Immunological Markers of Protective Immune Reconstitution in HIV Infected Persons Sensitised by *Mycobacterium tuberculosis*

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For Nani and Nana

Declaration

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“You are your biggest investment” – Nishtha Jhilmeet

Summary

Tuberculosis (TB) is the leading cause of death from a single infectious agent worldwide and HIV-1 co-infection is the leading cause of susceptibility to tuberculosis. Sub-Saharan Africa has a high incidence of TB-HIV-1 co-infection and the risk of TB in HIV-1 infected people is increased at all stages of the infection. Antiretroviral treatment (ART) is the most effective way to reduce the risk of TB in HIV-1 co-infected people. By studying the protective, ART induced immune reconstitution in HIV infected individuals sensitised by *Mycobacterium tuberculosis (Mtb),* we can identify correlates of protection against tuberculosis in the form of transcriptomic, soluble or cellular biomarkers. This thesis focuses on characterising *Mtb*-specific reconstituting CD4 T cells as well as soluble and transcriptomic markers in HIV infected persons, sensitised by *Mtb*, by analysing samples collected longitudinally during 6 months of ART.

Analysis of peripheral blood mononuclear cells by 14-colour flow cytometry revealed the proportion and numbers of central memory $CD4^+$ T cells significantly expanded in HIV infected persons on ART, while the proportion and numbers of effector memory and terminally differentiated effector $CD4^+$ T cells decreased significantly. Additionally we noted a significant decrease in the proportion of activated $CD4^+$ T cells, and IL-2 single producing $CD4^+$ T cells in HIV infected persons at 6 months of ART, while polyfunctional Mtb-specific $CD4^+$ T cells secreting IFN-γ, IL-2 and TNF-α simultaneously, proportionally increased.

Analysis of soluble markers in the plasma of HIV infected persons revealed an overall decrease in pro-inflammatory cytokines during 6 months of ART. A significant decrease in IFN-γ, IL-1α, IL-1β, IL-6, IL-17A and TNF-α was observed, and concentrations of these cytokines fell towards those observed in HIV uninfected persons.

Transcriptomic analyses of 30 genes normalized to 3 different housekeeping genes, showed an overall increase in the expression of T cell memory specific genes, illustrating the regeneration of the memory T cell pool in HIV-infected adults on ART. Larger number of central memory specific genes showed increased expression when normalised to at least two housekeeping genes, as compared to effector memory specific genes. These results support the reconstitution of central memory CD4 T-cell specific response at 6 months of ART.

Our data provides insight into the reconstituting immune response to latent TB infection in the context of HIV infection and identifies potential correlates of decreased susceptibility to TB. We also show decreasing soluble and cellular factors indicative of decreasing immune activation in HIV infected persons receiving ART.

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List of Abbreviations

ACTB	β-actin
AF	Alexa-Fluor
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
AM	Alveolar Macrophage
ANOVA	Analysis of Variance
APC	Allophycocyanin
APC-Cy	Allophycocyanin-Cyanine
ART	Antiretroviral Therapy
BAL	Bronchoalveolar Lavage
BCIP/NBT-plus	Nitro-Blue Tetrazolium and 5-bromo-4-chloro-3'-Indolyphosphate substrate
BFA	Brefeldin-A
BH	Benjamini-Hochberg
BSC	Biosafety Class II Hood
BV	Brilliant Violet
CCL	C-C Motif Chemokine Ligand
CCR	C-C Chemokine Receptor
CD	Cluster of differentiation
cDNA	complementary Deoxyribonucleic Acid
CFP-10	Culture Filtrate Protein 10
CM	Central Memory

CO_2	Carbon Dioxide
cm	Centimeter
Ct	Cycle threshold
CTL	Cytotoxic T-lymphocyte
CXCR	α-Chemokine Receptor
DC	Dendritic Cell
d.f.	Degrees of Freedom
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme Linked ImmunoSpot assay
EM	Effector Memory
ESAT-6	Early Secretory Antigenic Target 6
FCS	Fetal Calf Serum
FGD	Fludeoxyglucose
FITC	Fluorescein Isothiocyanate
FGF	Fibroblast Growth Factor
FMO	Fluorescence Minus One
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
g	Gram
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GNBL2	β-2-microglobulin

GNLY	Granulysin
GZMA	Granzyme A
GZMB	Granzyme B
GZMK	Granzyme K
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen – antigen D Related
HLY	Listeriolysin
HREC	Human Research Ethics Committee
ICOS	Inducible T-Cell Costimulator
ICS	Intracellular Staining
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IGF1R	Insulin Like Growth Factor 1 Receptor
IGRA	Interferon-gamma Release Assay
INH	Isoniazid
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
IQR	Interquartile Range
IU/ml	International Units per milliliter
ITK	Tyrosine-protein kinase
kDa	Kilodalton
LEF	Lymphoid enhancer-binding factor
LTBI	Latent Tuberculosis Infection
M	Months

MCP-1	Monocyte Chemotactic Protein-1
MDR-TB	Multi-drug Resistant Tuberculosis
MHC	Major Histocompatibility Complex
MCP-1	Monocyte Chemoattractant Protein 1
MIP-1α	Macrophage Inflammatory Protein-1 alpha
MIP-1β	Macrophage Inflammatory Protein-1 beta
mg	Milligram
ml	Milliliter
mM	Millimolar
MFI	Mean Fluorescence Intensity
MOTT	*Mycobacteria* Other Than Tuberculosis
Mtb	*Mycobacterium tuberculosis*
Mtb WCL	*Mycobacterium* Whole Cell Lysate
NaHep	Sodium Heparin
NFκβ	Nuclear Factor-Kappa Beta
NHLS	National Health Laboratory Services
NK	Natural Killer
NIR	Near InfraRed
ng	Nanogram
nm	nanometer
ns	not significant
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor

PET-CT	Positron Emission Tomography - Computed Tomography
PFA	Paraformaldehyde
PMT	Photomultiplier Tubes
PRKCA	Protein Kinase C Alpha
QFT	Quantiferon® Gold In-Tube assay
PE	Phycoerythrin
PE-Cy	Phycoerythrin-Cyanine
PerCP-Cy	Peridin Chlorophyll Protein-Cyanine
PHA	Phytohaemogglutin-P
pg/ml	Picogram per milliliter
RA	Receptor Antagonist
RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
RBC	Red Blood Cell
RIN	RNA Integrity Number
RORC	Related Orphan Receptor C
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RR-TB	Rifampicin Resistant Tuberculosis
RT	Reverse Transcriptase
S	Svedberg
SAg	Super Antigen
SATVI	South African Tuberculosis Vaccine Initiative

SD	Standard Deviation
SEB	*Staphylococcus aureus* Enterotoxin B
SELL	Selectin
SFC/million	Spot Forming Cells per million
SI	Stain Index
SPICE	Simplified Presentation of Incredibly Complex Evaluations
SSC-A	Side Scatter Area
SSC-H	Side Scatter Height
TB	Tuberculosis
TB7.7	Rv2654c antigen of *Mtb*
TCF7L2	Transcription Factor 7 Like 2
TGFβ1	Transforming Growth Factor Beta 1
T_H	T-helper
TEM	Terminal Effector Memory
TNF-α	Tumor Necrosis Factor-alpha
UCT	University of Cape Town
VEGF	Vascular Endothelial Growth Factor
VL	Viral Load
WHO	World Health Organization
xg	Gravity
°C	Degree Celsius
µg	Microgram
µL	Microliter
%	Percentage

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Chapter One: The Pathogenesis of Tuberculosis – An Overview

1.1. Introduction

Mycobacterium tuberculosis (Mtb) was first discovered in 1882 by Robert Koch [1], which was a critical moment in the history of Medicine as well as in our understanding of this deadly disease. Over the past century Tuberculosis (TB) research has facilitated our understanding of this disease, the mode of transmission and disease progression [2]. This was made possible through researchers and their findings; Devoto had recognised that healthcare workers were at risk of developing TB in 1920 while Riley had described the deposition of airborne bacteria in the lung in 1961, and the arterial dissemination of *Mtb* in the TB ward between 1960-1962 [3-6]. In 1964, Chapman had attributed social, amongst other factors, that were associated with the transmission of TB in TB-affected households [7]. Lastly, in the mid 2000's the phylogeographical classification of global *Mtb* strains and the advent of whole-genome sequencing for molecular tracking of TB outbreaks were achieved [2].

