type I IFN-driven mortality [103].

Also relevant to PGE₂ (*vide infra*), various cytokines are produced by Th17: IL-17A, IL-17F, IL-21, IL-22 [284]. While IL-17A and F share similar functions (inducing inflammatory cytokine production), human IL-17F may be less effective than IL-17A [284] [285]. Th17 are implicated in inflammatory bowel disease (IBD) susceptibility (likely in part due to altered gut mucosal integrity and translocation [285]), and are regulated by IL-1 β , PGE₂, and IL-23 [284]. Th17 production of IL-17A and IL-17F are differentially regulated by PGE₂ (via its EP4 receptor): in conjunction with IL-1 β and IL-23 it enhances IL-17A production (i.e. promotes a switch to an IL-17-dominant response), while IL-1 β and IL-23 alone preferentially induce IL-17F [284]. As mentioned, HIV depletes Th17, but patients also exhibit relatively fewer IL-17A-producing Th17, and relatively more IL-17F-producing Th17 [284] [285]. This could suggest higher levels of IL-1 β unopposed by PGE₂, and assisted by higher levels of IL-23, in persons with advanced HIV infection. It is notable that IL-1 β , PGE₂, and IL-23 are all also implicated in susceptibility to IBD (perhaps partly via Th17 regulation) [284].

7.6.3.4. Type II IFN Responses (via NO) Limit IL-1- and Neutrophil-mediated Immunopathology

In mice and non-human primates, months to years elapse before the "relatively large" Mtb burden manifests as active TB, likely concomitant with an increased rate of bacillary replication [92]. However, although quantitative burden does not consistently correlate with clinical progression, loss of appropriate regulation of the immune response to Mtb likely does, contributing significantly to pathogenesis [92].

Pulmonary Mtb infection robustly elicits IL-1 production and neutrophil infiltration [92]. While IL-1 is a key mediator of innate immunity (including during TB), IL-1 and neutrophil infiltration on their own are ineffective at infection control and eventually become associated with host tissue damage, e.g. during chronic infection [92]. Once adaptive immunity is established, IFN γ induces infected macrophage microbicidal (nutrient restriction, microbicidal substance production, autophagy), usually including

iNOS-mediated NO production, for direct bactericidal (still incompletely effective, but iNOS deficiency leads to extreme murine Mtb susceptibility) and immune regulatory signalling [92] [163].

Many gene expression changes observed in response to IFN γ exposure during macrophage Mtb infection are iNOS and/or NOX-dependent, and NO inhibits NLRP3-dependent IL-1 production, limiting excessive inflammation in infected mice [163]. Indeed, iNOS exerts broad control over the Mtb-infected macrophage transcriptome, enhancing antimicrobial and regulating pro-inflammatory cytokine gene expression [163]. For example, HIF-1 α - required for IFN γ -mediated Mtb control - depends on NO production, and a positive feedback loop between iNOS and HIF-1 α (which "regulate a largely overlapping set of genes" due to their co-dependence) enhances macrophage activation [163]. Furthermore, HIF-1 α and iNOS have opposing roles (induction and inhibition, respectively) in regulating key inflammatory cytokines and chemokines, including iNOS/NO usually suppressing prolonged NF κ B RelA activation-mediated hyperinflammation [163].

During Mtb infection, both IL-1 production and immunopathology are inhibited by IFN γ -induced NO, which - via thiol nitrosylation - inhibits NLRP3 inflammasome assembly, persistent neutrophil infiltration, and "progressive tissue damage" [156]. In addition, both Mtb and HIV inhibit phagocytosis and the respiratory burst, and HIV decreases myeloid cell cathepsin activity (e.g. via inducing the cysteine protease cystatin B, which also opposes Th1-type immunity by decreasing levels of activated STAT1, and - during the later stages of cell infection suppressing cathepsin expression) [286] [287]. HIV-mediated suppression of phagocytosis (K⁺ efflux), the respiratory burst, and cathepsin activity could directly inhibit NLRP3 inflammasome activation (and the consequences of lysosomal dysfunction), and this embargo may then be lifted ARV therapy.

7.6.4. Do Proteomically-inferred Metabolic & Inflammatory Changes (Chapters 4 & 5) Agree, & What Drives Them?

As noted above, leukocyte activity requires context-appropriate metabolic reprogramming: immune activation generates highly context-specific metabolic signatures, and metabolic changes have immune consequences. Bidirectional metabolic and immune interactions encompassed by proteomic findings include the following. Since there is as yet no automated method for integrating metabolic and inflammatory proteomic findings, we attempt here to: do this manually (demonstrating convergence of results) and to use above contextual information to infer causality.

Proteomic findings suggest good (high) baseline IRIS group insulin sensitivity and both groups exhibit dominant baseline PKA signalling. As discussed, insulin and PKA pathways both have anabolic and anti-inflammatory effects, but IRIS group insulin sensitivity and both groups' PKA signalling declines in response to ARV therapy. Baseline findings suggest *inhibited* PI3K-AKT-mTORC1 (and inhibited AMPK, despite likely oxidative stress; perhaps due to inhibition by angiotensin II) signalling in both groups, with PI3K-AKT signalling becoming further inhibited during ARV therapy (though evidence is insufficient, the CTRL group may attempt to repress mTORC1/activate AMPK, while the IRIS group may experience mTORC1 activation). As discussed, myeloid activation (including under the influence of type I IFN) usually activates inflammatory response-facilitating and anti-autophagic PI3K-AKT-mTORC1 signalling (to potentiate rapid ATP provision via glycolysis, while supporting nucleic acid precursor provision, ER expansion, and gene expression), favouring it over anti-inflammatory and pro-autophagic AMPK signalling (which would provide ATP more slowly, via potentiating oxphos). Together, these findings suggest that exposure to ARV therapy initiates a switch away from baseline relative homeostatic anabolism, including metabolic changes increasing conduciveness to inflammation, both likely a product of cells responding to activatory environmental cues.

It is notable that mTORC1 can only achieve full activation in the presence of sufficient Leu, pro-inflammatory Arg, and Trp, and that IFN γ -induced cellular activity (e.g. IDO expression) depletes Trp in order to oppose translation of viral proteins [242] [254]. Under normal circumstances, inflammatory myeloid activation (via NF κ B) also induces expression of iNOS (which converts Arg to NO) and COX-2 (which facilitates prostaglandin synthesis). NO further induces COX-2- and mPGES2-mediated production of PGE₂, which inhibits type I IFN production, elicits IL-17A production by Th17, supports induction of T_{reg} and immunoregulatory MDSC phenotypes (which use Trp/Arg/Cys metabolites for

immunosuppression), and is pro-apoptotic [254]. For these reasons, certain viruses favour PGE₂ production [254]. Generation of NO is favoured by mTOR itself, which is inhibited by immunoregulatory IL-10. As mentioned above, both IL-1 and IL-1-elicited PGE₂ oppose synthesis and pro-mycobacterial effects of type I IFN, minimising immunopathology (although type I IFN reciprocally antagonise the IL-1/PGE₂ axis). Thus, inflammatory myeloid activation and type I IFN will only effectively activate mTORC1 in the absence of IL-10 and if IFN γ and NF κ B signalling have not significantly depleted Trp and Arg, respectively. Similarly, PGE₂ will only be effectively elicited to combat type I IFN-induced immunopathology if both NF κ B function and NO production are preserved.

Another enzyme able to deplete Arg (via conversion to ornithine and urea) is ARG1, inducible by hypoxia/lactate and Th2 cytokines. Similarly, Trp can also be depleted by the enzyme IDO (via conversion to immunosuppressive kynurenine metabolites), inducible by both type I and II IFNs [254]. T-cells are dependent on both Arg and Trp for normal functioning. Herein, at baseline, proteomic results suggest that the CTRL group consumes both Arg and Trp (for ammonium detoxification and NAD synthesis, respectively), while the IRIS group consumes Arg in an apparent attempt to detoxify ammonium (although ARV exposure enhances IRIS group ammonium production, its detoxification appears incomplete). Thus, in both groups, Arg depletion may impair NO synthesis (recall that type I IFN also oppose iNOS expression), and this – along with the likely altered NF κ B function and eicosanoid cascades suggested by proteomic results – may result in impaired PGE₂ synthesis and decreased opposition to immunopathology.

Although neutrophils are typically rich in glycogen for anaerobic (inflammatory site) energy provision, both groups exhibit decreased glycogen flux at baseline, perhaps indicating that they are not primed for activity at inflammatory sites. As discussed, both antiviral/type I IFN- and Th1/IFN γ -type inflammatory programmes enhance glycolysis and can either suppress or enhance oxphos (supporting FAO-driven oxphos in the case of type I IFN, or inhibiting mTORC1 in the case of IFN γ , though the latter also inhibits SIRT1-driven mt biogenesis), while Th2-type responses to host damage (e.g. by parasites) largely favours PPAR γ -mediated TAG uptake and FAO-driven oxphos. Pro-inflammatory Th1-type cytokines, especially, often favour a switch away from oxphos to anaerobic glycolysis, with metabolic reprogramming featuring: enhanced glucose uptake, enhanced glycolysis and PPP flux (the latter in order to generate NADPH and ribulose-5-phosphate to maintain redox homeostasis), TCA cycle suppression (allowing pyruvate to be diverted into HIF-1 α -LDHA-mediated lactate production), enhanced FA synthesis (for membrane expansion and repair), and enhanced survival (suppressed apoptosis). Such cytokines suppress the TCA cycle in ways that favour accumulation of succinate (stabilising HIF-1 α and also enhancing mtROS generation) and citrate (converted by ACLY into acetyl-CoA and OA for FA synthesis and ROS/NO production). Although HIF-1 α would favour anaerobic glycolysis (which also inhibits spontaneous neutrophil apoptosis, enhancing the potential for secondary necrosis), neutrophil recruitment, AMP expression, and microbial phagocytosis, it also suppresses the respiratory burst (microbicidal) and modulates NF κ B activity.

At baseline and during ARV therapy, both groups exhibit proteomic findings suggestive of enhanced glycolysis (and thus glyoxal stress) and PPP flux. However, a baseline decrease in G6PD in both classes suggests decreased PPP-mediated NADPH generation, and only the CTRL group exhibits an increase in G6PD levels during ARV therapy, suggesting relatively poorer maintenance of antioxidant capacity in the IRIS group. At baseline, apparently only in the CTRL group is glycolytic pyruvate diverted towards production of lactate (for anaerobic ATP production) rather than acetyl-CoA (for entry into the TCA cycle or lipid synthesis). Indeed, observations noted only in the IRIS group suggest ARV exposure further increases glycolytic driving of the TCA cycle (cellular monosaccharide uptake is also enhanced, and increased Asp production may enter the dicarboxylic acid cycle for Ser-based gluconeogenesis). It must be remembered, however, that acetyl-CoA is not only produced from glycolytic pyruvate: it can also derive from FAO and from certain amino acids; all resulting mitochondrial acetyl-CoA is transported to the cytosol via the ATP-dependent citrate shuttle (acetyl-CoA + OA $\rightarrow$ citrate, which is transported to the cytosol to recombine with CoA). Proteomic finding-suggested increased baseline OA production only in the IRIS group may contribute to the citrate shuttle or to gluconeogenesis (additional indications suggest the IRIS group may favour FA over carbohydrates as an energy substrate).

At baseline, both groups exhibit TCA cycle enzyme alterations suggesting α -KG buildup. This intermediate may feed elevated glycolysis/PPP flux as an energy source in the absence of baseline

glucose (an event which would require TCA cycle replenishment, likely by FAO-derived succinyl-CoA), or may be converted (via malate) to pyruvate to supplement NADPH production. Indeed, TCA cycle intermediates are antioxidants, reacting with hydrogen peroxide (incl. during ammonium/aldehyde-induced oxidative stress) to form succinate (higher α -KG or Fe^{2+} levels also favour nuclear translocation of HIF-1 α), and α -KG also helps replenish antioxidant (incl. GSH) levels to prevent mtDNA oxidation as well as opposing cyanide-induced oxidative stress and mitochondrial toxicity. Recall that in terms of signalling, α -KG favours anabolic mTOR signalling and (as a Gln homologue) myeloid phagocytosis, microbicidal activity, cytotoxicity, and cytokine production (however, it also inhibits NF κ B and favours PXR-mediated induction of xenobiotic detoxification).

Typically, neutrophils favour anaerobic glycolysis over oxphos: recall from prior chapters that even under normal conditions, the extensive neutrophil mitochondrial network exhibits lower activity of complex I (still critical to preventing ROS/lactate production), normal activity of complex II, normal Q10 levels, lower activity of complex III (upon which neutrophils are nonetheless *reliant for maintaining mt potential* and preventing lactate production), lower cytc levels, only residual activity of complex IV, and normal activity of complex V. Specifically, neutrophils rely more on complex III (which can transfer electrons from FADH₂ and the G3P shuttle *directly* to O₂) and V (which can cooperate with mtANT to maintain mt potential during mt stress) than on the proton-motive force to maintain mt potential, and more on complex V (which, during stress, engages alternate energy-production strategies) than on ATP generation for energy production during mitochondrial dysfunction.

Proteomic findings indicating decreased complex I activity in only the CTRL group suggest increased ROS and lactate production (indeed, there is evidence of baseline lactate production in this group, which may compensate for decreased oxphos-mediated ATP production), while increased baseline complex V activity is likely also ATP-compensatory (although this is lost after ARV exposure, at which time lower complex III activity suggests increased susceptibility to mt depolarisation). Proteomic findings indicating decreased baseline complex II and especially III activity in the IRIS group suggest earlier predisposition to mt depolarisation (relative to the CTRL group). Increased complex V activity is likely ATP-compensatory and continues after ARV exposure. Baseline type I IFN-induced RSAD2-mediated inhibition of sterol (and thus Q10) synthesis may further impair oxphos in both classes. Decreased oxphos facilitates funneling of TCA intermediates into biosynthesis (e.g. of FA from succinate and citrate) [254], and ARV exposure appears to resolve the baseline α -KG buildup in both groups. Lactate is known to inhibit RLR-induced type I IFN production (via MAVS) [288], which may contribute to CTRL group inflammation regulatory mechanisms. Factors underlying poor representation of key respiratory chain proteins are unclear, but may potentially be related to damaged mtDNA and/or suppression of mt biogenesis/mitophagy pathways and/or unavailability of synthetic enzyme co-factors or redox factors required for incorporation into respiratory chain proteins. Another possibility is that cytokine context suppresses oxphos; however, additional metabolic changes suggest enhanced FAO-driven oxphos (i.e. via delivery of FAO-derived FADH₂ to complex II) rather than suppressed oxphos (*vide infra*). Since both Mtb products and efavirenz induce mitochondrial toxicity (the latter especially affecting complex I), and type I IFN promote oxidative stress [254]: if mTORC1 is indeed more active in the IRIS group (e.g. activated as part of inflammatory myeloid cell activation, including support by type I IFN and ROS in the absence of restraining AMPK), inhibition of mitophagy would exacerbate the consequences of mitochondrial dysfunction (e.g. decreased ATP production/increased reliance on anaerobic ATP production, enhanced mtROS production, altered mt transport dynamics of pyruvate/malate/Asp/Glu/adenine nucleotides – all indeed suggested by proteomic findings to be perturbed, and initiation of apoptosis).

Whether it is the the outer or inner mitochondrial membrane that becomes permeabilised contributes significantly to whether an apoptotic or necrotic programme executes [289]. The two major apoptotic pathways both converge on caspase activation: extrinsic (ligation of surface death receptors by TNF/Fas-L/TRAIL triggers CASP8-mediated nuclear disassembly) and intrinsic (induced by mitochondrial stress) [290] [291]. Various stressors (see Chapter 6, fig. 6) can activate the classical intrinsic pathway: pro-apoptotic Bcl-2 proteins Bak and Bax insert into the outer membrane, producing mitochondrial *outer membrane permeability* (MOMP) and efflux of pro-apoptotic proteins such as cytochrome C (cytC) from the inter-membrane space into the cytosol [290] [291]. Cytosolic cytC binds APAF1, triggering apoptosome assembly for (initiator) CASP9 activation, leading to (executioner) CASP3 activation [290] [291]. Alternate caspase activation (apoptotic) pathways also exist: ER stress (involving upstream caspase-2 or -12) and the T cell-mediated perforin/granzyme B pathway (converges on the

same terminal mechanisms as the extrinsic and intrinsic pathways) [290]. In contrast, certain other stressors (e.g. Ca^{2+} , phosphate, and/or ROS overload) open the mitochondrial permeability transition pore (mPTP), producing transient/low-conductance or prolonged/high-conductance *inner membrane permeabilisation* [289] [292]. The identity of the mPTP remains unknown, but various proteins are implicated in architecture (e.g. ATP synthase – the strongest contender – and TSPO) and regulation (e.g. CypD – confirmed component, ANT, VDAC, SPG7, PiC, HK, CK, and PBR) [293] [289] [294]; it is interesting to note that various ATP synthase subunits, ANT, and VDACs are dysregulated in the current dataset. Persistent/irreversible/high-conductance opening of the mPTP causes inner membrane depolarisation (including respiratory uncoupling, increased ROS production, impaired oxphos-mediated ATP production, possible net consumption of ATP in an attempt to maintain membrane potential, and metabolic collapse), and eventual osmotic swelling, outer membrane rupture, and necrotic cell death (including cell rupture) [293] [295] [289] [292] [296]. Although the mPTP-necrosis pathway is independent of apoptosis (CypD deletion protects against Ca^{2+} /ROS-mediated mPTP opening without preventing apoptosis), considerable cross-talk exists between mitochondrially-mediated apoptotic and necrotic pathways [289]. For example, phagocytic failure to clear apoptotic cells results in necrosis-like *in situ* degradation (secondary necrosis) [297].

Under stress conditions, various mechanisms (e.g. depolarisation itself and low ATP levels) prevent fusion of severely-damaged mitochondria to the existing mitochondrial network, but apoptosis may be delayed/averted through chaperone-mediated protein quality control, the UPS (e.g. due to Pink1/Parkin recruitment to damaged and depolarised mitochondria), and autophagy (including mitophagy of fragmented mitochondria) [292]. All three known mitophagy types require lysosomal fusion: in type I (induced by nutrient deprivation, is fission-associated, and requires PI3K) cup-shaped phagophores engulf mitochondria, in type II (induced by mitochondrial damage/depolarisation and involves Pink1/Parkin recruitment but is fission- and PI3K-independent) autophagic structures coalesce on the mitochondrial surface, and in type III (induced by accumulated mitochondrial protein damage) mitochondrial vesicles containing oxidised proteins bud off and are internalised by multivesicular bodies [298]. In the current dataset, the IRIS group indeed exhibits up-regulated chaperone (including mitochondrion-specific chaperone) levels, as well as altered UPS components (ubiquitin ligases, deconjugating enzymes, and proteasome components). While no dysregulation of classical (type II) mitophagy proxy proteins is noted in the current dataset, type III mitophagy is most likely (given proteomic indications of mitochondrial and oxidative stress).

At baseline, the CTRL group is likely more resistant to mt/oxidant stress, but both groups do exhibit proteomic indications of inhibited (especially internally-initiated) apoptosis, with the IRIS group possibly being more prone to necrosis *in situ* rather than to efferocytosis. During ARV exposure, the CTRL group again appears more resilient to mt stress and its consequences (including mtROS production and mtDNA damage), while the IRIS group exhibits evidence supporting a greater degree of mt stress and increased susceptibility to mt membrane permeabilisation. While (still in response to ARV exposure) the CTRL group exhibits proteomic indications of a further increase in resistance to (both routes of) apoptosis, the IRIS group exhibits the opposite (especially an apparent proneness to internally-initiated mt-BAX-induced apoptosis, and possibly even pyroptosis, lipid-peroxidation-mediated ferroptosis, or DNA damage-induced necroptosis). Indeed, both groups exhibit proteomic changes suggestive of altered Fe metabolism at baseline, including enhanced Fe-S cluster/protein synthesis, intracellular Fe sequestration (CTRL group only), and decreased conversion of heme to biliverdin (IRIS group only). It is notable that both groups also exhibit increased xenobiotic (including AhR-induced) metabolism (NFkB is one of AhR's inducers, and biliverdin is one of AhR's ligands). ARV exposure alters xenobiotic responses (perhaps also via PXR), which decrease in the CTRL group but continue in the IRIS group (e.g. prevention of lipid peroxidation, enhanced heme catabolism/AhR activation), which also now exhibits intracellular Fe sequestration but concomitant decreased ALDH activity (and thus acetaldehyde breakdown as well as activation of pro-resolving vitamin A, which also impacts lipid metabolism) – perhaps attributable to AMPK inhibition by angiotensin II. The Cys-containing ALDH enzyme is also “redox sensitive”, being inhibited by oxidative reactive sulfur species (not H_2S , but sulfane-containing species such as sulphides/persulfides) [299]. Cyanide metabolites also inhibit ALDH [243]. (Keep in mind these hypotheses concerning *reactive sulfur species and **cyanide metabolites when reading related sections below). Fe overload would induce mt membrane phospholipid peroxidation to

produce lipid aldehydes (e.g. malondialdehyde) and would decrease the ability of ALDH to detoxify it (resulting in mitochondrial dysfunction) [300]. Indeed, ALDH under-activity increases oxidative stress, which should, under normal circumstances, activate both NRF (triggering the antioxidant response) and AMPK [248].

At both timepoints in the current study, both classes exhibit proteomic evidence of high oxidative stress and altered antioxidant response pathways. Oxidative stress is probably exacerbated by pre-existing low levels of NADPH, GSH, and GPx/Trx, especially in the IRIS group (in which all three amino acid GSH precursors are likely depleted during ARV exposure, while the CTRL group exhibits evidence of increased GSH recycling, though both groups at baseline – based on proteomic findings - may lack PLP required for conversion of Cys to GSH/GPx/Trx reductase). During ARV exposure, both groups exhibit evidence of decreased xenobiotic conjugation to GSH for elimination. At baseline, both groups also exhibit evidence suggesting higher pro-inflammatory homocysteine levels (likely due to ROS/lack of charged folates inhibiting reconversion back to Met, and lack of PLP preventing trans-sulfuration via Cys to GSH or H₂S, synthesis of the latter also requiring GSH), but this apparently resolves on ARV exposure, at least in the CTRL group. Although both groups at baseline exhibit evidence suggesting attempted increased H₂S generation via later steps (including those producing Ser and persulfides/thiosulfate), a decrease in the initial step (Cys → pyruvate) and depleted GSH suggests that these attempts are unsuccessful and perhaps result in increased *persulfide (which may inhibit ALDH) or thiosulfate levels (however, only the CTRL group exhibits evidence of increased reconversion of thiosulfate to sulfite, simultaneously detoxifying cyanide in a B12-consuming manner). Relatively increased **cyanide levels (perhaps resulting from muscarinic cholinergic signalling) in the IRIS group may also contribute to ALDH inhibition, while depleted B12 would contribute to lack of charged folate regeneration. Both groups exhibit proteomic evidence of increased baseline attempts (likely unsuccessful) at Q10- and GSH-dependent H₂S catabolism. During ARV exposure, H₂S synthesis attempts (but in the likely absence of Cys, GSH/Trx) and likely cyanide detoxification become further enhanced.

Regarding potential oxidative nuclear/mtDNA damage, even at baseline in both groups, nucleotide metabolism is dysregulated, with evidence suggesting cannibalism of pyrimidines to replace oxidatively-damaged guanine (purine) and abnormally-trafficked adenine (purine) nucleotides (although in the CTRL group G3P- plus Glu/α-KG-derived Ser is apparently used for glycerophospholipid/ceramide synthesis instead of recharging folates for purine synthesis). During ARV exposure, both groups (but especially the IRIS group) exhibit evidence suggesting enhanced DNA repair. However, while there is some normalisation of pyrimidine cannibalism and adenine/adenosine metabolism in the CTRL group, these abnormal effects continue in the IRIS group (which likely also starts prioritising using Ser for glycerophospholipid/ceramide synthesis instead of folate recharging). Due to low mtDNA copy numbers and high metabolic rates, neutrophils may be especially susceptible to NRTI ARV-induced mtDNA depletion and its effects.

Switching focus to lipid metabolism (which also involves and impacts mitochondrial function), both groups exhibit proteomic evidence of simultaneously enhanced FA synthesis and oxidation (including FAO-driven oxphos) at baseline, with ARV exposure having opposite effects in each group: further enhancing FA synthesis in the CTRL group (but decreasing it in the IRIS group), and further enhancing FAO in the IRIS group (while decreasing it in the CTRL group). This would be consistent with type I IFN-/Th2-dominant baseline signalling normalising after ARV exposure in the CTRL group, but increasing further after ARV exposure in the IRIS group (simultaneous enhancement of both FA synthesis and oxidation is more characteristic of type I IFN than Th2-type cytokine effects, though in the CTRL group there may also be a contribution by IFN γ induced HIF-1 α -dependent FASN-mediated FA synthesis, supported by elevated ACLY-mediated conversion of higher citrate levels to acetyl-CoA as a substrate for this process, although hypothesised higher citrate levels do also support translocation of mt acetyl-CoA into the cytosol). In the CTRL group, vitamin B5 depletion (suggested by proteomic evidence) likely decreases CoA production and evidence indicates that pyruvate is converted to lactate rather than to acetyl-CoA, suggesting that acetyl-CoA in this group must indeed instead derive from citrate, FAO, or ketogenic amino acids. In the IRIS group, acetyl-CoA also likely derives from FAO rather than glycolysis. Higher levels of oxidative stress converting higher levels of α-KG to succinate may enhance provision of this substrate for increased baseline FA synthesis.

Proteomic evidence suggests further-enhanced FA synthesis during ARV exposure in the CTRL group despite likely further-decreased acetyl-CoA production from pyruvate and FAO, suggesting this FA

precursor must instead derive from citrate or from ketogenic (e.g. branched-chain) amino acids (and indeed there are indications of elevated branched-chain amino acid catabolism). What drives this further increase in FA synthesis is unclear. Proteomic evidence suggests diminished FA synthesis during ARV exposure in the IRIS group despite likely elevated provision of acetyl-CoA by both glycolysis and FAO. Since type I and II IFN signalling likely continue in this setting, decreased FA synthesis may be attributable to the more rapid fall in VL-induced type I IFN signalling and/or to resumption of TCA cycling (i.e. resolution of citrate, α -KG, and succinate buildup, which may also simultaneously enhance susceptibility to oxidative stress), although type II IFN should continue to favour FASN- and citrate buildup-driven FA synthesis (whereas Th2-type signalling would favour uptake of TAG as the FAO substrate, as well as PPAR γ -dependent adipogenesis, especially in the presence of PPAR γ ligands such as certain HETEs and HODEs).

Regarding cholesterol synthesis, we have already discussed (pages 33 and 34) that isoprenoid intermediates are required for normal immunometabolic signalling (e.g. RHEB prenylation), and that cholesterol is required for incorporation into enlarging/repairing membranes (cholesterol-containing membrane regions being requisite for normal signal transduction and phagocytosis), as well as being a precursors for steroid hormone (including corticosteroid and vitamin D) and Q10 synthesis. Inflammation typically induces expansion of cholesterol-containing plasma lipid membrane regions, but since virion production requires FA and cholesterol for membrane synthesis, type I IFN – while enhancing FA synthesis – potentially interfere with cholesterol synthesis (e.g. via inhibition of SREBP2, preventing expression of the rate-limiting early cholesterol synthetic pathway enzyme HMG-CoA reductase). Such inhibition of cholesterol synthesis would decrease production of both isoprenoid intermediates and downstream products such as oxy-, desmo-, and cholesterol levels (as well as synthesis of cholesterol-requiring molecules and cell structures). In the presence of lower sterol levels, LXR remains unactivated (i.e. failing to enhance: SREBP1c-mediated FA synthesis, cholesterol efflux, and pro-resolving efferocytosis of spontaneously apoptotic neutrophils; and failing to suppress: NF κ B- and AP-1-mediated pro-inflammatory gene induction). However, such low sterol levels would enhance SREBP1 and -2 activation, including SREBP1-mediated FA synthesis, LDL uptake, and cholesterol synthesis (although SREBP2 activity may remain inhibited by type I IFN). Incidentally, LXR-unopposed NF κ B, too would induce SREBP1a-mediated lipid synthesis. On the other hand, Th2-type cytokines would induce PPAR γ , whose ligation (e.g. by Th2-type cytokine-elicited 13-HODE and 15-HETE) would favour CD36-mediated enhancement of pro-resolving efferocytic clearance of spontaneously apoptotic neutrophils (preventing secondary necrosis, neutrophil recruitment, and inflammatory neutrophilia). However, as previously specified, both LXR and PPAR require vitamin A-dependent RXR in order to execute their activities. Without the efferocytosis-supporting activities of LXR and PPAR, there would be no repression of myeloid IL-1 β , TNF α , and IL-12 production, and no repression of the IL-23/IL-17/G-CSF axis that favours granulopoiesis and neutrophilia. Recall (pages 34, 37, and 38) that while Th1-type IFN γ would metabolically favour enhanced anaerobic glycolysis and lipid synthesis and inhibit mTORC1 to enhance oxphos (while inhibiting mt biogenesis), Th2-type IL-4 and IL-13 would metabolically favour PPAR γ -driven FAO of exogenously-derived TAGs (as well as PPAR δ expression and enhanced oxphos), and type I IFN would (in a PPAR α -dependent manner) also metabolically favour FAO (but of of endogenously-produced FAs), sterol synthesis inhibition, and increased activation of vitamin D.

Here, both groups exhibit baseline evidence not only of FA/glycerol buildup, but also of enhanced HMG-CoA reductase-mediated mevalonate production concurrent with the type I ISG RSAD2 interrupting downstream conversion of isoprenoid IPP to isoprenoid FPP, likely resulting in IPP buildup (and thus interfering with protein prenylation-mediated signalling, and with sterol synthesis, though the latter may be relatively preserved in the CTRL group), with increased efflux (HDL export) in the IRIS group only. Incidentally, such isolated inhibition of cholesterol synthesis can also *induce* type I IFN generation via the cGAS-STING-TBK1-IRF3 pathway; cholesterol levels/lipid rafts may affect ER-resident STING signalling, and IFN-induced cGAS is a viral/host DNA sensor [254]. These findings suggest low intracellular sterol levels in both groups, resulting in failure of LXR-induced cholesterol efflux, efferocytosis, and suppression of NF κ B and AP-1 signalling, but in enhanced SREBP1/2-mediated LDL uptake and cholesterol synthesis (although type I IFN may continue to suppress SREBP2 activity, suggesting that HMG-CoA reductase expression must be induced by some other stimulus in this context). Indeed, although LXR-activating NRF1 may be active in the CTRL group, it will only activate LXR provided the ER is cholesterol-overloaded, which is unlikely here. Furthermore, as suggested in Chapter 5, while ARV therapy appears to increase the CTRL group's capacity to respond to vitamin A (and corticosteroids), it achieves the opposite in the IRIS group, which also exhibits decreased levels of

ALDH (and thus potentially activation of vitamin A). This may suggest a decreased capacity for LXR/RXR- and PPAR/RXR-mediated pro-resolving efferocytosis, thus favouring neutrophilic inflammation. As mentioned above, in the event that any LDL taken up is of a modified (e.g. oxidised) form, it could induce lysosomal dysfunction (including NLRP3 activation). Here, ARV exposure relieves RSAD2-mediated interference with isoprenoid/sterol synthesis in both classes, perhaps facilitating farnesylated RHEB-mediated mTORC1 activation in the IRIS group (although it would also activate AMPK, another concurrent factor may prevent this: *vide infra*).

At baseline, both groups apparently experience increased sensitivity (via elevated receptor expression) to (decreased production of) pro-inflammatory angiotensin II (normally elicited during hypotension through the actions of renal renin and pulmonary ACE) and bradykinin (normally liberated from kininogen during inflammation), and only the CTRL group becomes less responsive to angiotensin II on ARV therapy exposure. This suggests a context in which there is predisposition to angiotensin II-mediated inflammation, and only the CTRL group recovers from this during ARV therapy, again perhaps facilitating heightened inflammation in the IRIS group through angiotensin II-mediated induction of NFκB and p38 MAPK-AP-1 signalling and thus increased production of IL-6 and TNFα. Both angiotensin II and TNFα may contribute to decreased appetite, cachectic-type weight-loss (including UPS-mediated muscle degradation), and mitochondrial dysfunction (via PP2Ca-mediated AMPK inhibition) with ATP depletion (in such contexts in mice, AMPK agonist AICAR restores ATP levels and blocks muscle wasting, the latter potentially via AKT-mediated FoxO1-UPS inhibition). Recall that angiotensin II also favours resistance to anti-inflammatory insulin (consistent with potentially-decreased sensitivity to insulin in the IRIS group), induces NOX and mitochondrial ROS production, and activation of Ca²⁺-dependent enzymes (e.g. calpain, which can contribute to necrosis) and CASP3. During BCG-induced granulomatous pulmonary inflammation, ACE is known to generate angiotensin II (supporting inflammation) and degrades bradykinin (preventing bronchoconstriction and angioedema) [301]. In a context in which angiotensin II contributes to overzealous inflammation, an ACE inhibitor may help ameliorate inflammation, but in this particularly context it could also potentially worsen bradykinin-mediated effects.