1.1.1. The Epidemic of Tuberculosis

Fast forward to 2017 and TB still remains a global health problem, with it being the ninth leading cause of death worldwide. The burden of disease is an estimated 10.4 million persons contracting TB in 2016, of whom, 10%

were people living with HIV [8]. Additionally, there were an estimated 1.3 million deaths among HIV uninfected persons (compared to 1.7 million in 2000), with a further 374 000 deaths amongst HIV infected persons. Although there is still a high rate of mortality globally, the mortality rate has since fallen by an estimated 3% globally [8]. The incidence of TB is falling by an estimated 2% but this needs to increase by 2-3% more per annum, in order to reach the first milestone of the “End TB strategy” by 2020. The WHO European region has the fastest decline in TB incidence (from 3% in 2015 to 4.6% in 2016) with the decline since 2010 in high TB burden countries also exceeding 4% per year [8]. Since 2010, the WHO in European and the Western regions had the fastest decline rate in TB mortality with 6% and 4% respectively, while other high TB burden countries have rates exceeding 6% per year since 2010 [8]. The proportion of people who develop TB and die from the disease (also known as the case fatality ratio) needs to be at 10% by 2020 to meet targets, but was estimated to be at 16% in 2016. TB treatment has prevented a predicted 44 million deaths amongst HIV uninfected persons, and TB treatment in conjunction with ART has prevented an estimated 9 million deaths in HIV infected persons between 2000 and 2016 [8]. Finally the threat of drug resistant TB is persistent, with an estimated 490 000 cases of multidrug-resistant TB (MDR-TB) in 2016. An additional 110 000 cases were susceptible to isoniazid (INH) but resistant to Rifampicin (RR-TB), the most effective first line anti-TB drug, with almost 50% occurring in China, India and the Russian Federation.

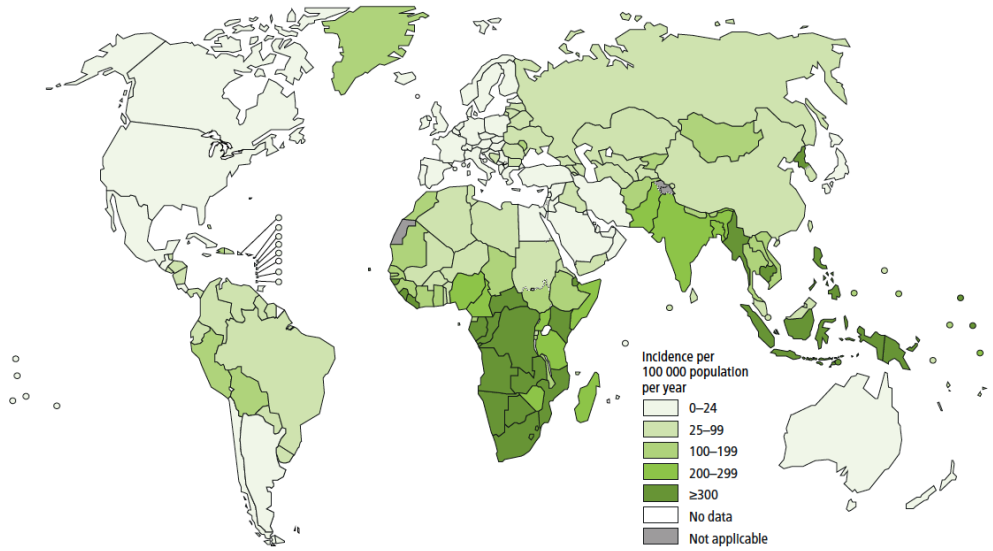

Figure 1. 1: Estimates of the incidence of Tuberculosis in 2016. This map highlights the incidence of TB worldwide. Sub-Saharan Africa, has the highest number of TB-related deaths in HIV infected persons, with more than 300 persons per 100 000 population infected with TB. (Image sourced from WHO: Global Tuberculosis report 2016 [8]).

1.1.2. The TB burden in South Africa and its implications

South Africa has an estimated population of 56 million people, with an estimated 438 000 persons living with TB and an estimated 123 000 people dying as a result of TB in 2016 [8]. Of the 438 000 people that are infected with TB, 182 000 (~42%) are females and 256 000 (~58%) are males. HIV/TB co-infection accounts for a higher mortality rate than those that are infected with TB alone, 181 per 100 000 people compared to 41 per 100 000 people respectively, with an estimated 100 000 people dying from HIV/TB co-infection in 2016 [8]. TB infection may be categorised into two clinically defined conditions, namely active TB and latent tuberculosis infection (LTBI). A diagnosis can be made for active TB when a patient presents with clinical

signs and symptoms of TB in addition to having evidence of *Mtb* infection [9]. Pulmonary TB may be characterised by a chronic cough (with or without the presence of blood), fever, sustained weight loss and wasting. *Mtb* is cultured from sputum or the microorganism is identified by nucleic acid testing, or acid-fast staining, for microbial confirmation and diagnosis of TB disease. LTBI is typically characterised by the presence of immunological sensitisation to mycobacterial antigens in the absence of clinical symptoms and can be ascertained by using a tuberculin skin test or interferon-γ release assay (IGRA) [9].

1.1.3. Early events in TB infection and the subsequent adaptive immune response to TB infection

TB infection is most commonly acquired by the inhalation of aerosolized bacteria. These bacteria-containing small particles are carried via the airstream and are inhaled into the lungs [10]. The mycobacteria are taken up by alveolar macrophages and upon appropriate activation the bacteria may either be destroyed, or if not destroyed, may begin to replicate after a lag period of a few days depending on the host's resistance [11]. Following bacterial replication, cell-mediated immunity and the formation of granulomas ensues. The presence of granuloma formation within the lung is a hallmark in *Mtb* infection and is comprised of immune cells that provide a safe microenvironment to establish latency, while also halting the spread of *Mtb* infection [12]. The granuloma is highly structured and is composed of infected alveolar macrophages (AM) and epithelial cells and is surrounded by

activated macrophages as well as CD4+ and CD8+ T cells that function to restrict the spread of the mycobacteria [13]. Continuous bacterial multiplication leads to the death and disruption of host cells, resulting in the bacilli escaping AM containment. The bacilli and resulting necrotic cellular debris induce an inflammatory response and in turn attract phagocytic cells to the site of infection. When encountered the DCs engulf the bacteria, migrate to the lymph nodes and present the bacteria to T cells, in order for antigen priming to occur and to initiate the adaptive immune response [12]. T cells play a critical role in the protective immunity against *Mtb* due to the intracellular lifestyle of the bacterium [14]. Pathogenesis and protection are mediated by cellular responses and involve the interaction of phagocytes, macrophage lineage, and cells in TB [14]. The production of cytokines such a IFN-γ and TNF-α are imperative in establishing protective immunity against *Mtb* infection as both cytokines are able to activate macrophages, that in turn play a significant role in the control of *Mtb* [14].