After ARV exposure, both classes – surprisingly - exhibit evidence of decreased GSTK1-mediated conjugation of GSH to e.g. xenobiotics for elimination. The CTRL class exhibits additional evidences of a decreased xenobiotic response, while the IRIS class exhibits decreased ALDH-mediated acetaldehyde detoxification, but otherwise evidence of ongoing xenobiotic responses (including to prevent lipoprotein oxidation, and for increased heme breakdown/AhR activation).

Both groups exhibit baseline evidence of altered gene/protein expression, including decreased ER protein folding/glycosylation capacity, but enhanced chaperone capacity and flux through the UPS (with possible mTORC1-mediated suppression of autophagy, more so in the IRIS group). This situation persists in both groups during ARV exposure, but while the CTRL class exhibits increased secretory protein export from the ER, *decreased* entry of misfolded/fusion proteins into the UPS (including decreased IFN-induced immunoproteasome activity), and likely a *retained capacity* for the UPR, the IRIS group instead exhibits increased glycoprotein/lipid transport between the ER and Golgi (possibly retrograde, due to misfolding, or perhaps due to an increased requirement by stressed mitochondria for nascent proteins, or perhaps due to increased targeting of inflammatory secretory proteins to the cell membrane), *increased* entry of misfolded/fusion proteins into the UPS, and a likely *decreased capacity* for the UPR. Various fusion proteins are identified in the current dataset (e.g. HNRNPUL2-BSCL2, TMED7-TICAM2, HSPE1-MOB4, NME1-NME2, and RPS10-NUDT3), perhaps suggestive of dysregulated rapid gene expression (and perhaps contributing to aberrant protein folding). A prior study has shown that at three weeks post-ARV initiation translation initiation factor eIF2 (which initiates protein synthesis by ribosomes and is a critical mediator of the cellular integrated stress response) is enriched in ex vivo PBMC from both CTRL and IRIS groups, but is likely more active in PBMC from the IRIS group [302]. It is known that IL-10 (indicated by literature to be up-regulated at the time of TB-IRIS) typically enhances protein folding and UPS-mediated degradation of misfolded proteins.

7.7. Do TB-IRIS Literature & Current Study Proteomic Changes Agree?

In many ways, detectable neutrophil proteomic changes support and extend literature-derived hypotheses (Table 11).

For example, lower Hb may be a risk factor (suggesting heme breakdown and Fe sequestration - including by myeloid cells - due to chronic infection), and proteomic findings indeed support ferritin-mediated iron storage (at least at baseline in the CTRL group and after ARV exposure in the IRIS group).

Also in agreement with proteomic findings, literature (Table 7) specifically notes altered eicosanoid and cholesterol homeostasis both preceding and during pathogenesis and manifestation. At baseline, literature notes increased levels of HETE, which is consistent with proteomic findings suggesting inhibited ALOX5-mediated leukotriene synthesis (with arachidonate perhaps being driven instead down the ALOX15 arm of the cascade). During manifestation, literature notes increased expression of COX-1 (while proteomic findings suggest decreased COX-2-mediated prostanoid synthesis) and higher levels of 9-HODE (while proteomic findings suggest further-decreased ALOX5/15-mediated leukotriene and HETE/HPETE synthesis; although ALOX15 can be responsible for 9-HODE production, this oxidised linolenic acid metabolite can also arise spontaneously through interaction with reactive oxygen species and may represent a biomarker of lipid peroxidation [303]). Regarding altered cholesterol metabolism, proteomic findings specifically suggest (perhaps ISG-induced) buildup of the isoprenoid intermediate IPP, with decreased downstream cholesterol (and thus steroid hormone and Q10) synthesis (likely including corticosteroids and vitamin D). This may be construed as consistent with a disorder in which provision of corticosteroids contributes to prevention. Further in agreement with neutrophil proteomic findings, literature notes monocyte gene expression changes representing enhanced Trp conversion to NAD at the time of manifestation. Proteomic findings suggest the same, and that this NAD may be specifically required to support replacement of oxidatively-damaged purine bases.

Neutrophil proteomic findings suggest for the first time many additional neutrophil metabolic changes - likely in response to activation in the presence of a specific cytokine milieu, and likely differing between CTRL and IRIS due to differential availability of adaptogenic resources - not already addressed in literature, but consistent with a general understanding of immunometabolism. Specifically, these findings suggest baseline depletion of metabolic adaptogens (e.g. GSH, PLP/vitamin B6, NAD/vitamin B3, NADPH, ATP) and potential inhibition of certain adaptive stress responses (e.g. auto/mitophagy, mt biogenesis, the antioxidant response), which likely make cells more vulnerable to xenobiotic/activation-induced mitochondrial/oxidative/genotoxic stress and perhaps more susceptible to inflammatory types of cell death. The contribution of neutrophil pyroptosis, for example, to settings such as sepsis is just beginning to be recognised [304] (and in some ways TB-IRIS neutrophil behaviour resembles that of neutrophils from septic patients). Frustrated mitophagy can also lead to enhanced mt depolarisation and mtROS production, along with cellular extrusion of NLRP3/TLR-9-activating and interferogenic (also via cGAS-STING) oxidised mtDNA (concomitantly with a decrease in NAPA and PSEN, and an increase in STXBP5). Literature reports increased baseline circulating mtDNA in the IRIS group. Proteomic findings demonstrate that at baseline the IRIS group exhibits higher levels of NAPA than healthy volunteers (whereas the CTRL group does not), and that during ARV exposure, neutrophil NAPA becomes significantly down-regulated (even after multiple-testing correction) in both groups. Although PSEN and STXBP5 are not detected to be dysregulated, at baseline the CTRL group exhibits higher levels of STXBP3 than healthy volunteers (whereas the IRIS group does not), and during ARV exposure this becomes absent from the CTRL group. That so many key intracellular signalling molecule (calcium, ROS, ATP/ADP/AMP, acetyl-CoA, and NAD/NADH [294])-related pathways are apparently perturbed suggests comprehensive metabolic and signalling alterations. It is worth noting that NAD/NADH are central to mitochondrial bioenergetics: the TCA cycle produces NADH, recycled by complex I of the respiratory chain to NAD, with mitochondrial-cytosolic connections facilitated by the malate/aspartate and glycerophosphate shuttles [294]. Cytosolic NAD is also required by PARPs and sirtuins, with key roles in DNA repair and mitochondrial biogenesis respectively [294]; again, if it is lacking, cell death will thus be more likely.

Furthermore, that so many pathways (leukotriene synthesis, antioxidant responses, possible purinergic P2X7R activation, xenobiotic detoxification, Fe metabolism, amino acid metabolism including Met-SAMe-Cys, the NO cycle, B-vitamin metabolism, H₂S metabolism and cyanide detoxification, and protein folding and degradation) reliant on the presence of GSH exhibit dysregulation seems unlikely to be coincidental (and literature agrees that intracellular GSH becomes depleted during advanced Mtb/HIV infection). Indeed, it was unclear from existing literature/knowledgebases that some of the

above pathways (e.g. Fe-S protein, Met-SAMe-Cys, H₂S, and ammonia metabolism) are even so prevalent within neutrophil metabolomics (and thus likely crucial to neutrophil functioning). That is, to the best of our knowledge, this is the first time the importance of these functional modules has been so clearly indicated in human neutrophils. Proteomic findings also suggest the possibility of increased intra-neutrophil NH₄ or cyanide levels during TB-IRIS manifestation, perhaps contributing to cell dysfunction and death. This has never before been speculated in TB-IRIS literature.

Cytotoxic oxidising activity of chloramines (RNHCl) may depend on ammonia: HOCl reacts with various endogenous nitrogen compounds (e.g. ammonia, taurine, amino acids in granule proteins/peptides) to produce N-Cl bond-containing molecules (RNHCl) of low toxicity, but that further react with ammonia to yield more cytotoxic *lipophilic* oxidant NH₂Cl [305]. Thus, RNHCl derivatives may reserve oxidizing equivalents until a relative increase in ammonia (as likely occurs in IRIS2 class neutrophils) favours conversion to toxic NH₂Cl [305].

Regarding the effect of oxidative stress on lipids, both LOX and ROS peroxidate PUFA, generating products (e.g. 9-HODE) which degrade to form toxic aldehydes (e.g. malondialdehyde – a peripherally-detectable marker of lipid peroxidation). Peroxidation of arachidonate, specifically, generates isoprostanes (non-enzymatically formed prostaglandin-like compounds that are mediators and markers of oxidative stress, also reflecting lipid peroxidation) [306]. Phagocyte HOCl can also oxidise HDL. Higher Fe levels increase lipid oxidative stress, elevating levels of malondialdehyde and 8-isoprostane, at the expense of significant proportions of cellular arachidonate, linoleic acid, vitamin A, and vitamin E (over 50% reductions for the former two in *in vitro* macrophages) [307] [308]. Peroxidated lipids increase LDL oxidation and induce IL-1 β secretion (both 9-HODE and aldehydes are able to perform the latter function) [308] [309]. In the absence of lipid-deperoxidating GPX4 (and/or GSH), ferroptosis results, due to accumulation of reactive lipid species (e.g. Fe-dependent accumulation of lipid hydroperoxides); at least *in vitro* this is preventable by provision of vitamin E [307]. Although coronary artery disease patients exhibit a positive correlation between serum ferritin and malondialdehyde levels, ferritin is also induced as part of the acute-phase response [310]. Although vitamin B5 deficiencies are rare, acute processes (e.g. inflammation) may acutely deplete it. For example, a higher dietary intake is associated with "lower or attenuated increases in CRP" [311] and in ischemia-reperfusion injury it "increases ATP and decreases lactate and malondialdehyde" [312]. Similarly vitamin B6 deficiency decreases cellular antioxidant capacity [313], for example aggravating homocysteine-induced oxidative stress, decreasing GSH synthesis, decreasing GPx activity, increasing malondialdehyde levels, and increasing glucocorticoid receptor-dependent [314] transcription. Indeed, in the context of inflammation, circulating PLP is low, correlating inversely with inflammatory markers and predicting chronic inflammation-related disease risk [315]. Dietary supplementation of vitamin B6 partially ameliorates inflammatory abnormalities in deficient humans and animals [315]. Hyperhomocysteinemia would activate: mTOR (impairing autophagy), ER stress and the UPR, and lysosomal dysfunction (e.g. decreased LAMP2, v-ATPase ATP6V0A1, and cathepsin D), thus "exacerbating cellular injury during oxygen and glucose deprivation" [316]. Since virulent Mtb suppress all-source ROS production (thereby suppressing lysosome permeabilisation) ATT may inadvertently up-regulate this potentially cell death-inducing process.

Regarding oxidative nucleic acid damage, local inflammation-induced HOCl produces oxidised pyrimidines (e.g. 8-halodeoxyguanosine, which appears in urine within 2-3 days, compared with later oxidative stress markers such as 8-hydroxydeoxyguanosine). Interestingly, Mtb inhibits phagosome maturation and phagolysosome fusion, preventing acidification (by decreasing vacuolar H⁺ ATPase levels) and engaging in molecular exchange with the plasma membrane to allow transferrin receptor-mediated import of Fe *into the endosome* [317]. Although mechanisms of Mtb inhibition of phagosome maturation are incompletely understood, the production of ammonia or envelope *glycolipids* (perhaps major phenolic glycolipid PGL-Tb1) may contribute [317].

Although literature hints at immunity baseline-*primed for over-activation* at the time of TB-IRIS manifestation (e.g. IL-27-mediated sensitisation to IL-12, lack of capacity to restrain classical complement pathway activation, etc.), proteomic findings provide a host of additional potential mechanisms priming for neutrophil over-activation (absence of certain inflammatory receptors – e.g. LTB₄ – in the CTRL but not the IRIS group, additional decreased complement inhibition capacity, absence of various inhibitory receptors only from the IRIS group, increased Lyn-SHP1/2-mediated

inhibition of IL1R/TLR-MyD88 and JAK-STAT signalling with lesser PTPRC-mediated opposition to this inhibition – likely due partly to PTPRC inhibition by more advanced HIV, potentially elevated IPP which is pro-inflammatory at high extracellular levels, and increased expression of intra- and extracellular PRR – e.g. TLR-2 and CD14 – relative to the CTRL group, thereby sensitising cells to host- and pathogen-derived inflammatory molecules). Mice lacking MyD88 are highly susceptible to various bacterial pathogens (including failure to control Mtb H37Rv after aerogenic infection) and exhibit significantly down-regulated expression of Th1-type cytokines (IL-12, TNF α , IFN γ) and NOS2, suggesting that resistance to Mtb requires MyD88-mediated signalling. MyD88 protein was detectable in pilot studies for the current project, but was not demonstrably dysregulated between CTRL and IRIS at the time of typically TB-IRIS manifestation.

Indeed, literature notes mobilisation of activated myeloid cells (especially neutrophils) into circulation both at baseline and at the time of manifestation (with activation appearing especially potent at the latter time). In this context, activation refers to increased expression ($\pm$ shedding) of PRR, as well as to activation-induced cytokine expression. However, these cells may be less competent at normal function and antigen clearance at both time-points. Proteomic findings extend this idea, suggesting defective baseline recruitment (especially in the IRIS group), chemotaxis, adhesion, extravasation, possibly degranulation (although findings are difficult to interpret), phagocytosis, microbicidal substance production and the respiratory burst (especially in the IRIS group), and apoptosis. Specifically, for example, absence of NF κ B p65/RelA subunit may account for many motility/infiltration-related defects, and it is also possible that this as well as metabolic depletion of Arg impairs generation of microbicidal NO. Hyperlipidemia (as suggested by baseline proteomic results indicating increased glycerolipid synthesis) can also impair efferocytosis and antigen clearance.

In further agreement with literature, proteomic findings suggest that - during manifestation - IRIS group neutrophils become more activated (including up-regulation of certain inhibitory receptors – e.g. LILRB2, SIGLEC-5 – in response), now exhibiting enhanced recruitment, chemotaxis, adhesion, and possibly migration through the ECM, as well as phagocytosis (likely complement- and IgG-mediated uptake, given the literature suggesting continued enhanced monocyte C5a production, proteomic findings suggesting enhanced CD55-mediated opposition of complement pathway amplification in the IRIS group, and elevated Fc γ RII expression in the IRIS group). However, proteomic findings also suggest further-decreased respiratory burst activity, further Arg depletion, and decreased neutrophil cytotoxicity (although degranulation also appears further impaired, findings are difficult to interpret). As mentioned, Mtb is known to activate both the classical and alternative complement pathways, with C1q expression correlating with disease severity (it has also been hypothesised to contribute to TB-IRIS pathogenesis), as well as increasing Fc γ R expression and signalling during active TB; uptake of Mtb and its products via complement rather than Fc receptors may contribute to impaired neutrophil microbicidal functions. The IRIS group exhibits findings suggestive of decreased lipid synthesis (a change opposite to that observed in the CTRL group), perhaps due to continued hyperlipidemia/in response to ER stress. Proteomic findings also suggest low baseline sterol levels (i.e. decreased LXR-induced cholesterol efflux, efferocytosis, and restraint of pro-inflammatory NF κ B and AP-1 signalling) and thus a decreased capacity for these anti-inflammatory effects in *both* groups. However, ARV exposure likely relieves this sterol synthesis deficiency, and indeed an existing proteomic study of ex vivo PBMC at week 3 post-ARV therapy initiation has previously demonstrated that the LXR/RXR activation pathway is relatively enriched in the IRIS (versus the CTRL) group [302]. Interestingly, terpenes are able to bind and influence RXR (e.g. activating RXR α -PPAR γ but inhibiting RXR α -LXR) [251] [318]. Recall from Chapter 3 (page 21-22) that Mtb Rv3377c gene product (a diterpene cyclase which converts GGPP to tuberculosinyl pyrophosphate) may be more abundant in IRIS than in CTRL neutrophils, and that Mtb Rv3378c gene product conjugates tuberculosinyl pyrophosphate to adenosine to produce 1-tuberculosinyladenosine (a.k.a. substance A), which switches macrophage metabolism to a more permissive state. Indeed, it has been suggested that two abundant Mtb-specific terpene nucleosides (including 1-tuberculosinyladenosine) may serve as diagnostic markers of Mtb infection [319]. Given proteomic indications of altered isoprenoid synthesis (baseline IPP accumulation, relieved by ARV therapy to allow subsequent synthesis of FPP and GGPP to proceed), it is not impossible that stressed residual viable Mtb in the presence of high levels of GGPP produce high levels of terpenes which may interfere with normal activity of PPAR, LXR, PXR, and other RXR partner nuclear receptors.