CD4 T cells:

The CD4 T cell subset may be divided into T helper type 1 and 2 (T_H1 and T_H2) cells; with each subset possessing the ability to produce distinctive cytokines [11]. T cells, B cells, neutrophils and Natural Killer (NK) cells are involved in the immune response to *Mtb*, with the CD4 T_H1 cells being the most researched and understood, while other interleukin (IL) producing T cells, such as CD8 T cells, are also involved in protection against *Mtb* [14]. Upon infection *Mtb* stimulates CD4 and CD8 T cells, as well as other immune cells, to secrete IFN-γ resulting in the production of a strong type 1 immune

response; with the CD4 cells being the primary source of IFN-γ production [14]. IFN-γ is important in activating the bactericidal action in the host macrophages and has shown to be vital in immune protection against TB in both mice and humans [15]. While T_H1 cells are able to control *Mtb* infection by producing IFN-γ and TNF-α, T_H2 cells are characterised by their ability to produce IL-4 and IL-10. IL-4 plays a role in the downregulation of T_H1 responses and mouse studies have demonstrated cytokines, IL-4 and IL-10, to promote infection with intracellular pathogens possibly due to macrophage inhibition [11]. The deleterious mutations in genes encoding proteins; IL-12p40 subunit, IL-12Rβ1 and IFN-γR1, IFN-γR2 and STAT1 are essential for the induction and function of T_H1 cells with the presence of these mutations increasing susceptibility to *Mtb* [16]. Additionally recent evidence supports the activation of T_H17 cells in TB with T_H17 cells being able to produce IL-17A, IL-17F, and TNF-α. The T_H17 cells have pro-inflammatory functions and are capable of mediating anti-microbial immunity against extracellular bacteria and fungi [16]. Through the use of high-dose aerosol *Mtb* infection in mice, it has been shown that T_H17 cells play an important role in the early phases of protection to *Mtb* infection [17]. CD4 T cells are pivotal in immunity against *Mtb*, however CD8 T cells also play an important role in immunity and protection against TB [18].

CD8 T cells:

There are multiple pathways culminating in the activation of CD8 T cells namely; (1) DC presentation to MHC-class I molecules, (2) active transmembrane transport to MHC-class I presentation pathway, (3) *Mtb*

infected cells undergoing apoptosis and forming apoptotic vesicles that are taken up by DCs and subsequently presented via MHC-class I molecules and (4) autophagy, which involves antigen presentation to and the cross priming of T cells in response to intracellular pathogens [19]. CD8 cells are able to produce cytokines with a T_H1 profile and function as MHC class I restricted cytotoxic effector cells [11]. *Mtb*-infected non-phagocytic cells are recognised by CD8 T cells. CD8 T cells also complement CD4 T cell immunity by surveying a larger number and range of cell types [16]. Furthermore, CD8 T cells are able to produce granules containing cytotoxic molecules such as perforin, granzymes and granulysin that are able to lyse host cells, with granulysin being able to kill *Mtb* directly [16]. In individuals with HIV infection, CD8 T cells may have a greater role to play in immune competence [20, 21] as CD4 T cells are depleted [22] thereby highlighting their important role in protective immunity to *Mtb*. Although there is limited knowledge about CD8 T cells in *Mtb*, multifunctionality has been considered in protection to *Mtb* whereby *Mtb*-specific CD8 T cells produce IFN-γ and IL-2 and were associated with protective host defence following curative TB treatment whereas CD4 T cell polyfunctionality may possibly be associated with protection, disease [23] or with antigen load [24, 25].

B-cells:

Although infiltration and activation of CD4 T_H1 cells and CD8 cytolytic lymphocytes are a requirement in the effective control of human TB, B cells have been shown to play a role [26]. Naïve and memory B cells have been shown to be present in TB granulomas as well as in the human lungs

resembling germinal centre-like secondary lymphoid structures [27]. B cells can present antigens to T cells, are involved in the production of cytokines and *Mtb*-specific antibodies upon antigen recognition [27]. Several studies that demonstrate the antigen-presenting capacity of B cells and thus establishing an important mechanism for T cell immunity modulation [28, 29]. B cells, like DCs, are capable of presenting antigen that can prime T cells as demonstrated in studies using *in vivo* mouse models [30]. A study by Pape *et al.* suggested that B cells participate in the events of the early phase of the immune response, such as antigen presentation and T cell priming [31]. Various mouse models of TB have revealed a potential role for specific antibodies in the host defence against *Mtb* [32]. The induction of humoral immune responses in animal and human models of LTBI and active TB respectively have suggested that B cells play a significant role in determining the clinical outcome of *Mtb* infection [32]. As mentioned above, in infectious disease models, the antigen-presenting capability of B cells have been linked to the modulation of T cell responses and protection but the ability of B cells in the regulation of T cell function is not limited to antigen presentation [27]. Upon activation, B cells are also capable of producing cytokines and antibodies (immunoglobulins), both of which play an important role in the maturation of antigen presenting cells, and thereby influence the development of T cell immunity [33]. B cell development may be divided into 3 distinct subsets, namely, B effector 1 (Be1), B effector 2 (Be2) and IL-10 expressing cells, each of which influence the cytokine milieu and the way they interact with antigens and T cells [27]. Be1 cells produce IFN-γ and IL-12. Additionally TNF-α, IL-10 and IL-6 develop once B cells are primed in a

T_H1 environment, whereas priming in a T_H2 cytokine milieu will result in the development of the Be2 subset that is capable of producing IL-2, lymphotoxin, IL-4, IL-13, IL-10 and IL-6 cytokines [27]. IL-12 and IL-4 producing B cells may prejudice the development in vitro of T_H1 and T_H2 responses respectively [34, 35]. Together this demonstrates the important role that cytokines, produced by B cells, play in modulating immunity. Furthermore, our understanding of the role B cells and the cytokines produced by B cells play in infectious disease is still at early stages.

1.2. Disease progression

1.2.1. Progression from latent tuberculosis infection to active disease

TB disease severity varies greatly and shows diverse patterns of lung involvement encompassing a range of infection outcomes [36-39]. Individuals with asymptomatic LTBI possess the ability to contain the bacteria, although persisting, and prevent the infection from spreading. Thus asymptomatic LTBI ranges from clearance of the disease to low-grade TB [36]. The concept of a spectrum of TB infection for LTBI allows for the stratification of risk of LTBI reactivation in individuals, prioritizing preventative treatment and emphasizing heterogeneity of host responses to *Mtb* [9, 40]. As expected individuals that are able to contain the infection well are less likely to reactivate infection compared to individuals with LTBI harbouring low-grade sub-clinical infection, with the latter at a higher risk of reactivation and eventually requiring treatment [9, 40]. Esmail *et al.* [41] demonstrated that patients with signs of subclinical TB disease, had a higher risk of developing

active TB compared to patients that had no evidence of subclinical pathology. In this study 4 patients developed active TB out of 10 that showed signs of subclinical TB; while the remaining 6 of these patients did not develop active disease thus demonstrating variability in disease progression. Additionally Berry *et al.* [42] conducted a study in 2010 in which they reported a signature unique to patients with active TB disease (in both intermediate and high burden settings) in comparison to patients with LTBI and healthy controls. Briefly this transcript signature indicated increased transcription of IFN-inducible genes in neutrophils, isolated from blood, sampled from patients with active TB. This finding also correlating with lung disease that was assessed by radiograph [42, 43]. Although some patients with LTBI did not express the transcript, approximately 10-25% of LTBI persons expressed a similar transcriptional profile to individuals with active TB. Additionally a portion of patients that received TB therapy displayed notable gene expression differences compared to TB treated individuals. This further highlights the heterogeneity that is intrinsic to the TB disease spectrum. The findings of these studies suggest that the site of infection, bacterial burden as well as the host response to infection, are linked to illness. Additionally a study in adolescents with LTBI demonstrated that a transcriptional signature in whole blood correctly identified individuals at risk of developing active TB up to 12 months prior to clinical diagnosis thus further supporting the concept of a differential risk of reactivation in populations [9, 44]. These findings in humans were previously observed in the macaque model of TB, in which a spectrum of disease was also noted [45, 46]. Lin and colleagues sought to characterise the histopathology,

pathology and immunological characteristics of macaques that were infected with a low dose of *Mtb*. Differences observed between LTBI macaques and macaques with active disease included bacterial burden and the degree of pathology [46]. Heterogeneity was noted in both groups with some monkeys with clinically active disease having bilateral lobe involvement and pulmonary cavitation compared to other monkeys with active disease that had a pathology in the thoracic lymph nodes and a single lung lobe [46]. Moreover this heterogeneity was also observed in latently infected macaques in which some macaques had only one lymph node involvement while others were classified as having an intermediate disease state. Similar findings in humans were observed in the study by Esmail *et al.* [41]. Studies in both macaques and humans support the concept of heterogeneity of TB and further suggests that patients may be segregated according to disease risk.

The pro- and anti-inflammatory signals that are initiated upon *Mtb* infection are imperative in granuloma formation [47, 48]. Careful control is required as these mediators possess the ability to limit and promote bacterial dissemination [47]. A favoured pro-inflammatory state results in the remodelling of the granuloma and subsequent destruction of the surrounding lung parenchyma [9]. Prolonged release of pro-inflammatory mediators culminate in active disease [49] which is in turn vital for the successful transmission of *Mtb* [50]. Conversely a shift toward the anti-inflammatory state favours a better host outcome, a reduced risk of activation and a better long-term prognosis following treatment [49, 51, 52]. Malherbe *et al.* found that in patients that appeared cured of pulmonary TB residual inflammation

was associated with the presence of *Mtb* mRNA in sputum and bronchoalveolar lavage (BAL) fluid samples as assessed by Fludeoxyglucose (FDG) Positron Emission Tomography - Computed Tomography (PET-CT) [52]. Patients in South Africa and South Korean mirrored this observation even after a year of treatment [52]. This study highlighted the variable host outcomes even after patients had received treatment as well as the heterogenous role of inflammation in TB outcome [9]. Another study by Marakalala *et al.* [53] examined the proteomes of granulomas from TB-infected persons and rabbits. They illustrated that centres of granulomas consist of pro-inflammatory eicosanoids, anti-microbial peptides and reactive oxygen species, while the surrounding tissue had an anti-inflammatory signature [53]. These findings were similar to studies conducted with human and macaque granulomas in which the compartmentalization of inflammatory mediators were organised around the different macrophage populations [54]. Marakala *et al.* also proposed that the balance of inflammatory mediators, and the localisation, have an effect upon the fate of the granuloma fate and in turn the host's outcome [53]. Together these studies suggest that intervention at the granuloma level could skew granuloma responses in way to favour host resolution as opposed to active disease [9].

The heterogeneity of the TB immune response directly influences the local granuloma microenvironment. Firstly, many granulomas form due to a single bacterium, that in turn multiplies within the granuloma for up to 4 weeks post infection [55]. At this point, the adaptive immune response is initiated in

which case bacterial killing is observed in the majority of granulomas leading to approximately 10% of granulomas being sterile by 11 weeks [9]. A pilot study by Subbian *et al.* [56] illustrated variation in the extent of fibrosis in granulomas. Moreover, they observed variation in the density of T cells despite having similar numbers of macrophages in lesions. These variations were in accordance with bacterial load and immune activation [9] and further suggested that these variations may be driven by the different immune profiles observed from granuloma to granuloma, which are indicative of the maturation state of the granuloma [56]. A study by Gideon *et al.* found that the total number and phenotype of T cells varied substantially, even within the same animal. The range of cytokine profiles and bacterial burden within each granuloma also varied [57]. Of interest was the finding that less than 10% T cells within the granuloma were able to produce cytokines in response to *Mtb* antigen stimulation, with majority of them only producing one cytokine, of which IFN-γ, IL-2, TNF, IL-10 and IL-17 were dominant [58]. They illustrated that entire granulomas were functioned as multifunctional cytokine environments and that various T cells contributed to the cytokine repertoire [9]. They concluded that both pro and anti-inflammatory cytokines, produced by T cells, such as IL-10 and IL-17 is necessary for a balanced cytokine response in order to achieve bacterial clearance [59].

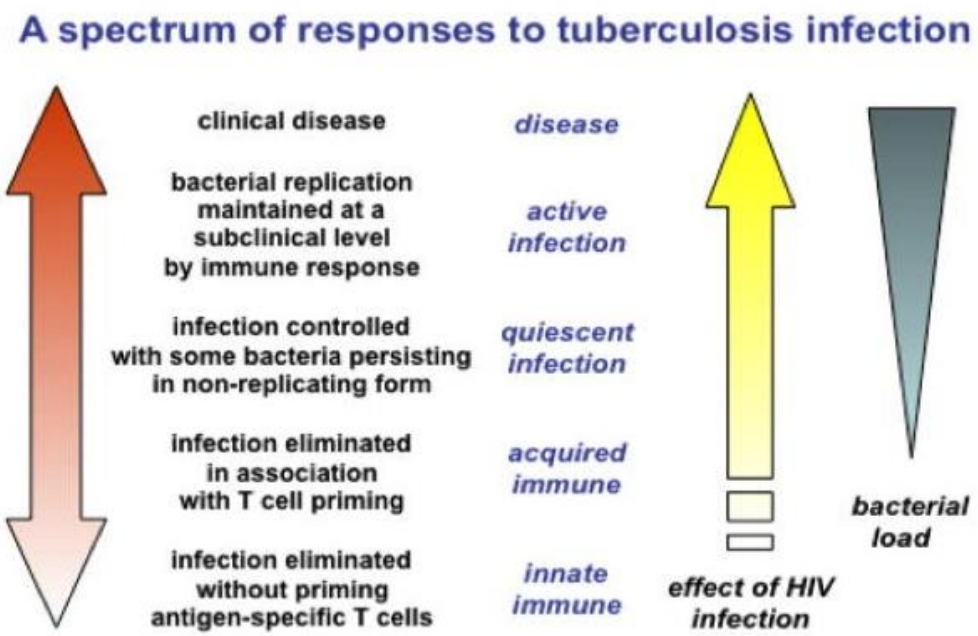

Figure 1. 2: The spectrum of TB from early *Mtb* infection to active TB disease. The diagram depicts a model of the spectrum of responses to TB infection and the effect of HIV infection on TB disease susceptibility. (Image sourced from Barry *et al.* 2009 [40].)

1.2.2. Susceptibility to tuberculosis and factors influencing TB/HIV co-infection

The probability that *Mtb* can be transmitted from one individual to another is dependent on many factors that may be exogenous or endogenous [60]. Individuals with a severe case of pulmonary TB may emit a higher number of infectious droplets by producing droplets at an elevated rate, with the rate of droplet production being affected by the frequency and vigour of coughing in addition to pathology related factors thereby allowing pathogens to escape into the airway [61-63]. A person that has a sputum positive result is able to

infect as many as 10 individuals per annum, with the possibility of each smear positive case in turn leading to two new cases of TB, with at least one being infectious [64]. Although some individuals may be a smear negative, they may also be able to transmit infection with the infection dose of *Mtb* being as little as one to 10 bacilli [65, 66]. The risk of infection is increased when a susceptible individual is within close proximity and for a prolonged duration of time with an infectious individual [60] with household contacts and care givers/healthcare workers being at a higher risk of becoming infected with *Mtb* and the development of active TB [67].

Delays in diagnosing patients as well as delays in initiation of treatment of TB results in an increased prevalence of infectious TB, in turn resulting in the increased probability of onward transmission [68]. Additionally, factors that contribute to delays in treatment are inadequate diagnostics, the behaviour of individuals in seeking healthcare treatment, structural barriers to healthcare access and the timeous diagnosis and treatment initiation within the healthcare system [60].

Environmental factors also play a role in increasing infection by *Mtb*. Closed indoor spaces with limited air ventilation and minimal UV light exposure provide the ideal environment for airborne particles containing *Mtb* bacilli to remain viable, and infectious, thereby increasing the risk of infection [61]. In developing countries, solid fuels are still used for cooking with the firewood or biomass smoke being an independent risk factor for TB disease [69].

Host factors play an important role in an individual's risk of progression to active pulmonary disease after infection. Host related determinants of risk include HIV infection, diabetes, smoking, excessive alcohol use and malnutrition [60] with the degree of infectiousness of the inoculum influencing the likelihood of disease. HIV co-infection is the greatest risk factor for the development of active TB disease [70] and HIV also increases the chances of reactivation of latent TB infection [71]. Malnutrition has been shown to increase the risk of TB due to an impaired immune response and TB disease exacerbates this due to a decreased appetite and changes in metabolic processes [72]. Diabetes has also been shown to increase the risk of active TB as emerging evidence has demonstrated that the immune response to type II diabetes is hyperinflammatory. Smoking is also a cause of increased risk to TB disease, with an additional risk of death in persons with active TB [73]. It is believed that the nicotine in cigarettes impair clearance of mucosal secretions, reduce phagocytic ability of alveolar macrophages and results in a decreased immune response [74-76]. Alcohol has also been recognised as one of the strongest risk factors for TB as it alters the immune system, specifically altering the networks and molecules that play vital roles in cytokine production [77]. Although young children and infants are at a higher risk of rapid progression and may act as sentinel populations for ongoing transmission, they rarely contribute to ongoing transmission as a result of their lower infectiousness [78, 79].