A previous whole blood transcriptomic study suggested elevated inflammasome and IL-1R/TLR-MyD88-NFκB signalling in the IRIS group, although the cellular source(s) of such transcripts (and whether they were released due to preferential lytic death of those cellular sources) was unclear. Proteomic findings indeed also suggest that neutrophil activation is likely via TLR, inflammasomes, and other intracellular NA/cholesterol precursor sensors (e.g. RIG-MAVS, cGAS-STING). However, at baseline the p65/RelA subunit of NFκB is apparently absent from both classes, while NFκB subunit dimerization-promoting MPTN is unique, suggesting production of non-RelA-containing NFκB dimers with altered functionality. Since RelA is not detected to be dysregulated in response to ARV therapy, there is a possibility that this altered NFκB functionality may persist at the time of TB-IRIS. Proteomic findings also exhibit the unique presence of PTPRC ligand LGALS3 (which may also bind Mtb major capsule constituents - mycolic acids - as an opsonin, but also interfering with LGALS3 binding Fas to favour apoptosis [320]) at the time of manifestation in the IRIS group, *suggesting lifting of baseline Lyn-SHP1/2 inhibition of MyD88-NFκB (i.e. TLR- and IL-1R-initiated) signalling in this group only. At the time of manifestation, proteomic findings also demonstrate that only the CTRL group experiences increased expression of inhibitory receptor CD300LF (which signals via SHP1/2) and decreased expression of PGLYRP1 (an inflammation-amplifying TREM-1 ligand).*

Furthermore, although inflammasomes sense intracellular PAMPs, DAMPs, and HAMPs (homeostasis-altering molecular processes) [321] - including LGALS3 [322] - and NLRP1, NLRP3, AIM2, and NLRC4 are likely present (based on literature and proteomic data), CASP1 is apparently absent from neutrophils at the time of IRIS manifestation (perhaps due to non-induction via malfunctioning NFκB, or perhaps due to high ROS levels regulating NLRP3 inflammasomes via inhibition of CASP1 [323]), suggesting it is not via the NLRP3-CASP1 pathway that gasD, IL-1β, and IL-18 are matured. However, there may instead be increased direct activation of CASP4/5, causing gasD-calpain-mediated pyroptosis (or ER stress/TRAIL-induced apoptosis). Indeed, TLR4-NFκB-IFNγ and TLR4-IRF3-type I signalling can elicit CASP4/5 expression [324] [325] [326]. Since literature I-FABP findings suggest that LPS lipid A is likely absent in this context (or at least present at levels equivalent to those in the CTRL group), CASP4/5 activation may instead be due to structurally-similar Mtb LAM [327], oxidized phospholipids [328], TRAIL [324], or host cell UPR induction [329], and may be mediated by a final common pathway of abundant neutrophil lysosomal cathepsin CTSG leakage into the cytosol [330]. It is also possible that viral and/or bacterial elicitation of the ISGs GBP4 and GBP5 (both up-regulated during TB-IRIS manifestation) contributes to inflammasome activation. Interferon-drives expression of GBP (which is both antiviral and antibacterial [331]); IFNγ "induces a quantitative switch in GBP levels, redirecting pyroptotic and apoptotic pathways" [to be under GBP control] [331]. GBPs simultaneously but independently: directly facilitate inflammasome assembly [331] (including activation of CASP1 and CASP11 [332], the murine homolog of human CASP4/5, with contributions to cell death [327]) and mediate lysis of cytosolic bacteria, thereby releasing pathogen products including DNA, with resultant induction of cGAS-STING-mediated type I IFN synthesis [331] [327]. During infection with certain intracellular pathogens, GBPs are a pre-requisite for AIM2 inflammasome activation [331]. GBP5 also favours cGAS-STING activation, and STING appears to be absent at baseline from the CTRL group only, suggesting that such a pathway may yet be active in the IRIS group.

Although non-canonical CASP4/5-mediated activation of NLRP1, NLRP3, and NLRC4 would usually also cause intracellular maturation of IL-1β and IL-18, absent CASP1 precludes this (though CASP4/5 may *directly* mature IL-1β and IL-18 [324] [325] [326]). Furthermore, FasL engagement of Fas can induce neutrophil release of CASP1-independent but ASC/CASP8-dependent mature IL-1β and IL-18 (and during ARV exposure, pro-apoptotic CASP8 is absent from the CTRL but not from the IRIS group). Finally, lytic neutrophil death also releases pro-IL-1β and -IL-18, which can be *extracellularly* matured by non-caspase proteases, including neutrophil proteinase-3, elastase, MMP9, granzymes A and B (the latter noted by the literature to be elevated), and mast cell-derived chymase. This can lead to significant sterile neutrophilic inflammation via IL-1R/IL-18R-MyD88 signalling [333]. Inhibiting IL-1R-MyD88 signalling may be able to alleviate sterile cell death-mediated inflammatory tissue damage without "compromising tissue repair or host defense" [333]. Here, the apparent lack of CASP1 may also prevent degradation of IL-33, thus favouring a more Th2-type response (e.g. featuring IgE); as previously

mentioned, IL-1 β can also favour a Th17 response, but IL-18 in the presence of IL-12 and IL-15 favours a Th1 response.

Cellular 'overactivation' during IRIS may occur due to supraphysiological myeloid activation stimuli (e.g. IFN γ , TNF α) as persistent uncleared antigens continue to stimulate immune responses. Likely-decreased activity of host-protective autophagy, NO, PGE $_2$, and endogenous corticosteroids (as suggested by proteomic findings) may also contribute to immunopathology in the IRIS group.

The literature-reported baseline cytokine profile (elevated IL-1 β , IL-18, IL-8, IFN γ , and IL-27, but CTRL-equivalent TNF α) suggests host stress- rather than pathogen-induced inflammation (e.g. perhaps mediated by MAPK-AP-1 rather than via MyD88-NF κ B signalling), but by the time of manifestation the cytokine profile (elevated IL-1 β , IL-18, IL-6, TNF α , IL-8, IFN γ , and IL-10; increasing IL-12 and MCP-1; but CTRL-equivalent IFN α) suggests pathogen-induced inflammation. Baseline proteomic findings exhibit elevated STAT3/6 (and ARG1) in the IRIS group only, suggesting elevated Th2-type and/or regulatory (IL-10/TGF β /IL-27) rather than Th1/cytotoxic/Th17-supporting signalling (including STAT3-mediated enhancement of the IFN response and ARG1 expression). Proteomic findings during pathogenesis in the IRIS group suggest increased type I IFN and inflammatory cytokine (IL-1 α , TNF α , IL-6) production, and this group also exhibits higher STAT1 and STAT3 levels, the former suggesting ongoing responses to IFN and the latter suggesting ongoing responses to type I IFN, IL-6, IL-18, and/or IL-10 (STAT6-mediated responses to Th2-type cytokines likely continues). Neutrophil proteomic findings also suggest that in response to ARV therapy the CTRL group likely *decreases* IL-1 α , IL-6, IL-10, and TGF β signalling, while the IRIS group likely *increases* TNF α , IFN γ , and TGF β signalling. Literature has not examined TGF β levels at either time-point. Unfortunately type I IFN levels at baseline are unknown (but are predicted to be higher due to more advanced Mtb/HIV-coinfection), but at the time of manifestation literature reports they are apparently equivalent (suggesting a more rapid fall if they were indeed higher at baseline, consistent with the more rapid fall in VL observed in these patients). Neutrophil proteomic findings suggest dominant (although of a slightly differential quality) baseline type I IFN signalling (including elevated STAT1/2 expression) in both groups, with ARV exposure unsurprisingly decreasing ISG in the CTRL group, but *increasing* certain ISG (likely induced by IFN γ) in the IRIS group. Literature reports that matrix-remodelling MMPs (e.g. MMP-8) and indicators of increased coagulation (e.g. TF at both time-points, D-dimer at baseline) are elevated at both baseline and the time of manifestation. In agreement, neutrophil proteomic findings exhibit elevated MMP-8 and MMP-9, as well as PLAUR, at the time of manifestation in the IRIS group. A prior proteomic study of ex vivo PBMCs also demonstrated that at three weeks post-ARV initiation the prothrombin activation pathway (and various other prothrombin-relevant pathways) is enriched in the IRIS group (relative to the CTRL group) [302].

Recalling (from page 18) that HIV co-infection results in a less-induced DosR regulon (concomitantly with lower myeloid microbicidality-activating IFN γ and higher microbicidality-opposing IL-10 levels), myeloid cell immune reactivation (including within tissues, e.g. in granulomas) after ARV exposure would likely produce a more hypoxic environment characterised by higher levels of oxidative stress, to which any residual live Mtb would respond by inducing the DosR regulon (including AC1, to which TB-IRIS patient T-cells have been shown to be responsive ex vivo).

Literature reports that $\delta 2$ T-cells are likely more prone to activation at both timepoints, and that likely IL-18-conditioned NK cells exhibit increased and decreased degranulation marker expression at baseline and during manifestation, respectively. Elevated circulating granzyme B noted by literature during manifestation may derive from neutrophils (although it is not proteomically detected here). Literature demonstrates that baseline CD4 counts are more depressed (due to more advanced HIV), as is the anti-PGL-Tb1 antibody titre, suggesting either decreased expression of this molecule by Mtb, or decreased responses to it by B-cells. Literature notes that during manifestation, Mtb-specific polyfunctional Th1 reactivate (but likely along with incompatible Th2, Th17, and ineffective T $_{reg}$ responses), while anti-PGL-Tb1 titres remain lower (recovery post-IRIS suggests that B-cell responsiveness to this molecule recovers). Neutrophil baseline proteomic findings common to both CTRL and IRIS groups suggest dominant type I IFN signalling (as mentioned), otherwise-decreased support for Th1/cytotoxic/Th17 induction, and increased support for Th2/T $_{reg}$ induction. In response to ARV exposure (in both CTRL and IRIS groups) this switches to increased support for Th1/cytotoxic/Th17 induction, as well as increased Th2-type cytokine signalling. Only the IRIS group demonstrates proteomic changes

suggestive of increased TGF β signalling (as mentioned). As such, neutrophil proteomic findings concerning T- and B-cell function do not contradict – and in some cases agree with - literature.

The above proteomics-informed model of neutrophil molecular immunological activity during TB-IRIS manifestation is summarised in fig. 7 (*vide infra*).

Table 11 Comparison of TB-IRIS Literature-derived Hypotheses & Current Study Proteomic Findings

Hypotheses Derived from Literature	Associated Neutrophil Proteomic Findings (group)
more advanced Mtb/HIV coinfection	na
more pronounced type I IFN &/or Th2 cytokines	dominant type I IFN signalling incl. $\uparrow$ STAT1 & STAT2 (both) $\downarrow$ support for Th1/cytotoxic/Th17 induction (both) $\uparrow$ support for Th2/Treg induction (both) incl. $\uparrow$ STAT3 & STAT6 (IRIS), & ARG1 (both)
higher levels of non/viable Mtb/antigens sequestered in myeloid phagocytes of granulomas	na
ineffective microbicidal/antigen clearance inefficacy (improperly activated myeloid phagocytes)	$\downarrow$ complement inhibition capacity (IRIS) $\downarrow$ phagocytic activity (both) $\downarrow$ microbicidal substance production (IRIS) Arg depletion may impair NO production (both) $\downarrow$ respiratory burst activity (IRIS) $\downarrow$ apoptosis (both)
ATT stress generates additional antigens	$\uparrow$ xenobiotic metabolism incl. AhR signalling (both) $\uparrow$ late glycolysis & PPP, incl. $\downarrow$ G6PD & thus NADPH production (both) $\uparrow$ glycolytic pyruvate $\rightarrow$ lactate, not acetyl-CoA (CTRL) $\uparrow$ provision of TCA cycle-driving acetyl-CoA (IRIS) $\uparrow$ TCA cycle a-KG buildup (both) FAO passes NADH to CI (CTRL) & FADH2 to CII (both) $\downarrow$ CI, $\uparrow$ CV function (both) $\downarrow$ CIII function, likely mt depolarisation (IRIS) likely $\downarrow$ Q10 synthesis likely $\uparrow$ mt stress & mtROS (esp. IRIS) $\uparrow$ Fe loading (CTRL) & $\downarrow$ heme breakdown (IRIS) $\uparrow$ *oxidative stress, likely mtDNA damage (both) $\uparrow$ pyrimidine catabolism for NAD-dep. damaged guanine synthesis (both), but only CTRL exhibits $\uparrow$ NAD provision $\uparrow$ Ser synthesis for pyruvate/glucose, lipid, Met/folate, or purine synthesis (IRIS) $\uparrow$ NRF1 antioxidant response & mt biogenesis (CTRL) $\uparrow$ mt Fe-S protein synthesis ?due to RSAD2 (both) ?mTORC1 inhibits auto/mitophagy (both), but only IRIS exhibits $\downarrow$ auto/mitophagy capacity $\uparrow$ proneness to necrosis (IRIS) *only CTRL exhibits $\downarrow$ vitB5 recycling; B5 required for antioxidant response & CoA synthesis
lower myeloid phagocyte capacity for metabolic (mt/oxidative) stress (e.g. lower glutathione, NADPH, NAD, mitophagy) with predisposition to inflammatory death	$\uparrow$ FA synthesis & oxidation (both) FAO-derived acetyl-CoA for TCA cycle or lipid synthesis $\uparrow$ G3P $\rightarrow$ glycerolipid (IRIS)
	Δ protein expression, $\uparrow$ chaperone & UPS activity (both) $\uparrow$ protein damage/misfolding/fusion with failure of compensatory mechanisms (IRIS)
	$\downarrow$ PLP synthesis & PLP-dep. metabolic reactions (both) likely $\uparrow$ homocysteine, incl. ROS- $\downarrow$ conversion to Met (both) $\downarrow$ vitB5/PLP-dep. conversion to Cys/GSH (esp. CTRL) $\downarrow$ GSH-dep. conversion to H ₂ S (esp. IRIS) $\downarrow$ pyruvate-dep. early- but $\uparrow$ GSH-dep. late-pathway H ₂ S synthesis (both) $\uparrow$ H ₂ S catabolism via thiosulfate to GSH persulfide (both) $\uparrow$ thiosulfate buildup, no vitB12-dep. cyanide detox (IRIS) $\uparrow$ late urea cycle Arg $\rightarrow$ ornithine + urea (both)
	$\downarrow$ complement inhibition capacity (IRIS) $\uparrow$ capacity for inflammatory signalling (esp. IRIS) incl. $\uparrow$ SHP1/2 & Lyn, & $\downarrow$ PTPRC (both) $\downarrow$ ALOX-mediated leukotriene synthesis (both) $\downarrow$ sensitivity to LTB4 (CTRL) absent NFkB p65/RelA subunit but $\uparrow$ NFkB dimerization (both), suggesting $\downarrow$ myeloid chemotaxis, adhesion, extravasation, phagocytosis, & survival indeed: $\downarrow$ recruitment (IRIS), chemotaxis (both), adhesion (both), & extravasation (both) $\downarrow$ degranulation (both) IFN-induced FA/glycerol/mevalonate/IPP buildup (both) i.e. $\downarrow$ protein prenylation & $\downarrow$ cholesterol/steroid hormone/vitD/Q10 synthesis (only CTRL exhibits attempts to preserve steroid hormones)
higher circulating/intracellular ATT/ARV/drug metabolite concentrations	?baseline $\downarrow$ OAT (PLP-dep.) drug uptake activity baseline $\downarrow$ ABCB1 expression & drug efflux (both) despite Mtb/Rif-mediated induction post-ARV conditions (incl. PLP recovery) favour $\uparrow$ OAT activity & oppose ABCB1-mediated ARV efflux $\downarrow$ xenobiotic conjugation to GSH (esp. CTRL) ongoing additional xenobiotic responses (IRIS), perhaps due to heme product-mediated AhR activation
ARV therapy directly or indirectly (e.g. via TNFa/IFNy) stresses mitochondria, triggering co-ordinated myeloid phagocyte inflammatory death, with release of stress molecules & sequestered Mtb/antigens (incl. via MMP), mimicking new infection & overactivating remaining myeloid phagocytes,	$\uparrow$ anti-inhibitory PTPRC ligand LGALS3 & inflammatory TREM-1 ligand PGLYRP1 (IRIS) $\uparrow$ recruitment (IRIS), chemotaxis (IRIS, opposite), adhesion (IRIS, opposite), & ECM migration (IRIS) further $\downarrow$ degranulation (IRIS) $\uparrow$ Fc γ RIA, MMP-8, & MMP-9 (IRIS)
	$\uparrow$ monosaccharide uptake (IRIS) & total glycolysis (both) $\uparrow$ PPP-derived NADPH (CTRL) $\uparrow$ glycolytic pyruvate- & FAO-derived acetyl-CoA (IRIS, opposite; despite CTRL vitB5 recovery facilitating CoA synthesis)