1.3. Antiretroviral therapy (ART) induced immunity

1.3.1. ART induced immunity to intracellular pathogens

HIV is able to enter cells via the CCR5 and CXCR4 chemokine receptors as well as the CD4 receptor, expressed on CD4 T cells, and inducing uptake of the viral particle. Upon uptake the virus undergoes replication thus leading to cell death, including CCR5 expressing CD4 T cells during acute HIV infection, when predominantly memory and effector CD4 T cells are depleted [80]. CCR5 antagonism prevents entry of HIV into the cell by blocking the CCR5 co-receptor on the surface of CD4 T cells, thus preventing viral replication and in turn decreases the number of infected CD4 T cells.

Antiretroviral therapy (ART) is administered to HIV infected persons with the aim of viral load suppression leading to immune restoration. When HIV replication is inhibited, the previously affected CD4 T-helper cells are able to avoid infection by the virus thus allowing their normal functions to be carried out. Immune recovery is a gradual process and the time required for this to occur, varies from person to person and is also dependent upon the current stage of the infection.

Following the initiation of therapy HIV infected persons experience a dramatic increase in numbers of CD4 T-helper cells for the first few months, a slower more gradual increase in subsequent months, eventually reaching a plateau after years of receiving ART. The initial increase in the numbers of CD4 T-helper cells may be attributed to the redistribution of existing cells

from the lymph nodes into the bloodstream. These new cells are produced by clonal expansion of existing cells and consist of memory cells, that have been primed based on previous antigen exposure, and naïve cells, that have the ability to encounter new pathogens and antigens.

In HIV infected persons a better quality of life and improved overall health and survival, by reducing the incidence and severity of opportunistic infection and death, is expected once CD4 cell counts reach high enough numbers. However this can take a long time, especially in HIV infected persons with advanced disease and the numbers of CD4 T-helper cells rarely reach levels observed in HIV uninfected persons [81].

Conversely, HIV infected persons initiating ART may experience Immune Reconstitution Inflammatory Syndrome (IRIS) in which exhibit clinical deterioration despite evidence of viral suppression [81]. This occurs when the immune system begins to recover but additionally responds to an antigen already present with an overwhelming inflammatory response [82, 83]. Interestingly, measurements of cytokines in serum point to the innate immune system as the source of cytokines, as IL-8 and IL-6 cytokines and C-reactive protein (CRP) have been shown to have the highest levels regardless of the underlying pathogen [84-88].

1.3.2. ART induced immunity to Mycobacterium tuberculosis

It has been well established that HIV infected persons are more susceptible to TB than HIV uninfected persons irrespective of their CD4 T cell count [89,

90]. Additionally HIV infected persons with decreasing CD4 T cell counts have an increased susceptibility to TB, and individuals having counts of less than 200 CD4 T cells/μL blood are more susceptible to TB than individuals with more than 500 CD4 T cells/μL blood [91]. HIV infected persons with active TB disease contain fewer CD4 T-helper cells at the site of infection [92] thus resulting in poor containment of *Mtb* bacilli. It is therefore anticipated that the overall decline in the number of CD4 T-helper cells, due to HIV infection, would further result in a decline in the number of *Mtb*-specific CD4 T-helper cells [93, 94]. However HIV infection also affects other cells and further affects the balance of cytokines [22]. This in turn also prevents persons with LTBI from controlling the infection. It has also been shown that the initiation of ART results in a partial recovery of *Mtb*-specific CD4 T-helper cells however despite receiving reconstituting treatment, the numbers and proportions of cells are not comparable to those observed in HIV uninfected persons [95, 96]. ART is the best way to protect HIV infected persons developing TB, despite the fact that it lacks antimicrobial activity. In HIV infected persons the increase in CD4 T cells, due to ART, correlate with a decrease in susceptibility to develop active TB, thus suggesting that these T cells play an important role in protection to TB [97]. Thus the characterisation of *Mtb*-specific CD4 T cells that expand during ART, in HIV infected persons sensitised by *Mtb*, will aid in understanding ART induced protection to TB and aid in the identification of more reliable correlates of protection.

1.3.3. The evolution of ART leading to an increased life expectancy

The year 1995 marked the beginning of a new era in which ART treatment was revolutionised. ART drug regimens were designed to incorporate the inhibitors of two HIV enzymes, namely reverse transcriptase (RT) and protease. The inclusion of two drugs that targeted both enzymes, as opposed to either enzyme, resulted in a significant reduction in viral load (VL) and a corresponding CD4 T cell count increase [98-101]. Collier and colleagues illustrated that the combined effect of several drugs simultaneously increased the effectiveness of ART by drastically reducing viral replication and subsequently increasing CD4 T cell counts [98]. Also supporting these findings were Gulick and colleagues in which the results of this double-blinded study indicated that at least 6 months of treatment with a regimen consisting of 3 drugs (indinavir, zidovudine and lamivudine) resulted in significantly reduced HIV RNA levels compared to HIV RNA levels observed in patients receiving monotherapy or bitherapy. A corresponding increase in CD4 T cell count was also registered, although not the highest in the trial, and this persisted for up to 52 weeks [101]. Lastly, in 1997 Hammer and colleagues conducted a study in which HIV infected persons received either monotherapy (indinavir) or bitherapy, consisting of zidovudine and lamivudine. Results of this study revealed that the proportion of persons receiving bitherapy had a lower chance of progressing to AIDS or death, compared with the proportion of persons receiving monotherapy. Additionally the group of persons receiving monotherapy had a higher mortality rate compared with those persons receiving bitherapy [99]. These studies prompted questions relating to immune restoration and its mechanism.

1.4. Correlates of protection to TB

1.4.1. Vaccines – Past and present

Successful vaccines have been shown to target pathogens against which humoral immunity is sufficient to achieve eradication and protection, however in order to provide sufficient protection and *Mtb* eradication, TB vaccines need to drive the cellular compartment of the immune system [16]. The only vaccine available against TB is Bacille Calmette-Guerin (BCG), which was developed by Albert Calmette and Camille Guerin in 1921, and is routinely administered to infants worldwide in order to provide protection against severe forms of TB [16]. However BCG has been shown to provide minimal protection to pulmonary TB, the transmissible form of TB, in adults [16]. BCG plays a role in protection against *Mtb* by inducing CD4 and CD8 T cell responses [102]. For ideal host protection against TB there is a need for the induction of both the T_H1 and T_H17 responses, however BCG only induces a T_H1 response thus leading to the inferior efficacy of the BCG vaccine [103].

The development of new TB vaccines ought to aim to replace BCG by improving recombinant BCG (rBCG) or by genetically attenuating *Mtb* [16], with rBCG being more effective than the parental BCG due to the introduction of genes that have been lost during *in vitro* attenuation [104]. Horwitz *et al.* [105] and Horwitz and Harth [106] produced the first rBCG, rBCG30, that overexpressed antigen Ag85b and has been shown to provide protection against TB in animals. rBCG30 is able to significantly increase Ag85b-specific T cells that inhibit intracellular mycobacteria as compared

with parental BCG [107]. VPM1002, the second rBCG vaccine candidate [108] is formed using the gene encoding Listeriolysin (Hly) inserted into the genome of BCG to form rBCGUre:CHly, has been shown to provide higher protection against *Mtb* challenge through aerosol infection in mice. rBCGUre:CHly is currently in clinical trials due to its enhanced protection against TB [109].