including concomitant metabolic changes & exacerbated mitochondrial/oxidative stress (on background of continued low GSH, NADPH, mitophagy)	↓ TCA cycle α-KG buildup (both) NADH passed via ↑ mt malate-Asp shuttle to CI (IRIS) FADH₂ passed to CII (CTRL) further ↑ CV function (IRIS, opposite) ↑ mt ADP uptake & ATP-alternative energy (IRIS) ↑ folding QC of mt proteins imported under stress (IRIS) likely ↑ mt stress, mtROS, attempt to maintain mt polarisation ↑ Fe loading (IRIS) & ↑ heme breakdown (IRIS) ↑ attempt to prevent (likely Fe-induced) lipoprotein & lipid peroxidation (IRIS) to form toxic aldehydes → ferroptosis, but ↓ ALDH-mediated aldehyde detox/vitA activation (IRIS) likely ↑ oxidative stress & mtDNA damage (esp. IRIS) Δ antioxidant responses; GSH (both) & NADPH (IRIS) may limit (CTRL recovery of vitB5 recycling may assist) ↑ DNA repair (esp. IRIS): ATP/purine>pyrimidine synthesis (IRIS) recovery of NAD provision (IRIS) ↑ nt-derived Ser, for pyruvate/glucose/lipid> Met/folate/purine synthesis (IRIS) ?RHEB activates mTORC1, inhibiting auto/mitophagy (IRIS) (CTRL may experience greater mTORC1 inhibition, perhaps due to greater baseline Trp depletion) ↑ ex/internally-initiated apoptosis (IRIS, opposite) incl. granzyme/TNF/Fas-CASP8-CASP10-CASP3/7-induced (CASP8 absent from CTRL, CASP10 elevated in IRIS) & mt-BAX-CytC-induced ↑ proneness to mt membrane permeabilisation (IRIS) ?↑ NLRC4-CASP4/5-mediated pyroptosis &/or lipid peroxidation-mediated ferroptosis &/or DNA damage-induced necroptosis (IRIS only) ↓ lipid synthesis (?↑ TG uptake), further ↑ FAO (IRIS, opposite) ?↑ acetyl-CoA feeding into TCA>lipid synthesis (IRIS) resulting ↑ OA for gluconeogenesis or citrate shuttle transport of mt acetyl-CoA into cytosol (?FA>carbohydrate as energy substrate) ↑ sterol synthesis, de-repressing FPPS to relieve IPP buildup, facilitate prenylation, & recover cholesterol/steroid hormone/vitD/Q10 synthesis (both) prenylated RHEB (elevated in IRIS) ?activates opposing AMPK & mTORC1 ↑ but altered ER protein processing (IRIS) ↑ glycolipid/protein transport between ER & Golgi (IRIS) further ↑ chaperone & UPS activity (both) ↑ UPS degradation of misfolded/fusion proteins (IRIS) ↓ IFN-stimulated immunoproteasome activity (CTRL) likely inhibited autophagy (IRIS) ROS↓ homocysteine → Met likely persists normalised PLP synthesis & PLP-dep. reactions (both) but ↓ homocysteine → Cys/GSH (IRIS): while CTRL exhibits ↑ GSH recycling, IRIS exhibits likely limiting levels of all 3 AA precursors to GSH resulting ?further ↓ homocysteine → H₂S (IRIS) indeed, ↑ H₂S catabolism (IRIS, opposite: CTRL ↑ H₂S synthesis) ?↑ cyanide detox (both) ↑ Asn → Asp (may feed into dicarboxylic acid cycle converting Ser-derived pyruvate into glucose) + NH₄ (not fully detoxified by urea cycle) (IRIS)
persistent defective myeloid-mediated antigen clearance (e.g. depleted glutathione, CR- > FcR-mediated uptake, threshold-exceeding TNFα, & IL-10 inhibiting phagocytosis & microbicidality) & thus immune stimulation + ineffective regulatory mechanisms leading to chronic inflammation	↑ phagocytosis (both; ?efferocytic in CTRL, but complement/IgG-mediated in IRIS) further ↓ respiratory burst activity (IRIS) ↓ Arg (both) may impair NO synthesis ↓ cytotoxicity (IRIS) further ↓ LOX-mediated leukotriene & ↓ HETE/HPETE ↓ COX-2-mediated prostanoids (?incl. PGE₂) (only CTRL exhibit preserved PAF production; IRIS exhibits ?PAF inactivation) likely ↓ protective NO, PGE₂, & steroid hormone signalling (IRIS)
activated (partially effective) innate cytotoxic lymphocytes ?of 62 by CD1-presented host IPP/Mtb phosphoantigens in presence of Th1 IFNγ	
reactivation & expansion of Mtb-specific poly-functional Th1 (provided sufficient remaining Mtb-specific memory despite HIV, &/or sufficiently-compromised lymphoid architecture)	↑ support for Th1/cytotoxic induction (both)
induction of multiple incompatible response programmes, including partially-effective FoxP3⁺ T_{reg} & probably FoxP3⁺ Tr1 & myeloid regulatory cells	↑ support for Th17 induction (both) ↑ TGFβ signalling (IRIS) ↑ IL-4/13 signalling (CTRL) ?↓ extracellular Trp (both) may inhibit T-cells
myeloid-derived cytokines are responsible for fever, malaise, weight loss, an acute-phase response (with ↑ haptoglobin & pentraxins e.g. CRP), myeloid products induce hypercoagulability, & myeloid infiltrates mediate airway inflammation as well as local tissue damage via lysed cell products (proteolytic enzymes, complement, TNFα, granzymes, etc.)	↑ TNFα, IFNγ, & TGFβ signalling (IRIS) ↓ IL-1α, IL-6, IL-10, & TGFβ signalling (CTRL) ↑ coagulability (IRIS)
antigen deposition & lymphocyte expansion in lymphoid organs is responsible for lymphadenopathy (± hepatosplenomegaly)	na

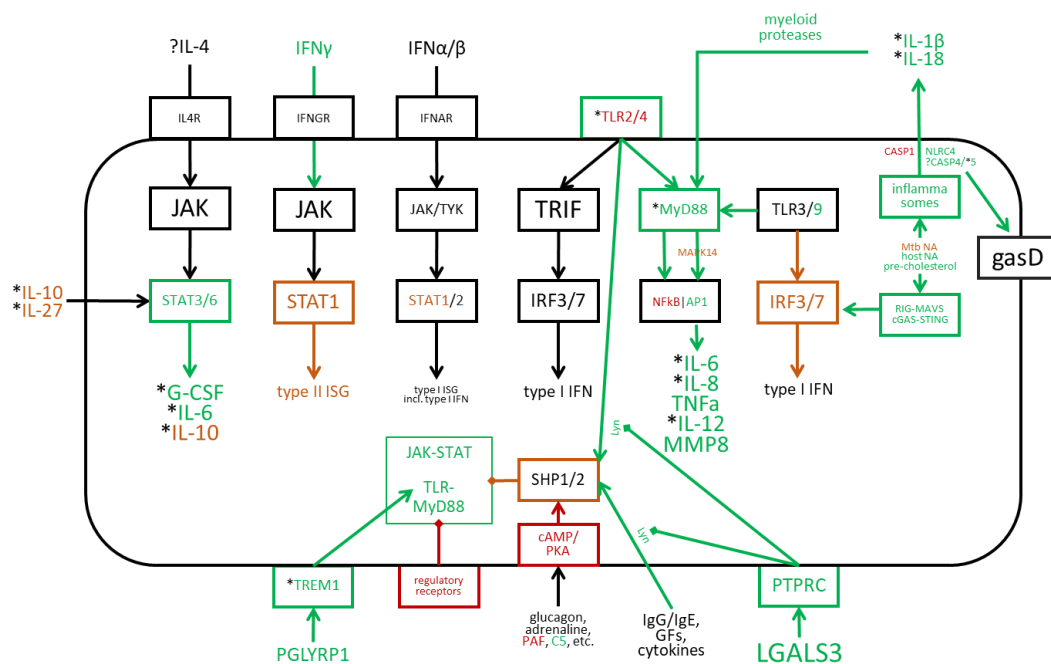


Figure 7 Simplified Neutrophil Immune Signalling during TB-IRIS Manifestation: Predictions Based on Proteomic Data
green increased, red decreased, orange potentially increased (*literature-indicated relative cytokine levels)

When considering that neutrophils act not in isolation but embedded within a web of interactions with other leukocytes and somatic cells, a broader model may be envisioned in which ARV therapy brings about redistribution Mtb-specific Th1 from the memory to the effector compartment, facilitating $\gamma\delta$ T-cell recovery and responses to phosphoantigens (whether host- or pathogen-derived or both), after which activated δ 2 cause monocytes to induce (ARV therapy-recovered) Th17 activation [154], restoring mucosal immunity with IL-22-elicited (IFN γ -supported) production of β -defensin, resulting in further enhancement of already largely neutrophil-mediated immunity (δ 2 can also directly activate neutrophils via MCP-2 [154]). Simultaneous stress-induced necrosis of myeloid cells at sites of previous TB disease (also brought about – whether directly or indirectly - by ARV therapy), as well as delayed/ineffective antigen clearance, contribute to triggering and sustaining this dysregulated inflammation.

The ways in which proteomic findings complement and extend existing models in the literature are summarised in fig. 8.

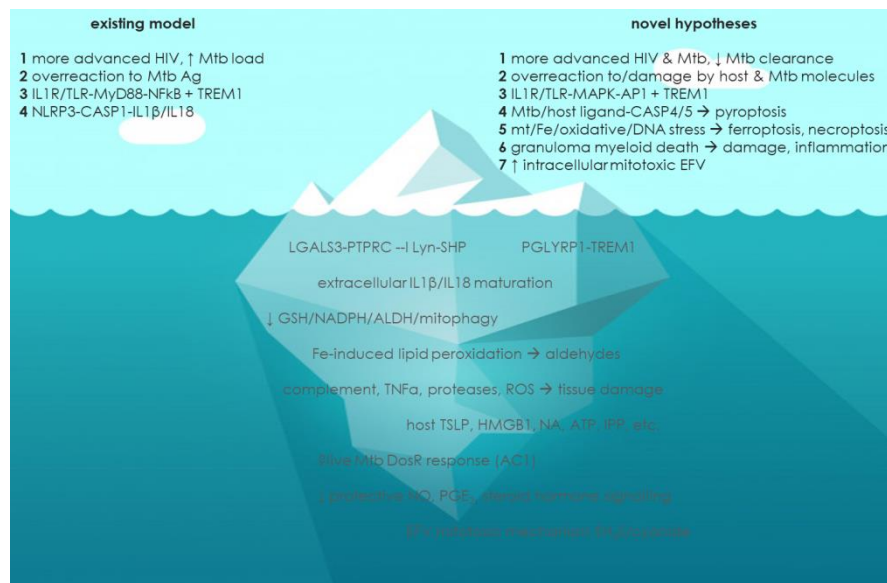


Figure 8 Selected Novel Hypotheses Derived from Integration of Pre-existing Literature & Neutrophil Proteomic Data

7.8. Are Live-cell Microscopy Results Congruent with the Hypothesis that ARV Therapy May Contribute to Mitochondrially-Initiated Myeloid Cell Death?

As an initial attempt at validating arguably the most controversial above hypothesis, we examined the effects of NNRTI efavirenz on *ex vivo* neutrophils.

As discussed in Chapter 6, live-cell fluorescent microscopy confirms that addition of an *in vivo*-equivalent efavirenz concentration rapidly causes mitochondrial fragmentation and swelling (commitment to death) in neutrophils derived from healthy volunteers. Mechanisms are unknown and remain to be investigated; presumably any sufficiently mitotoxic stimulus (e.g. another mitotoxic ARV drug or events inducing oxidative stress) may have the same effect. We cannot directly extrapolate these findings to neutrophils from TB-IRIS prone patients without repeating the live cell microscopy experiment using *ex vivo* neutrophils from such patients. However, it is tempting to speculate that metabolically-stressed neutrophils from TB-IRIS prone patients may react similarly to efavirenz (or the combination of Mtb/HIV-coinfection, ATT, and ARV therapy) or other mitotoxic stimuli (including other ARV drugs) *in vivo*, pending validation.

Although efavirenz was the only ARV investigated in the present study, all NRTIs are known to be mitotoxic, mainly via inhibiting DNA polymerase- γ -mediated mtDNA replication and synthesis. Invalid source specified.. Resultant impaired oxphos compromises ATP production, inducing an anaerobic metabolic shift, including increased lactate production/lactic acidosis, *decreased* FAO, triglyceride accumulation, and *decreased* ROS neutralisation. Tenofovir, specifically, may engage in additional mitotoxic mechanisms independent of DNA polymerase- γ (e.g. inhibition of mitotoxic drug clearance) [334]. However, of the NRTIs, tenofovir exerts relatively minimal mitochondrial toxicity [334]. It does not impact transcription of complex IV (cytochrome C oxidase) constituents COXII and IV Invalid source specified. and increases lactate production by <20% Invalid source specified., but does elevate mtDNA content and mitochondrial number, as well as altering mitochondrial morphology Invalid source specified.. Emtricitabine appears not to impact mitochondrial parameters [334]. Both tenofovir and efavirenz alter the functionality (including mitochondrial) of various cell types (e.g. hepatocytes, renal epithelia, and neurons).

A previous study in our laboratory [335] implicated efavirenz metabolites (and potentially CYP450 polymorphisms) as mediators of efavirenz-induced neurotoxicity. In that study the CYP3A4 inhibitor ketoconazole was able to ameliorate this neurotoxic effect [335] (indeed, many azoles inhibit CYP3A4

Invalid source specified., though no association was found in the current cohort between prior azole exposure and altered TB-IRIS susceptibility: Chapter 3). Extrapolating from this idea, CYP2B6 polymorphisms producing slower efavirenz metabolism (e.g. CYP2B6*6) may drive higher levels of efavirenz down the CYP3A4 metabolic pathway (both enzymes are inhibited by ritonavir, which featured in the previous first-line S.A. ARV regimen). An association also exists (in Chinese Invalid source specified. and Brazilian Invalid source specified. populations) between CYP2B6 polymorphisms and ATT-induced hepatotoxicity. While rifampicin is a prototypical PXR ligand and thus CYP3A4 (and CYP2A) inducer, azoles exhibit differential effects on PXR-mediated CYP3A4 expression (in a manner dependent on their impact on PXR interaction with various co-activators/repressors) [252]. For example, ketoconazole inhibits CYP3A4 induction by interrupting cross-talk between PXR and steroid receptor coactivator (SRC)-1, whereas oxiconazole enhances PXR-mediated CYP3A4 expression [252]. Rifampicin also induces the drug-transporter P-glycoprotein (encoded by the ABCB1 gene) [336]. Interestingly, ketoconazole (and miconazole) also antagonise(s) glucocorticoid receptor-mediated expression of PXR and CYP450s [337]. Inflammation, viral/bacterial products, and oxidative stress decrease glucocorticoid receptor functionality, with PI3K and MAPK pathways (the latter dephosphorylated by DUSP1, which contributes to steroid-mediated inhibition of inflammatory genes) implicated in steroid resistance [338]. Since both glucocorticoid receptor and PXR are also critical regulators of many immunometabolic pathways, the impact of azoles (and other drugs) on their function has more general biological implications not just limited to altered CYP450 activity [337].

Notably, hepatic and extra-hepatic CYP expression becomes down-regulated by inflammation Invalid source specified., as does availability of Fe (an essential cofactor for CYP function) Invalid source specified.. Early and systemically-acting inflammatory molecules and cytokines such as LPS, IL-1 β , IL-6, TNF α , and IFNs impact CYP expression levels Invalid source specified. Invalid source specified.. For example, LPS and the above cytokines inhibit hepatocyte expression of CYP2A3 and CYP2C8 at the mRNA level, while IL-6 and IFN both down-regulate CYP2B6 mRNA levels Invalid source specified.. Such inhibition of CYP expression may produce "elevated plasma drug concentrations" Invalid source specified.. This suggests that patients exhibiting a higher baseline level of inflammation (or perhaps those who more recently become inflamed) may be more/less prone to TB-IRIS because of CYP2B6 down-regulation and consequent higher efavirenz/lower metabolite levels.

7.9. Hypotheses Generation: Repurposing Existing Agents for Safe & Effective TB-IRIS Prophylaxis/Therapy

Should it be confirmed that: (1) efavirenz (and perhaps genetically-determined efavirenz disposition) contributes to mitochondrial stress, (2) that this effect contributes to myeloid cell inflammatory death, and (3) that such a mechanism may contribute to TB-IRIS pathogenesis, patients could perhaps be risk-stratified prior to ART initiation, and various clinical strategies may be prophylactic in the high-risk group, including but not limited to: lowering the efavirenz dose, delaying ART initiation (provided CD4 counts are within acceptable levels and no other indications for immediated ARV initiation are present), employing an alternative non-mitotoxic ARV regimen (e.g. incorporating darunavir, raltegravir, and rilpivirine Invalid source specified.), or perhaps pre/co-administration of substances which may assist cells in mounting adaptive stress responses to ameliorate the cytotoxic effects of increased inflammatory metabolic demand and/or efavirenz and its metabolites. Although the S.A. first-line regimen is currently replacing efavirenz with dolutegravir, unfortunately neither darunavir nor rilpivirine are pharmacologically compatible with rifampicin.

Such strategies are not clinically unprecedented, and some are supported by recent research. Indeed, plasma efavirenz levels (1-4mg/L) achieved on a 600mg per day efavirenz regimen exceed the required plasma IC₅₀ (0.51 μ g/L [249]) and suggested therapeutic range (1-4 μ g/mL) by over a thousand-fold [249]. The ENCORE1 study demonstrated that a daily efavirenz dose of 400mg did not compromise virologic efficacy, and some evidence indicates that dose-reduction lowers the rate/severity of adverse effects (though the frequency of these events remains high, at 37%) Invalid source specified.. Furthermore, a 2017 Ugandan study investigating "genotype-based efavirenz dose

recommendations" during adult TB-HIV coinfection (in the presence of rifampicin-based co-treatment) suggested that a daily efavirenz dose of 450mg is sufficient (250mg for individuals homozygous for CYP2B6*6) Invalid source specified..

Regarding identification of TB-IRIS prophylactic or therapeutic compounds, the classical drug discovery and development pipeline focuses on screening large libraries of existing compounds for bioactivity (followed by *ad hoc* identification of potential applications) or - more usefully - on identifying novel molecular targets to agonise/enhance or antagonise/inhibit (followed by development of/screening for compounds capable of the desired activity), and subsequent pre-clinical mechanism validation (cell-free assays, *in vitro* culture, and animal work), and finally validation of safety and efficacy in human subjects (clinical trials) [339] [340]. Novel drug target identification is informed by existing literature and databases (include phenotypes resulting from genetic alteration), as well as by new studies (e.g. those screening for gene function through knock-out/interference, or those comparing biomolecule expression patterns or levels between healthy and diseased patient tissues) [339]. The classical pipeline suffers from many bottlenecks and shortcomings (e.g. incompletely rational drug/vaccine design, and a reliance on the availability of early- and easily-measurable biomarkers for indication of successful target pathway modulation and a positive therapeutic response [339]), spans many years, and costs many millions of dollars. Given the significant resource investments involved, it is unacceptable that many therapeutics entering clinical trials ultimately do not meet efficacy and/or safety end-point targets (and this failure rate has been increasing for efficacy) [341] [342] [343]. Between 2006 and 2009, for example, phase II clinical trial success rates fell from 28% to just 18% [341]. Between 2008 and 2010, 51% of failures were due to insufficient efficacy [341], and, similarly, between 2007 and 2010, 66% of phase III and submission failures were due to insufficient efficacy [341].

Furthermore, it is, at best, irresponsible to attempt manipulating incompletely understood systems (in which the scope of ramifications resulting from propagation of perturbation through the system is unpredictable), although this is exactly what classical drug development does [339]. Truly successful novel target identification for complex disorders requires a deep understanding of physiology and molecular pathogenesis [339], facilitated by appropriate analysis of integrated systems biology datasets [339]. In fact, it is arguably much more important to elucidate the molecular pathogenesis of a disease (e.g. to facilitate prevention) than to develop novel therapeutic agents (especially in complex disorders such as TB-IRIS, where polypharmacy is already a concern).

As research philosophy and techniques mature, it is becoming increasingly evident that many existing (proven safe and effective) drugs in fact do display powerful - and useful - off-target effects (e.g. anti-inflammatory statins) which may be advantageously leveraged. Thus, an alternate potential shortcut to maximising therapeutic utility is more complete characterisation of the effects of existing drugs, and subsequently exploiting these effects for therapeutic intervention for additional indications (requiring only stage II and III clinical trials) rather than developing drugs *de novo* (requiring execution of the entire development pipeline).

For example, many existing agents are already known to target mitochondria, lysosomes, and the ER/protein quality control. One in particular – rapamycin, by virtue of its target mTOR (which it inhibits) being shared among multiple response pathways – stands out as having the potential to *simultaneously* therapeutically target all of these. As mentioned, mTOR is a key cellular sensor, and mTORC1 – in response to signals such as availability of nutrients, amino acids, and growth factors – regulates cell growth. Activation of mTORC1 favours (protein, pyrimidine, lipid) anabolism and opposes catabolism (e.g. autophagy). Because cytosolic mTORC1 impacts pleiotropic processes (e.g. “protein translation, immune responses, nucleotide and lipid synthesis, glucose metabolism, autophagy, and lysosomal biogenesis”) [344], rapamycin-mediated inhibition of mTOR necessarily has pleiotropic effects, including on mitochondrial, lysosomal, and ER function/protein homeostasis. Mitochondria, lysosomes, and the ER independently act as nutrient/energy-sensing cellular hubs, integrating information from multiple metabolic pathways to determine ultimate appropriate cellular responses, but their combined activity determines cellular fate. Mounting evidence associates aging and disease with the above cellular

processes, and their pharmacological modulation for the purposes of health optimisation and disease prevention/cure are under intense scrutiny.

Rapamycin exhibits therapeutic potential in all examined mitochondrial disease models, concomitant with metabolic reprogramming (e.g. oxphos restoration and/or "a shift from glycolysis to amino acid catabolism") [344]. In murine models rapamycin impacts the mitochondrial integrated stress response (ISR; a "complex ... pathway involving transcriptional and metabolic adaptations"), including "induction of mitokines FGF21 and GDF15, one-carbon metabolism, and the mtUPR", and "significant changes in liver amino acid, carbohydrate, fatty acid, cofactor, and nucleic acid metabolism, without affecting key mitochondrial functional pathways (e.g. glucose, lactose, pyruvate, and β -hydroxybutyrate metabolism)" [344].

Data support a key role for mTORC1 in regulation of lysosomal distribution, activity, and biogenesis Invalid source specified.. High amino acid levels induce recruitment of mTORC1 to the lysosomal membrane, an event required for mTORC1 activation by farnesylated RHEB Invalid source specified.. Lysosomal lumen amino acid level, too, modulates mTORC1 activity via vacuolar H⁺-ATPases Invalid source specified.., whose acidification of lysosomes is also associated with mTORC1 activity Invalid source specified.. Plentiful nutrients support mTORC1 activity, promoting "retention of TFEB and TFE3 in the cytosol, inhibition of the ULK1/2 complex and the ATP-sensitive Na⁺ channel", and peripheral localisation of lysosomes Invalid source specified.. When nutrients are lacking, mTORC1 inhibition causes TFEB and TFE3 nuclear translocation (such TFEB activation is necessary but insufficient for lysosome activation Invalid source specified..), "induction of lysosomal biogenesis, lysosome membrane potential changes, and autophagy activation" (possibly by facilitating autophagosome-lysosome fusion) Invalid source specified.. The lysosome is required not only for the degradation phase of autophagy, but also for its induction Invalid source specified.. Thus, mTOR inhibition - whether via starvation, Torin1, or PP242 (but not rapamycin) - activates lysosomal function Invalid source specified.. This effect co-occurs with mTORC1 (but not mTORC2) suppression Invalid source specified.. Niclosamide, another mTORC1 inhibitor, inhibits lysosome degradation, increases lysosomal permeability, and causes cytoplasmic acidification via release of lysosomal protons Invalid source specified.. Inhibition of mTORC1 - whether via amino acid deprivation, Torin1, rapamycin, or niclosamide - activates autophagy Invalid source specified..

Rapamycin promotes autophagy and protein clearance, with "chronic administration reducing amyloid burden and improving cognitive function in a murine Alzheimer's Disease model" [345]. Rapamycin also "relieves ER stress and reverses lysosomal dysfunction" induced by excessive homocysteine [316]. High homocysteine levels induce the UPR, inhibit autophagy, and "exacerbate cellular injury during oxygen and glucose deprivation as well as reperfusion and oxidative stress", effects which can be ameliorated by vitamin B-mediated alleviation of ER stress, restoration of autophagic flux, and reversal of lysosomal dysfunction [316].

As with all complex diseases, however, the inter-linking of complex biological systems complicates causal inference and identification of potential therapeutic interventions which are both safe and effective. Pharmacological targeting of key nodes in these interconnected networks "has high therapeutic potential", but it is crucial that these mechanisms (including their regulation, interactions, and functions) be more completely understood *prior to* more specifically-targeted pharmacological modulation, to prevent unforeseen consequences given system complexity [346]. For example, lysosomal dysfunction can impair mitophagy (and concomitant removal of excessive ROS), causing cellular dysfunction that can hypothetically be ameliorated by "correction of the primary lysosomal defect or neutralisation of mitochondrial oxidative stress" Invalid source specified.. However, *it is important to clearly identify the most proximal pathology-causing deranged step or component of an effector pathway "before attempting to intervene"* [347] [345]. Especially in this type of interconnected and inter-dependent system (where many branches involve second messengers common to multiple signalling pathways, and where most branches can also lead to cell death), the problem of multiple off-target effects can lead to significant unintended consequences.

Conceptually, for example, preventing apoptosis would *only* be beneficial in the case of *simultaneous alleviation of cell stress and induction of adaptive biological programmes which maintain normal cellular function*. Similarly, pharmacological autophagy induction would only be beneficial if it were the early steps of the autophagy pathway that were compromised (if later steps, such as endolysosomal degradation, were deficient, autophagy induction may “further overload the cellular degradation system”) [347]. Response inter-dependence, too, can give rise to unintended consequences (e.g. suppressing/enhancing function of the UPS invokes enhanced/suppressed autophagy, and vice versa).

However, a benefit of such complex system interconnection is emergence of a new therapeutic paradigm (particularly evident in the anticancer chemotherapeutic field), whereby a drug does not specifically target a dysregulated pathway, but acts as a cell stressor which *leverages certain subsequently-induced adaptive compensatory mechanisms to combat the pathology of interest* (a kind of host-directed metabolic rather than immune therapy). For example, mild ER stress provokes an adaptive autophagy response, producing neuroprotective effects in a Parkinson's Disease (PD) model [347]. In such cases, however, it is crucial that the cells stressed by the therapeutic agent exhibit sufficient availability of resources to mount successful adaptive stress responses, rather than the stressor inducing unintended (potentially inflammatory/host-damaging) forms of cell death.

The above observations - combined with the general robustness of networks to perturbation - may indicate yet another alternate therapeutic approach: targeting “multiple nodes in parallel functional cascades” responsible for the disease phenotype (although an optimal evaluation method to determine such targets has not yet been established, iterative node removal may assist) [348]. This could even *indirectly* influence critical nodes through targeting nodes that modulate them [348] (e.g. identified by betweenness measures [349]). The logic of a multiple-target versus a highly-selective approach must be considered on a per-pathology basis [339]. A drug combination that simultaneously targets a number of molecules may well be more suited in complex or multi-tissue diseases such as cancer or immunoinflammatory pathologies (indeed, such combinations are already the clinical gold-standard in many therapeutic areas e.g. cancer, type II diabetes mellitus, infections, and asthma) [233]. Combination therapies are also less susceptible to drug resistance, since biological systems are less able to adapt to the larger perturbation [233].

However, drug combinations require additional clinical trials to test a constrained number of drug pairs [233]. While *in vitro* drug combination screens with a “pathway-focused approach to target discovery” can identify unknown multi-target mechanisms and synergies (not to mention “unexpected interactions between disease-related pathways”, and an improved understanding of molecular pathogenesis) for expedition to clinical trials [5], they also (non-trivially) require culture systems true to “the essential elements” of the pathology (the “complexity of the diseases system must be reproduced *in vitro* for discovery screening”) [233]. Instead of a drug combination, then, it is also possible to develop single but multi-target drugs (conceptually similar to rapamycin). Nevertheless, while targeting multiple pathways has met with some success, the more targets affected by a single drug (i.e. the less selective it is), the higher the risk of undesired or adverse events [339].

Based on the proteomic and microscopy findings of the present study, various existing compounds which may prove useful in TB-IRIS prophylaxis/management are identified (Table 12), with the most promising candidates being those which would facilitate a return to near-physiological functioning rather than further perturbing the system. Indeed, many of these compounds fall under the banner of host-directed therapy (in which the body's own mechanisms are leveraged to prevent/treat pathology; one fringe benefit to targeting host systems is that microbes cannot develop resistance to the drug). Indeed, it has previously been suggested that combining mTOR pathway modulation (in such a way as to boost autophagy) with the standard ATT regimen may enhance TB outcomes [350]. Many listed compounds (e.g. formoterol, doxycycline, corticosteroids, statins, vitamin A, vitamin D, vitamin E, L-theanine) also exhibit *multiple different* mechanisms (i.e. appear multiple times throughout the table) which may be beneficial in preventing/treating TB-IRIS (based on our hypotheses). Given the Chapter 2 suggestion that smoking may be protective against TB-IRIS, it is interesting to note that

nicotine appears on this list (in the present study, ARV exposure down-regulates TTC38, known to be part of a smoking-induced gene signature [351]). Clearly, more preclinical (*in vitro*, animal model) evidence would be required in order to warrant TB-IRIS-oriented clinical trials for most of these compounds, but overwhelming favourable safety profiles of some (e.g. NAC, L-theanine, B-complex) may be sufficient to support small early add-on studies.

Table 12 Compounds with Potential Utility in TB-IRIS Prophylaxis/Management

Compound	Mechanism of Potential Utility	Evidence-based Hypothesis Relevant to TB-IRIS	Potential Caveats
adrenaline (epinephrine) or β -adrenergic agonists (e.g. formoterol)	anti-inflammatory, pro-resolving via multiple mechanisms	$\downarrow$ circulating adrenaline/ $\downarrow$ sensitivity to adrenaline	may be too short-acting; sensitising cells to endogenous adrenaline may be a better strategy
alarmin (ATP, HMGB1, oxmtDNA, etc.) antagonists/chelators	$\downarrow$ unnecessary inflammatory not reflective of active infection	overzealous activation of inflammatory pathways	$\uparrow$ antigen clearance
ALDH agonists e.g. Nashi Pear	detoxifies oxidant-induced aldehydes, converts vitamin A to active form (ATRA)	$\uparrow$ aldehyde toxicity $\downarrow$ vitamin A activation	?outcomes of clinical trial for ALDH agonist were never reported
amino acids e.g. likely-depleted Arg	Arg is required for NO production to $\uparrow$ antigen clearance, $\downarrow$ immunopathology	depletion of certain amino acids required for various metabolic functions	na
anti-oxidants e.g. NAC/glutathione, MitoQ, Q10, tea polyphenols such as *L-theanine, vitamin C/**E, Nrf agonists, etc. *also enhances mitochondrion/lysosome/ER function **also inhibits ALOX15 expression	enhanced mitochondrial respiration, antioxidant response, & antigen clearance	$\uparrow$ mitochondrial & oxidant stress, $\downarrow$ antigen clearance	NAC can only $\uparrow$ glutathione in the absence of factors preventing Cys usage/glutathione synthesis (e.g. PLP deficiency) although human NAC usage is safe, <i>in vitro</i> it can exacerbate or rescue HeLa cell death during mild versus severe p38 MAPK/JNK-induced ER stress Invalid source specified.
antiprotease inducers/protease inhibitors e.g. doxycycline (inhibits MMPs)	oppose activity of released myeloid proteases	protease-mediated tissue damage	may further impair antigen clearance
anti-complement/cytokine (IL-1, IL-6, TNF α) MAbs	oppose pro-inflammatory/antigen clearance-impeding complement and cytokine effects	$\downarrow$ antigen clearance, $\uparrow$ tissue damage, cytokines directly contribute to TB-IRIS manifestation	unfavourable adverse effect profile
ATRA (vitamin A)	pro-resolving, supports autophagy, required by RXR	$\downarrow$ autophagy, altered PPAR/LXR/RXR-regulated lipid metabolism	unfavourable adverse effect profile
autophagy agonists e.g. AMPK agonist metformin, mTORC1 inhibitor rapamycin	improved mitophagy AMPK also $\uparrow$ ATP production & $\downarrow$ ROS	$\uparrow$ mitochondrial stress, $\downarrow$ mitophagy	na, unless pathogenic problem is downstream of these regulators
B-vitamins e.g. B5, B6, B9, B12	metabolic enzyme cofactors	abnormalities in various metabolic pathways requiring these cofactors, which may be depleted	na
cholesterol	steroid hormone, Q10, vitamin D synthesis	$\uparrow$ pro-inflammatory IPP, $\downarrow$ sterols, corticosteroids, Q10, vitamin D	can be pro-inflammatory
corticosteroids	pro-resolving, anti-inflammatory via multiple mechanisms (low concentrations favour innate over adaptive immunity); also enhance protein folding and UPS-mediated degradation of misfolded proteins		already in use for prophylaxis and therapy, with only partial efficacy
cyclosporine	immunosuppressive CypD inhibitor which opposes mPTP opening & consequent mitochondrial swelling [56]	mPTP opening is final cell death pathway shared by many pathologies; other antagonists incl. adenine nucleotides, acidic pH, divalent cations (e.g. Mg ²⁺)	cell death may be the most adaptive response to supra-physiological stress
efavirenz disposition modulators e.g. OAT (uptake) inhibitors ABCB1 (efflux) inducers CYP2B6/3A4/3A5/2A6 modulators	$\downarrow$ intracellular efavirenz concentration	$\uparrow$ intracellular efavirenz concentration	$\uparrow$ virological efficacy, drug-specific adverse effects
eicosanoid cascade modulators e.g. PLA ₂ agonist COX-2 inhibitor (e.g. aspirin)/inducer ALOX5 inhibitor (e.g. zileuton) exogenous PGE ₂	$\downarrow$ immunopathology aspirin $\downarrow$ TXA ₂ & activates AMPK-mTORC2-AKT/ERK [352] zileuton $\downarrow$ LTB ₄ , thereby $\uparrow$ PGE ₂	altered eicosanoid cascade with $\uparrow$ arachidonate provision, $\downarrow$ leukotriene/lipoxin production & $\uparrow$ PGE ₂ production	not enough known about eicosanoid perturbation in this context, ALOX5 already exhibits lower levels
ferroptosis inhibitors e.g. desferrioxamine (Fe chelator) ferrostatin-1 liproxstatin-1	$\downarrow$ lipid peroxidation & thus ferroptosis	$\uparrow$ Fe-dependent accumulation of lipid hydroxides (implicated in degenerative disease) leading to ferroptosis	unknown
galectin-PTPRC inhibitors	prevents opposition to Lyn-SHP1/2-mediated inhibition of MyD88-NF κ B & JAK-STAT inflammatory pathways	$\downarrow$ Lyn-SHP1/2-mediated inhibition of inflammatory pathways	$\uparrow$ inflammation
inflammasome modulators e.g. NLRP3 (colchicine, P2X7R inhibitors) NLRC4 inhibitors CASP4/5 inhibitors gasD antagonists	$\downarrow$ inflammasome-induced inflammation & pyroptosis	overzealous inflammasome activation & pyroptosis	CASP1 is already absent: may need to target other inflammasomes, $\uparrow$ inhibiting cell death may worsen cell dysfunction