DNA vaccines express the *Mtb*-specific antigens Ag85A, ESAT-6, Ag85B, PstS-3, MPT-64 and the 65kDa heat-shock protein. In infected mice, these proteins were found to inhibit growth of *Mtb* [110]. Ad5Ag85A is a viral vectored adenovirus serotype 5 vector vaccine expressing Ag85A and has been through Phase 1 clinical trials without any serious vaccine related adverse effects. Ad5Ag85A had immunogenicity in BCG naïve and previously BCG vaccinated groups but a more effectively boosted CD4 and CD8 T cell immunity was demonstrated in the group that had previously received the BCG vaccine compared to the group that did not receive BCG [111]. The MVA85A vaccine was the first new TB vaccine to enter clinical efficacy trials and was a strong contender by eliciting T_H1 responses [112]. However MVA85A failed to induce any protection against TB infection in phase IIb trials in both infants and HIV-infected adults [113]. Other viral vector vaccines are the MVA58A combined with Crucell Ad35 vaccine (Phase 1 trial) and a recombinant influenza vaccine (TB/FLU-04L) that have completed phase 1 trials and have a phase IIa trial planned [114].

In 2013, MTBVAC entered phase 1 of clinical trials and is the only live-attenuated *Mtb* vaccine that has entered phase I trials [115]. MTBVAC has been able to replicate the same safety profiles as BCG, and has also been shown to provide a superior form of protection [116]. Recombinant protein-based vaccines develop less reactogenicity, and are considered more potent, safe and are better characterised [14] therefore, the correct identification of *Mtb*-specific antigens secreted in culture fluid is important in the establishment of protective immune response against TB, and for better vaccine design [104].

Adjuvants are compounds and molecules, or macromolecular complexes, that are able to boost the potency and effective duration of specific immunological responses to antigens [117]. Subunit vaccines that contain adjuvants are therefore able to enhance immune responses. The adjuvants that are currently safe and approved for use in humans are Aluminum salts, AS03/04 and MF59 which promote humoral or T_H2 immunity as opposed to T_H1 immunity [118]. Hybrid 1 + IC31 is a subunit adjuvant vaccine that is a hybrid of ESAT6 and Ag85B antigen with IC31, and this vaccine had showed durable T_H1 responses in patients infected with HIV [119]. Hybrid 4 + IC31 vaccine contains the *Mtb* antigens Ag85B and TB10.4 in addition to IC31. In order to determine the immunogenicity and safety of this vaccine, a 3 arm, phase IIa study was announced by Aeras in 2014 [120]. The Hybrid 4 + IC31 vaccine has been shown to enhance protection to *Mtb* by lengthening and boosting immunity induced by BCG [121]. Other adjuvant vaccines include Hybrid 56 + IC31 which has been shown to not only contain the latent stage

of TB, but has also been able to control late stage TB infection. CD4 T cell responses that were specific to M72/AS01 indicated immunogenicity of the vaccine [122, 123].

Therapeutic vaccines are targeted to prevent latent infection or reduce the need of chemotherapy [124]. RUTI is composed of detoxified, fragmented *Mtb* cells delivered in liposomes. RUTI was able to control the latent form of TB infection in mice and guinea-pigs and induce a T_H1/T_H2 polyantigenic response following a short period of chemotherapy [125]. A phase I clinical trial of RUTI vaccine carried out in Spain in persons without a TB infection and prior BCG vaccination, indicated that four different doses of RUTI that were used were tolerable, however moderate pain was noted with higher doses [126]. There are approximately 16 vaccine candidates at various stages of clinical trials currently being carried out (Figure 1.3), however further research needs to be done in order to correctly identify and select antigens that may serve as novel and more effective vaccines against TB [14].

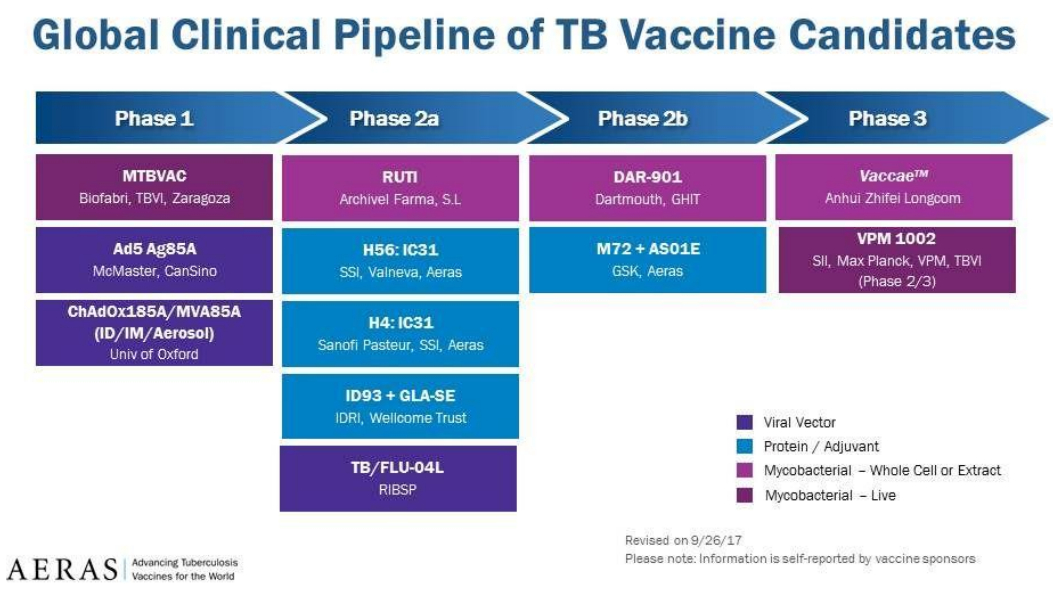

Figure 1. 3: Current TB vaccine candidates in the vaccine pipeline. The vaccine candidates currently in the pipeline are viral vector based vaccines (purple), protein/adjuvant based vaccines (blue), *Mycobacterial* whole cell or extract based vaccines (magenta) or live *Mycobacterial* based vaccines (dark magenta). (Image sourced from http://www.aeras.org/pages/global-portfolio).

1.4.2. Role of T-cells in protection against TB

CD4 T cells play a vital role in protection to TB disease via the production of pro-inflammatory cytokines. Both human studies and animal models support the notion that CD4 T cells playing a central role in the control of *Mtb*. In 1999 Caruso and colleagues demonstrated that mice lacking CD4 T cells displayed increased susceptibility to *Mtb* challenge compared to wild-type mice [127]. Additionally, mice lacking MHC class II molecules were more susceptible to *Mtb* infection than CD4 knockout mice [127]. The importance

of CD4 T cells in HIV infected persons was summarised by Lawn *et al.* highlighting that HIV infected persons with low CD4 counts had an increased susceptibility to TB disease [91]. CD4 T cells are able to further differentiate into T helper cell subsets depending on the cytokine stimulus received, the prominent transcription factor and the ability to produce certain cytokines.