calpain inhibitors			
IL-1R/IL-18R/TLR-MyD88-NFκB inhibitors e.g. TAK-242, chloroquine (TLR) T6167923 (MyD88), nicotine (TLR-MyD88-NFκB), D-JNKI-1 (JNK)	↓ MyD88-NFκB-induced inflammation nicotine (via nACh receptor-IRAK- STAT3 signalling) inhibits TLR signalling and NFκB nuclear translocation	overzealous MyD88-NFκB signalling	↑↓ antigen clearance (e.g. nicotine also inhibits NFκB-mediated iNOS/NO induction)
p38 MAPK (e.g. JNK)-AP-1 inhibitors e.g. T-5224 (AP-1)	↓ MAPK-AP-1-induced stress responses & inflammation [353]	overzealous MAPK-AP-1 signalling	↑↓ adaptive stress responses
Lyn-SHP1/2 agonists	↓ MyD88-NFκB & JAK-STAT inflammatory signalling	overzealous MyD88-NFκB signalling	
mitochondrial modulators: biogenesis (PGC1α, β2-adrenergic, PPARα) agonists e.g. formoterol fusion (Mfn2) agonists e.g. L-theanine, M1 hydrazine fission (Drp1) inhibitors e.g. MDIVI1	improved mitochondrial function	↑ mitochondrial stress	may be maladaptive if pathologic mechanism is downstream/mitochondrial toxicity-induced cell death is the most adaptive response, drug-specific adverse effect profiles
omega 3 fatty acids	↑ efferocytosis, pro-resolving	↓ antigen clearance, ↓ inflammatory regulation, ↑ chronic inflammation	pre-existing myeloid dysfunction
PPAR agonists: PPARα (e.g. fibrates) PPARγ (e.g. glitazones)	↑ peroxisomal FAO (PPARα) ↑ lipogenesis/lysosomal TAG oxidation, ↓ ROS, ↑ efferocytosis, pro-resolving, ameliorates murine neutrophilia/monocytosis (PPARγ)	altered lipid metabolism	not enough known about lipid perturbation in this context
purines e.g. organ meats	↑ NA synthesis & DNA repair ↓ necroptosis	oxidative DNA damage	may require other limiting factors (e.g. NA synthesis is NAD-dependent), exacerbates gout (↑inflammasomes)
statins	earlier inhibition of sterol synthesis pathway avoiding buildup of pro-inflammatory intermediates, anti-inflammatory (e.g. may inhibit TLR-4), inhibition of viral replication	abnormal isoprenoid buildup, ↓ sterol synthesis, ↑ inflammation	largely favourable adverse effect profile, may impact prenylation in unexpected ways
TNFR antagonists	↓ TNFα signalling & necroptosis	↑↑ TNFα, ↑↑ TNFα-induced necroptosis	potential for serious adverse effects
vitamin D	↑ antigen clearance, ↓ immunopathology	↓ synthesis, ↓ antigen clearance & immune modulation	na; toxic in large doses

Interestingly, ABCB1 inducers include drugs already present in TB-IRIS patients (e.g. rifampicin and ARVs). Others include actinomycin, doxycycline, corticosteroids, antifungal azoles, ivermectin, artemisinin, morphine/oxycodone, midazolam, antiepileptics, insulin, colchicine, cyclosporine, atorvastatin, propranolol, vitamin A, vitamin D, EGCG, quercetin, caffeine, rapamycin, genotoxic anticancer drugs, sildenafil, and verapamil.

As mentioned in Chapter 1, the IRIS phenomenon also arises in other diverse settings, such as in non-HIV-infected individuals following lifting of immunosuppressive factors (e.g. withdrawal of anti-TNF or corticosteroid therapy) [238]. These settings do, however, all share the following: immunosuppression, and presence of an antigen [238]. Furthermore, many pathogenic mechanisms implicated in TB-IRIS are also present in other, related, inflammatory disorders, and it has been hypothesised that their prophylactic/therapeutic strategies may thus potentially be applicable to TB-IRIS [238].

Ceva et al. (2019) suggest potential interventions aimed at: prevention (e.g. immunomodulating corticosteroids and statins, although these blunt CD4 reconstitution), attenuating excessive innate activation (e.g. both anti-IL-1 antibodies and IL-18BP are already in use for autoimmune/inflammatory disorders, and small molecule inhibitors of the NLRP3 inflammasome are under investigation), targeting hypercytokinemia and tissue damage (e.g. anti-TNFα and anti-IL-6 antibodies are already in use for autoimmunity, but are expensive and possess an unfavourable adverse effect profile, though host-directed therapy may be able to ameliorate neutrophil activation or MMP activity, e.g. doxycycline which suppresses TNFα secretion, MMP activity, and in vitro granuloma matrix turnover), or general immunosuppression for prevention/treatment (e.g. immunomodulating corticosteroids or statins – the latter shown to slow onset of human GvHD; although both blunt CD4 reconstitution, statins do not impede antigen clearance despite inhibiting APC MHC-II-mediated antigen presentation and co-stimulatory molecule expression, instead opposing neutrophil apoptosis, decreasing MMP levels, and inducing T_{reg}) [238].

That Ceva et al. (2019)'s independent model of TB-IRIS closely matches our own, and that their independent recommendations for potential prophylactic/therapeutic TB-IRIS strategies overlaps with ours lends additional credence to our findings and the resulting hypotheses.

7.10. Putting It All Together: Overall Thesis Conclusions

Although biological systems are complex and adaptive, the immune system behaves in a largely predictable manner during *normal* inflammatory responses. However, TB-IRIS arises against a background of host immunity modulated by additional layers of significant complexity (simultaneous bacterial and viral infections, as well as the presence of ATT and ARV). Resulting aberrant immunometabolic responses are difficult to predict, and complicate the disentanglement of cause and effect (*vide infra* Section 7.11).

Given the hypothesis-generating nature of this study, we have attempted to extract full utility from the neutrophil differential proteomic profiling approach, keeping in mind that is difficult without further experimentation to determine which findings are associative and which are causal. Although our experimental design cannot address all remaining questions laid out in Section 7.5, this work does address many questions regarding predisposition/prophylaxis, biomarkers, and pathogenesis/therapeutic intervention.

We have achieved the following: extensive literature review, appropriate study design, cohort assembly, sample collection, leukocyte isolation and storage, sample preparation and analysis, and data analysis. Data analysis identified various significantly differentially expressed proteins and over-represented biological functions/pathways between groups and classes, as well as 9 neutrophil-specific functional modules altered before and during TB-IRIS. We have identified putative risk stratification and diagnostic biomarkers, and - based on proteomic findings integrated with the most consistent and relevant/interpretable (i.e. *in vivo*) findings in existing literature - have constructed various plausible hypotheses regarding TB-IRIS predisposition and pathogenesis, as well as identifying various existing compounds (safe and effective for other indications) which may more specifically and effectively address prophylaxis/treatment of TB-IRIS (in ways seeking to re-establish physiological homeostasis, rather than further perturbing the system). We have partially validated one of our controversial hypotheses; although multiple evidences converge on common conclusions in large part agreeing with existing literature, all other hypotheses remain to be validated.

(1) What accounts for differential patient susceptibility to TB-IRIS? More advanced TB/HIV disease (including higher type I IFN levels and adaptogen depletion, possibly in combination with inadequate dietary compensation/GIT absorption) impedes myeloid microbicidal activity and antigen clearance, resulting in a higher tissue-sequestered Mtb load, an abnormal myeloid immunometabolic state (with a decreased capacity to adaptively counteract stress, partial activation by host alarmins, and priming for unrestrained, abnormal inflammation). Finally, partial ATT on a background of more advanced disease may prime cells for increased intracellular concentrations of mitotoxic efavirenz.

(2) What is the most proximal trigger for and nature of the TB-IRIS pathogenic cascade? Rapid removal of viral stimuli inducing type I IFN/ISG and restraining certain inflammatory pathways in the presence of increased mitochondrial, oxidative, genotoxic, and lytic stress on a background of decreased capacity to adapt to such stress results in improved pathogen-sensing and stress-mediated host cell over-activation, with proneness to lytic inflammatory death. Together, metabolically-induced ferroptosis and necroptosis, with immunologically-induced pyroptosis, may lead to coordinated myeloid inflammatory death within granulomas, with release of inflammatory host molecules (which may include extracellular ATP, oxidised mtDNA, HMGB1, cholesterol intermediates e.g. IPP, LGALS3, PGLYRP1, and peroxidated lipids, among others), inflammatory Mtb-derived molecules (although the identities of these remain largely unknown, they may include LAM, mycolic acids, PGL-Tb1, and AC1), and tissue-damaging molecules (ROS, proteases, TNF α , complement, and cytotoxic mediators). Activation of myeloid cells primed for detection of inflammatory molecules and recovering from inhibition of inflammatory pathways results in overzealous inflammasome (?NLRC4-CASP4/5), IL-1R/IL-18R/TLR-MyD88-NF κ B, MAPK-AP-1, and intracellular NA sensor signalling and supra-maximal levels of inflammation. Both myeloid and lymphoid cell activation (and activation of incompatible immunological programmes) is aberrant. Delayed clearance of Mtb and its products, as well as immune regulatory failure, contribute to chronicity of inflammation.

(3) Which downstream effectors are responsible for TB-IRIS clinical manifestation? As mentioned above, cytokines and released cellular contents are responsible for the signs and symptoms of TB-IRIS (fig. 9).

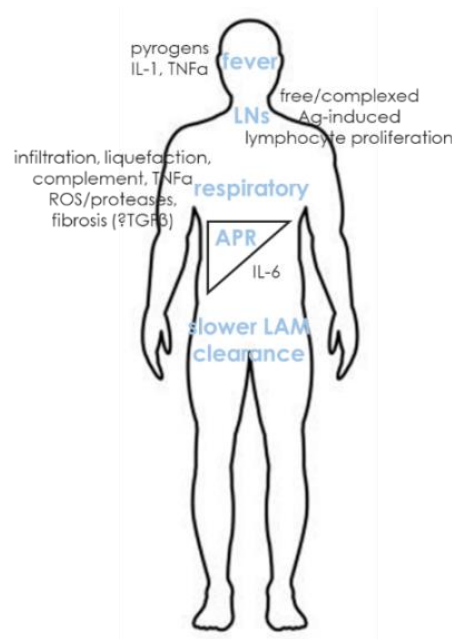


Figure 9 Effectors Responsible for TB-IRIS Manifestation

(4) Can we infer potential prophylactic/therapeutic interventions (with a focus on pre-existing safe modalities and/or host-directed therapy)? Yes, our improved hypothetical understanding of TB-IRIS predisposition and pathogenesis may suggest decreasing efavirenz dosages in patients at higher risk for TB-IRIS (although efavirenz is now being replaced by dolutegravir in the S.A. first-line regimen), and compounds with likely potential prophylactic/therapeutic utility are detailed in Table 12 (*vide supra*). Given the identities of these compounds, it is possible that dietary differences (e.g. in tea polyphenol, vitamin, or purine consumption) and incidental treatment of comorbidities (e.g. with statins, azoles, antihelminthics, β -blockers, etc.) for unrelated comorbidities may contribute to differential susceptibility to TB-IRIS.

Overall, results of this single study – integrated with the most consistent findings from ~20 years of pre-existing literature – provide potential answers to many remaining questions and allow us to construct a more comprehensive model of TB-IRIS pathogenesis (fig. 10).

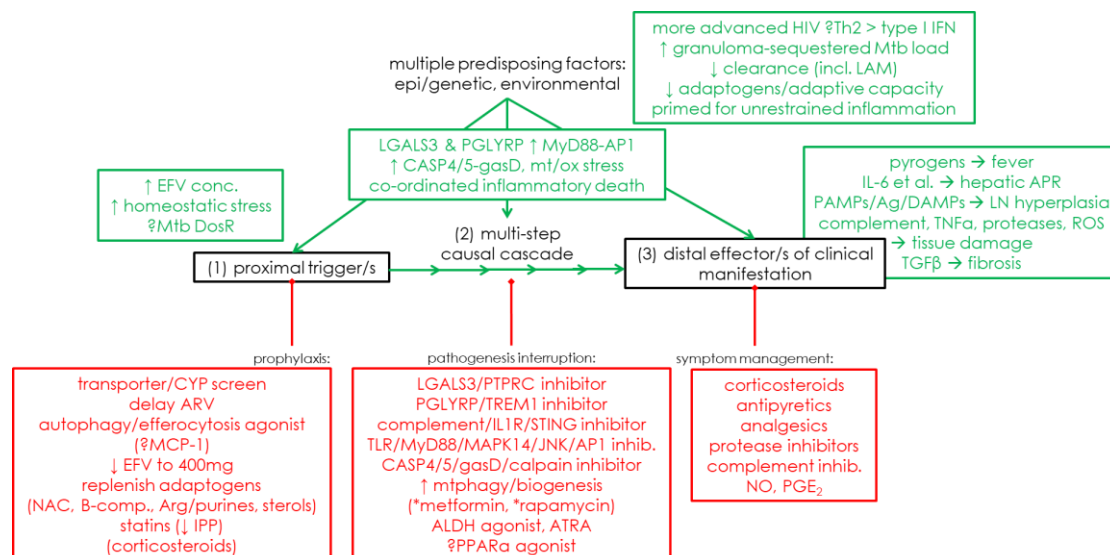


Figure 10: Selected Features of Hypothesised Comprehensive TB-IRIS Model Primary prophylaxis is the most desirable intervention, as it is the most specific and effective, removing underlying causes of the disorder (however, it is not always feasible). Intervening at the level of the multi-step causal cascade is less specific, and – especially if the intervention is early in the cascade where it would be most effective – represents the possibility for broad off-target propagation through the system, potentially leading to adverse events. Finally, intervening late during the causal cascade, or at the level of effectors directly responsible for clinical manifestation, is again more specific, with less likely off-target propagation, but this approach treats downstream symptoms, not underlying causes of the disorder.

(5) Are ideal biomarkers able to discriminate between healthy controls and partially ATT-treated co-infection, as well as between those who do and do not develop TB-IRIS (before ARV exposure and at the time of TB-IRIS)? The top identified biomarker candidates are provided in Table 13. None offer perfect sensitivity and specificity; it is likely that *detectable* versus *undetectable* markers, single pathogenesis-related present/absent marker *trajectories* (e.g. ISGs for discriminating/monitoring partially ATT-treated co-infection and STAT6 for risk stratification/diagnostics), or carefully-selected marker *panels*, will exhibit superior performance over single markers significantly differentially expressed between classes.

Table 13 Top Potentially Clinically-translatable Biomarker Candidates

	Candidate	Sensitivity & Specificity	Peripherally Available?	Specific to TB-IRIS?	Likely Involved in TB-IRIS Pathogenesis?
CTRL/HV	PGM1	100, 91	Y	N (MI)	Y (metabolic)
	DCTN1	89, 91	Y	Y	N
	PTPRJ	89, 100	Y	N (cancer, obesity)	Y (phosphatase)
IRIS/HV	PSMC4	100, 100	Y	N (infertility, Parkinson's, cancer)	Y (UPS component)
	GSTK1	100, 100	Y	N (cardiomyopathy, diabetes, stroke, infertility, cancer, Alzheimer's)	Y (peroxisomal glutathione transferase for cellular detoxification; associated with mitochondrial dysfunction)
	PSMA6	94, 100	Y	N (diabetic nephropathy, cancer, ischemia)	Y (UPS component)
IRIS/CTRL	LYN	82, 82	Y	N (cancer, steroids)	Y (inhibitory SFK)
	DCTN2	76, 73	RNA only	N (cancer)	N
	PTPN6	71, 82	RNA only	N (neutrophilic disease)	Y (inhibitory phosphatase)
CTRL/CTRL	ML12A	86, 68	Y	N (sepsis, cancer, dysmegakaryopoiesis)	?Y (DNA repair, apoptosis)
	G6PD	67, 86	Y	N (anemia, cancer)	Y (PPP enzyme generating NADPH)
	NAMPT	67, 82	Y	N (inflammation, cancer)	Y (rate-limiting NAD biosynthesis enzyme)
2 / -	NAMPT	85, 94	Y	N (inflammation, cancer)	Y (rate-limiting NAD biosynthesis enzyme)
	DYSF	90, 88	Y	N (muscular dystrophy)	?Y (membrane damage repair)

	HSP90AA1	75, 76	Y	N (sepsis, cancer)	Y (inducible chaperone involved in inflammation; favours TGFβ signalling)
IRIS2/CTRL2	HSP90AB1	85, 76	Y	N (cancer)	Y (STAT1 chaperone)
	GNAI2	85, 76	Y	N (cancer, hypertension)	Y (hormone-regulated adenylate cyclase function)
	PRKCSH	80, 71	Y	N (polycystic liver disease)	Y (ER protein processing)

Recall also that detectable/undetectable proteins may be better-suited to discriminating classes than are SDE. Various comparisons yield high numbers of such candidate biomarkers: **CTRL0 (vs HV)** 125 detectable, 114 undetectable; **IRIS0 (vs HV)** 97 detectable, 180 undetectable; **CTRL2 (vs CTRL0)** 53 detectable, 119 undetectable; **IRIS2 (vs IRIS0)** 156 detectable, 38 undetectable.

7.11. Study Strengths, Shortcomings, & Future Directions

The study was fortunate to have access to a uniquely relevant population, from which a well-characterised cohort was assembled by excellent clinical research teams, under guidance of some of the world's foremost TB-IRIS researchers. It was able to study the largest (generally under-studied) leukocyte population, responsible for initiation and shaping of innate immune responses, and implicated in both TB and TB-IRIS pathogenesis (but also under-studied in this context). Patient-derived leukocytes were directly analysed *ex vivo*, using techniques (mass spectrometry-based proteomics) most well-suited to a comprehensive understanding of neutrophil phenotype and function, as a step towards a deeper conceptual understanding of the complexity of immune functioning in this context. Having access to samples from two rationally-selected timepoints, as well as a small healthy volunteer cohort, has facilitated investigation of many different questions, and has provided more power to derive hypothetical answers to these than a single cross-sectional study would have been able to. Ultimately, findings are internally consistent, agree with and extend existing literature, and an attempt to partially validate one arising hypothesis was successful.

Neutrophils present in different biological compartments are known to exhibit differential phenotypes. Although we examine neutrophils derived from circulation rather than from the site(s) of disease, disease signals are still present systemically before and during TB-IRIS, and the impact of such differences on neutrophils was clearly discernible. Without further experimentation, we cannot know which proportions of the studied neutrophils have recently exited the bone marrow for recirculation through lymphoid and non-lymphoid tissues (i.e. less likely to be directly infected with Mtb/HIV and therefore with phenotypes conditioned largely by interaction with other cell types and soluble factors), and which have recently egressed from sites of disease. Nor can we know neutrophil maturity state or phenotypic sub-population composition. Furthermore, since it is important to interpret the hypothesised neutrophil causal narrative within context of the specific experimental system in question, it is tempting to speculate regarding neutrophil impacts on the broader immune interaction network, though this must be done with caution. A more complete picture would emerge in the presence of data deriving from all leukocyte types and cytokines/chemokines in all relevant biological compartments, but this is beyond the scope of a single PhD project (indeed, leukocytes also interact with somatic cells as part of immunity). However, in context, some degree of prediction regarding neutrophil interaction with other leukocytes and cell types is already possible from neutrophil data alone. Additionally, access to extracellular proteomic data (i.e. not just the neutrophil cellular proteome, but that of the circulation itself) would assist in maximising accuracy of data interpretation.

Although our healthy volunteer population is small, leading to potentially unbalanced comparisons with other groups, we randomly selected size-matched subsets of the other groups for balanced comparisons, whose outputs agreed with those of the full comparisons. Given the hypothesis-generating nature of this study (i.e. a greater concern with higher coverage of functional pathway members rather than with avoiding false positives), we employed multiple sequential t-tests rather than MANOVA. We believe this strategy to be sound, since any hypotheses arising from the study will ultimately be orthogonally validated. Applying an approach such as non-linear mixed effects modelling could better take into account the impact of all known variables (e.g. demographic and

clinical), and sub-group analyses (e.g. examining differences between corticosteroid exposed versus unexposed patients) could be very informative.

We have been mindful of specific data interpretation caveats at every level - arising from an understanding of LC-MS/MS, proteome functionality, the immune system, and complex disorders such as TB-IRIS – and have attempted to employ strategies mitigating these. For example, we understand that sample preparation methods significantly impact HPLC-MS/MS findings (and thus selected an optimised method aimed at the most unperturbed, unbiased, and deepest coverage of the human neutrophil proteome), that HPLC-MS/MS measures only a sample of the total proteome, and that non-detection of a protein does not necessarily indicate its absence (e.g. some proteins may be insoluble, not amenable to tryptic digestion, or produce peptides which do not efficiently ionise, are “outside observable mass range detection settings”, or produce spectra which are not easily interpretable by software). However, detection of a protein within many samples of one group but not in the samples of another *does* suggest the protein is either absent or present at levels below the limit of detection in the latter group. Furthermore, given similar sample types, consistent sample preparation, and consistent equipment performance during LC-MS/MS, it is likely that any technical influences were consistent across samples.

Regarding proteomics, additional data beyond protein identity and relative quantity (e.g. anatomical compartment, sub-cellular location, isoform identity, post-translational processing and modification status, and availability of interaction partners/co-factors) would be required to fully interpret findings of any proteomics experiment. Overall, a protein can only perform its predicted function(s) if it is present – in its mature form – in the correct cellular compartment, is activated (e.g. through phosphorylation of the correct residues), if the appropriate interaction partners are also available in their mature and functional forms, and if the correct co-factors are available. However, assessing complete proteomic data (e.g. *all* PTMs) is beyond the scope of even the most ambitious current proteomics project. Too, we have been mindful that the same protein/functional pathway may function differently within different tissue types (and have attempted to account for this by focusing mainly on literature relevant specifically to myeloid cell types), and that a rise or decline in the level of a protein in a cell lysate sample may be interpreted in a number of different ways (relating to transcription rates, transcript stability, translation rates, protein stability, protein subcellular location and interaction with other subcellular structures, and protein secretion/exocytosis/shedding), especially when considering receptor-ligand interactions (and especially when matched information about the interaction partner is unavailable), because cell sensitivity to ligands is regulated by alterations in receptor expression (either translation/degradation or translocation of pre-existing protein to/from the cell surface).

Furthermore: protein expression may be *incidentally* up/down-regulated if that gene happens to be part of a transcriptional cassette activated/deactivated in response to some other stimulus, immune responses often simultaneously activate *and* regulatorily oppose activation of functional modules, and it is not only protein up/down-regulation that matters, but also the degree and rate of such changes. Indeed, response kinetics can be helpful in data interpretation (e.g. degranulation is near-instantaneous, while secretion of pre-formed mediators or synthesis of new mediators can take minutes to hours); one of the many features that make time-course experiments particularly useful. Finally, protein-level regulation may be physiological (i.e. appropriate responses to stimuli, and therefore useful in inferring cell functionality) or pathological (i.e. unpredictable and not reflective of cellular needs, such as happens during chronic inflammation or malignancy, and therefore not useful in inferring cell functionality). Although proteomic data strongly suggest inferences regarding transcriptomic, lipidomic, and metabolomics states of the neutrophils under study, it would be ideal to directly confirm this more complete subcellular picture via simultaneous application of all relevant ‘omics technologies, followed by transomic data integration. This, too, is unfortunately beyond the scope of a single PhD project, however.

We are also cognisant of the shortcomings of existing knowledgebases, particularly those containing functional pathway data. Our partial solution is to focus on level changes in key, inference-friendly proteins (e.g. rate-limiting enzymes, transcription factors, and positive/negative regulators such as

kinases and phosphatases), as well as focusing on multiple lines of converging evidence. These approaches have allowed us to maximise data utility to achieve a more comprehensive understanding of neutrophil function under the conditions of interest, including to some degree circumventing the challenges associated with biased/subjective databases and interpretation of data given that neutrophils and TB-IRIS are significantly under-represented in existing knowledgebases.

Regarding the immune system (a dispersed, complex, adaptive system), various factors complicate disentanglement of cause and effect. Since common effectors are produced by different cell types, inference of the responsible source (from effector) is difficult. Since different effectors have pleiotropic (often overlapping) functions, inference of the responsible effector (from effect) is difficult. As alluded to above, effector function is dependent on isoform, post-translational state, biological/subcellular compartment, presence of interaction partners/co-factors/switch-combinations/decoys/antagonists, etc. Similarly to factors influencing protein abundance, factors influencing leukocyte counts (production, mobilisation/release, redistribution from various biological compartments, context-dependent variable lifespan, elimination/recycling, etc.) make the meaning of count changes more difficult to interpret (e.g. within circulation, cells may be performing steady-state surveillance, may be en route to infiltrating inflamed sites, or may be post-efferocytic and en route to disposal). Furthermore, cell function depends on where along the phenotypic continuum (e.g. from immaturity to maturity, including functional subgroup) that particular cell lies, as well as on the presence/absence of interacting cells. And yet, as mentioned above, normal immune function is to a large degree predictable, and becomes ever more so as we learn about homeostatic immune function and immune responses to various pathogens and other disturbances.

Regarding TB-IRIS itself, however, we have also mentioned that pathogen- and pharmaceutical drug-mediated modulation of the immune system from equilibrium adds additional, sometimes impenetrable, layers to the existing complexity, making it more difficult to extract a functional causal narrative.

Nevertheless, we have attempted to compile all existing high-quality human data regarding paradoxical TB-IRIS, and to integrate it with neutrophil proteomic findings of the present study to generate the most parsimonious hypothesis explaining the majority of results. Future directions resulting from this work largely include validation of major hypotheses. For example:

(1) The hypothesis that patients developing TB-IRIS may exhibit higher baseline levels of circulating type I IFN, Th2-type cytokine and IgE, TGF β , and HMGB1/IL-33 cytokines and higher at-manifestation levels of circulating angiotensin II, IL-21, IL-23, and IL-27 may be investigated using - for example - Luminex technology.

(2) The hypothesis that there is more severe glutathione (or other adaptogen) depletion from myeloid cells of patients who go on to develop TB-IRIS may be investigated by colorimetric assays determining ex vivo cell lysate glutathione levels, provided that reduced glutathione remains stable enough for detection (i.e. does not immediately become oxidised upon cell lysis). Even if glutathione does become oxidised, total glutathione levels may still be determined.

(3) The hypothesis that lipid peroxidation is the major mitochondrial stressor in this context may be investigated by assessing circulating levels of malondialdehyde (e.g. using colorimetric or mass spectrometry-based assays). Levels of F2 isoprostanes may also be investigated in support of increased oxidative stress. It may also be of interest to investigate within-granuloma myeloid cell iron content and DNA damage, but this is difficult in humans, and may require histological analysis of granulomas from animal models. As an oxidative DNA damage marker, serum/urine 8-oxoguanine levels may be determined. Similarly, leukocyte ALDH activity and circulating ammonia/cyanide levels may be assessed.

(4) The hypothesis that suppressed auto/mitophagy contributes to a decreased capacity for adaptive stress responses in the myeloid cells of patients going on to develop TB-IRIS may be investigated by examining molecular markers of this process.

(5) The hypothesis that co-ordinately granuloma myeloid cell death releases large quantities of inflammatory host and pathogen molecules may be investigated by monitoring serum/urine levels of HMGB1, IL-33, MMPs, IPP, and Mtb-derived molecules over time before, during, and after ARV therapy initiation in patients who do and do not go on to develop TB-IRIS. Ideally, within-granuloma onset of inflammatory forms of cell death would be directly confirmed, but this is difficult in humans, and may require histological analysis (including fluorescent detection of pyroptosis, necroptosis, and ferroptosis markers) of granulomas from animal models over the same time-period. Altered isoprenoid/sterol/steroid hormone homeostasis may also be further investigated.

More complicated would be determining which pathways (MyD88-NF κ B versus MAPK-AP-1) and inflammasomes/caspases (NLRP1/3, NLRC4, AIM2, CASP1, CASP4/5) or TNFR signalling pathways (CASP8, CASP10) are dominant in these cells. This could rely on transcriptomic and/or proteomic studies of the myeloid cells likely involved, but detection of the molecules of interest is not guaranteed. More difficult still would be validating the proposed model of the inter-leukocyte interaction web giving rise to TB-IRIS, but perhaps longitudinal analysis involving a rational combination of cytokine monitoring and flow cytometry-based leukocyte characterisation can contribute to this more comprehensive understanding.

However, it *would* be possible to assess performance of risk stratification/diagnostic biomarker candidates (or of a rational biomarker panel) in plasma/urine (e.g. using targeted mass spectrometry or ELISA) from an independent cohort of patients who do and do not go on to develop TB-IRIS. Assessing performance of pre-existing, safe compounds hypothesised to be of utility in TB-IRIS prophylaxis/therapy (e.g. N-acetylcysteine, L-theanine, B-complex, Arg, and doxycycline) may also be achieved by means of randomised, double-blinded trials in an appropriate cohort of patients at risk for TB-IRIS (provided of course that preclinical studies provide additional compelling evidence for relevance of these compounds in this context). If AMPK inhibition (with decreased ATP production) and mTORC1 activation (with decreased auto/mitophagy) are confirmed (especially if there are also indications that lysosomal stress-induced cathepsin leakage is contributes to inflammasome activation), metformin and rapamycin may also be of particular utility. If it is confirmed that higher circulating/intracellular levels of ARV drugs contribute to TB-IRIS pathogenesis, dosages decreased without affecting virological suppression in the most at-risk patients would be the simplest, most specific, and safest intervention.

We described in Chapter 2 that – at least in this cohort – a positive smoking history appears to decrease the risk of subsequent TB-IRIS by 60%, which we speculated may assist in elucidating the mechanisms of TB-IRIS. It is already known that smoking is protective against another complex immunological disorder (ulcerative colitis) in part via suppressing levels of T_H1/T_H17 cytokines (e.g. TNF α , IFN γ , and IL-17). However, smoking is also known to modulate many pathways we have here implicated as potentially being involved in the pathogenesis of TB-IRIS (e.g. enhanced expression of IL-33 and its receptor, altered MAPK and TLR2/4-NF κ B-COX2/NOS cascades, nAChR-STAT3-mediated inhibition of NF κ B translocation, enhanced IL-8/TNF α /LTB₄-mediated neutrophil recruitment and accumulation, inhibition of CASP3-mediated spontaneous neutrophil apoptosis, AhR signalling-mediated enhancement of T_H17-type responses, elevated IgE levels, and loss of T_{reg} homeostasis). It is worth noting that IL-4 induces nAChR expression. It is possible that smoking, over time, induces cellular responses gradually facilitating adaptation to such changes, and thus (if changes are indeed common to TB-IRIS pathogenesis) providing some degree of protection against TB-IRIS.

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