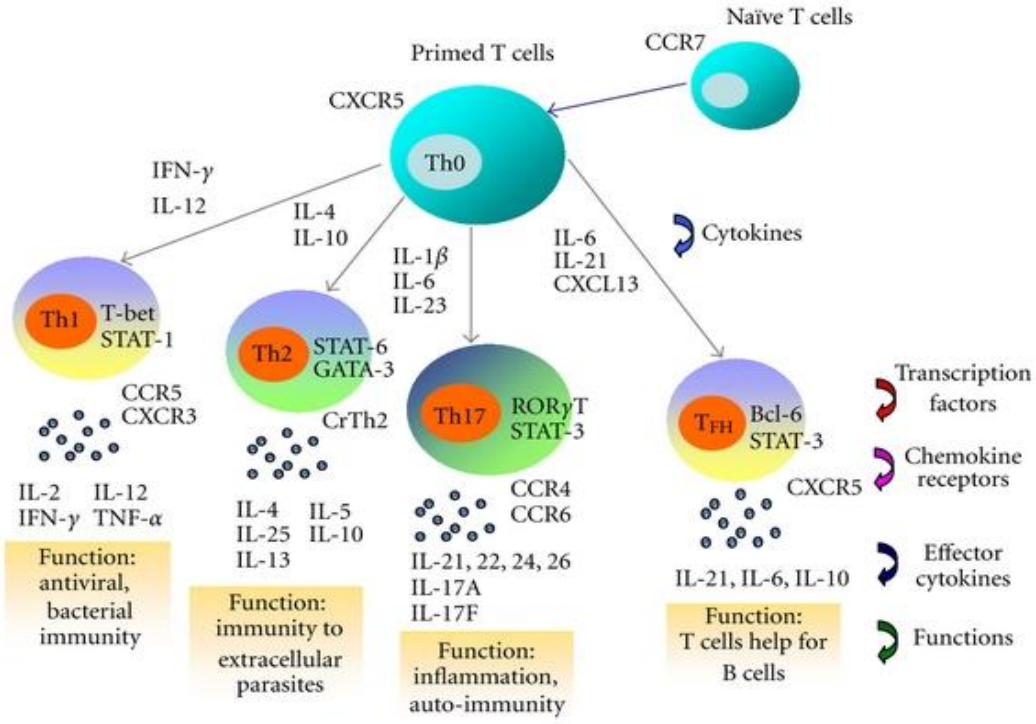

Figure 1. 4: Possible differentiation pathways for a naïve T cell, dependent upon the cytokine environment in which the naïve T cell is activated in. Naïve T cells, once primed by antigen, have the ability to differentiate into several different types of T helper cells. The master transcription factors of each subset and characteristic cytokines are also pictured. (Image sourced from Yu, Shui-Lian *et al.* 2012 [128]).

T_H1 cells have the ability to produce IFN-γ which, in conjunction with TNF-α, acts synergistically to activate macrophages. This was illustrated in the early 1990's, by Flesch and Kaufmann [129], in a study in which it was shown that IFN-γ stimulates anti-mycobacterial macrophage functions *in vitro*. Additionally it was shown that blocking the function of TNF-α led to reduced macrophage activation and that both cytokines were required in order for optimal macrophage activation [129, 130] as opposed to a single cytokine. It was further illustrated, by Flynn and colleagues, that gene knockout mice lacking the TNFR1 gene [131] exhibited impaired granuloma formation thus leading to exacerbated tuberculosis disease. In conjunction with IFN-γ, TNF-α is essential for the formation of granulomas during TB infection. This was illustrated by Algood and colleagues by neutralising TNF-α with antibodies in mice [132]. Mice were administered anti-TNF-α antibodies 4-6 weeks post infection, at which point mice were reinfected leading to their deaths. Of note was the loss of granuloma structure and the cell infiltrations throughout the lungs [132]. In humans Keane *et al*. found that the reported frequency of TB associated with anti-TNF therapy was higher compared to the frequencies of other opportunistic infections associated with anti-TNF therapy, thus indicating that anti-TNF therapy may re-activate TB in LTBI persons [133].

Sullivan and colleagues showed that mice lacking the T-bet transcription factor produced lower levels of IFN-γ [134]. T-bet is the master transcriptional regulator driving the differentiation of T cells to T_H1, IFN-γ producing cells. Thus, lower levels of IFN-γ results in a greater bacterial burden and ultimately death due to *Mtb* infection. Additionally Green *et al.*

(2013) illustrated the importance of IFN-γ produced by CD4 T cells, by showing that IFN-γ produced by cells other than CD4 T cells were insufficient to control *Mtb* infection in mice. Furthermore IFN-γ deficient mice had high bacterial loads, following *Mtb* challenge, and extensive necrosis leading to death [135, 136]. A study by Cooper and colleagues illustrated the importance of IL-12 in the T_H1 pathway. Since IL-12 is responsible for the T_H1 response, IL-12p40 deficient mice exhibited lower than usual IFN-γ levels indicating the importance of IL-12 in this pathway [137]. Similarly in humans, persons with IFN-γ autoantibodies exhibited an increased susceptibility to *Mtb* infection compared to persons with functional IFN-γ [138]. Also, humans with genetic defects in the production T_H1 of cytokines or transcription pathways display increased susceptibility to mycobacterial infections, compared to persons without T_H1 genetic defects [139, 140].

T_H17 cells are defined by their ability to produce IL-17. Once stimulated, T_H17 cells produce the cytokine, IL-17 that is responsible for attracting and activating neutrophils. T_H17 cells were also thought to produce IL-22, an equally important cytokine in TB protection, however recent evidence has indicated that IL-22 is produced by a distinct subset of T cells [141]. Additionally, T_H17 cells may be responsible for the recruitment of monocytes and T_H1 cells to the site of granuloma formation, and thus contributing to protection against *Mtb* [142, 143]. Multiple murine studies have illustrated the importance of IL-17 in TB – IL-17 deficient mice have been shown to have poor control of *Mtb* as a result of defective granuloma formation [142, 144]. In a study conducted by Scriba and colleagues, *Mtb*-exposed healthy

persons were found to have a high proportion of T_H17 CD4 *Mtb*-specific T cells in peripheral blood [141]. Sutherland and colleagues reported higher *Mtb*-specific IL-17 responses in latently infected persons compared to persons with active TB disease [96]. Together these data suggest an important role for IL-17 in TB protection.

1.4.3. Correlates of protection

The need to identify correlates of protection to TB disease is imperative as the potential use of these correlates is critical for the development and evaluation of an effective TB vaccine [145]. Many potential TB vaccines have been evaluated without a reliable correlate of protection however it is becoming increasingly complex to evaluate TB vaccines. The identification of such a correlate further has the ability to potentially determine early indication of efficacy, identify protective antigens and vaccine candidates, aid in optimising vaccine dose, provide insight regarding the schedule of immunisations and provide preliminary evidence of immunogenicity [145]. In the past *Mtb*-specific IFN-γ was thought to be good indicator of vaccine efficacy and was therefore thought to be a promising correlate of protection. This was supported by evidence obtained from mouse models that demonstrated the important role of IFN-γ in protection against *Mtb* infection in mice with IFN-γ disrupted genes [135, 136]. However, the use of *Mtb*-specific IFN-γ as a correlate of protection in humans was confounded as Hirsch *et al.* observed that individuals with active and advanced TB disease display decreased levels of *Mtb*-specific IFN-γ [146, 147] and it was unclear whether the decreased *Mtb*-specific IFN-γ was the cause or the effect of the

TB disease. Secondly, increased *Mtb*-specific IFN-γ was observed in persons with TST conversion [148, 149]. However recent evidence points to the fact that *Mtb*-specific IFN-γ alone may not be a suitable and reliable correlate of protection [150]. Two studies by Kagina *et al.* and Mittrüker *et al.* demonstrate that *Mtb*-specific IFN-γ from CD4 T cells does not correlate with protection against TB following BCG vaccination [151, 152].

Recently there has been mounting evidence suggesting that polyfunctional T cells have shown greater promise as correlates of protection to TB disease compared to *Mtb*-specific IFN-γ monofunctional T cells [153]. Polyfunctional T cells possess the ability to produce three or more cytokines, simultaneously. T_H1 polyfunctional T cells produce IFN-γ, TNF-α and IL-2 cytokines simultaneously. In mice, a high proportion of polyfunctional CD4 T cells correlate with protection against *Mtb* infection induced by vaccination [154, 155]. In humans, BCG-immunized infants generated multifunctional T cells however the polyfunctional profile observed did not correlate with protection mediated by BCG vaccination [151]. Hawkridge and colleagues also showed that the cytokine profile in BCG vaccinated individuals, administered a MVA85A booster dose, displayed a significantly increased frequency of polyfunctional T cells [156]. Together these findings suggest that polyfunctional T cells may play a pivotal role in protection against TB, however the function of polyfunctional T cells in TB disease still remains unclear [157].

Lastly it has been demonstrated that IFN-γ-independent mechanisms, mediated by CD4 T cells, may also play a role in protection against TB disease [153, 158]. This was partly illustrated by Gallegos and colleagues by adoptively transferring antigen specific CD4 effector T cells and illustrating the ability of these cells to confer protection to naïve hosts. This protection detailed an IFN-γ/TNF-α independent pathway [159]. Similarly, Wozniak and colleagues demonstrated that the adoptive transfer of BCG-specific T_H17 cells mediated protection to *Mtb*, in IFN-γ deficient mice [160]. The above studies indicate that perhaps IFN-γ is required for skewing CD4 T cell differentiation towards the T_H1 effector phenotype, after which IFN-γ may be dispensable [153].

1.4.4. Rationale for study

TB is the leading bacterial cause of death in Africa [8] and while there has been progress in diagnosing *Mtb* sensitisation, applicable to low incidence areas, and a number of new antitubercular drugs that have recently entered clinical trials, there remains an urgent need to understand protective immunity to tuberculosis in order to define correlates of protection that will help better design vaccines and host directed therapies.

HIV infected persons are 20-30 times more likely to develop TB than HIV uninfected persons who have a 10% chance of developing active TB in their lifetime [97]. HIV-1 co-infection is the leading cause of susceptibility to tuberculosis, and ART is the most effective way to reduce the risk of TB in HIV-1 co-infected people, reducing tuberculosis incidence by up to 67%

[161]. We hypothesized that longitudinal analysis of a highly susceptible group (HIV infected people with low CD4 counts), who undergo immune-reconstituting ART, thus become less susceptible to TB, is a novel approach to understanding protective mechanisms in human TB. Therefore, in a previous study conducted between 2005-2007 we longitudinally followed up 19 HIV infected adults with *Mtb* sensitisation over 48 weeks of ART and showed that the strongest correlate of increased ART mediated immunity was the expansion of central memory CD4 T cells [162]. Two recent studies addressing the mechanisms of action of promising vaccine candidates, using animal models have highlighted the importance of central memory T cells in protection against TB: the subunit vaccine containing Ag85B and ESAT-6 boosts $CD4^{+}KLRG1^{-}$ IL-2-secreting central memory T cells [163], and the recombinant BCG Δ*ureC::hly* vaccine mediated protection is also based on the expansion of central memory CD4 T cells that are CXCR5+CCR7+ and express low levels of the transcription factors T-bet and Bcl-6 [164]. Thus, the characterisation of *Mtb*-specific CD4 memory T cells that expand during 6 months of ART, in HIV infected persons that are sensitised by *Mtb*, will contribute to a better understanding of T cell mediated protection to *Mtb*. Therefore this study will contribute to the identification of correlates of protection that will help better design vaccines and host directed therapies.

1.5. Hypotheses

Hypothesis 1: Central memory T cells will significantly expand during 6 months of ART in HIV infected, *Mtb* sensitised individuals.

Hypothesis 2: Soluble factors associated with central memory T cell function will be detectable in plasma and their concentrations will increase, or decrease, over time on ART in HIV infected *Mtb* sensitised individuals.

Hypothesis 3: The transcriptomic signature associated with central memory T cells will increase over time in HIV infected persons on ART, while the signature associated with effector memory T cells will decrease.

1.6. Aims

The overall aim of this thesis was to define immunological markers of protective immune reconstitution in a cohort of HIV infected persons, sensitised by *Mtb*.

Specific aim 1: To investigate by FACS analysis the expansion of central memory T cell subset in peripheral blood mononuclear cells.

Specific aim 2: To evaluate soluble markers indicative of central memory T cell function in plasma from HIV infected, *Mtb* sensitised individuals on ART longitudinally using ELISA and multiplex assays.

Specific aim 3: To compare gene transcriptional signatures characteristic of central memory and effector memory T cells in 50 HIV infected persons starting ART and sampled at day 0, 1 month, 3 months and 6 months of ART.

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Chapter 2: Materials and Methods

Disclaimer:

Tissue culture procedures were performed under sterile conditions and using strict aseptic techniques. All experiments with unfixed cells were conducted in a class II bio-safety cabinet, in a bio-safety two-plus laboratory facility. Resultant waste generated was disposed of according to the University of Cape Town's bio-hazardous waste disposal standard operating procedure. Reagents were used and stored per manufacturer's instructions, unless otherwise stated. Lastly, laboratory equipment was serviced regularly according to the manufacturer's instructions, and a daily temperature monitoring system was in place for all necessary equipment.

2.1. Study Population and Sample Collection

Ethical approval for the study was obtained from the Faculty of Health Sciences Human Research Ethics Committee, University of Cape Town, HREC245/2009. Fifty antiretroviral (ART) naïve, HIV infected individuals were recruited from the Ubuntu Clinic in Khayelitsha, Cape Town during 2011. Individuals were sampled at baseline and after one, three and six months of receiving ART. These individuals had CD4 counts less than 250 cells/µL, as per the South African National Guidelines at the time. Blood was drawn for the Quantiferon® Gold In-Tube assay (QFT), Enzyme-Linked ImmunoSpot (ELISpot) assay, RNA extraction, Peripheral Blood Mononuclear Cell (PBMC) separation for storage as well as CD4 count and

viral load tests. Sputum was also collected to determine *Mtb* culture positivity. The control group, consisting of fifty-five HIV uninfected individuals, was recruited from the same site throughout 2012. These individuals were sampled once. Blood was drawn for the QFT assay, ELISpot assay, PBMC separation for storage and CD4 count determination. Although 55 HIV infected persons and 55 HIV uninfected persons were recruited for this study, not all patient samples were included for each study aim. This was due to: 1) incomplete sample sets from timepoints being available, 2) availability of samples varied for each aim as some samples were previously used for optimisation experiments, 3) poor sample quality prevented the inclusion of samples in certain experiments and 4) limited sample quantity prevented the inclusion of samples in experiments. The number of patients, in each cohort, included for each experimental aim will be noted in the patients characteristics sub-chapter in the chapters of this thesis.

2.2. Exclusion criteria

At the time of recruitment, the following exclusion criteria applied: evidence or suspicion of active tuberculosis (TB), current INH or TB chemotherapy, grade III-IV peripheral neuropathy, pregnancy, abnormal liver function, age <18 years, development of active TB during longitudinal follow up as determined by sputum culture at 1, 3 or 6 months of receiving ART. Six patients cultured positive for: *Mtb complex, M. fortuitum* and *Mycobacterium* Other than Tuberculosis (MOTT) these patients were referred to the TB services for treatment and sample collection was terminated. Samples

leading up to the time of TB diagnosis were stored and these samples were analysed separately.

2.3. Separation of Peripheral Blood Mononuclear Cells

12ml of blood was collected from HIV infected individuals, and 18ml from HIV uninfected individuals, in Sodium Heparin tubes. PBMC were isolated using the Ficoll-Hypaque (Sigma Aldrich) density gradient centrifugation method. Ficoll-Hypaque consists of ficoll which is a carbohydrate polymer that has a high density. The density of ficoll is higher than that of PBMC thus resulting in a layer of PBMC forming above the ficoll, following centrifugation, and thereby facilitating the separation of PBMC from plasma, platelets, and red blood cells. Briefly, the blood was diluted with 1x Phosphate Buffer Saline (PBS) (Sigma Aldrich) in a 1 to 1 ratio. The diluted blood was then carefully layered over 10ml of Ficoll-Hypaque and centrifuged for 20 minutes at a speed of 700xg (without brake) in order to generate a gradient of cells. The layer of PBMC was then removed using a 3ml pasteur pipette and transferred to a 15ml sterilin containing 5ml of RPMI. The cells were washed with RPMI followed by counting. 20µL of cells were added to 20µL 0.4% Trypan Blue and counted using a disposable counting chamber [1]. Viable cells were cells that were impermeable to the dye. Cells were enumerated according to the following formula:

$$Count\ per\ ml = \frac{total\ no.of\ viable\ cells\ counted}{no.of\ 4x4\ grids}\ x\ 10^4\ x\ sample\ dilution$$

The cells were washed again and frozen. For the freezing procedure, 0.4ml RMPI was used to resuspend the cell pellet after which 0.5ml freezing media was added in a drop wise fashion while rotating the tube containing cells. One to ten million cells were stored in a cryovial in a chilled Mr. Frosty at -80°C overnight, thereafter the cryovials were transferred to liquid nitrogen the following day.

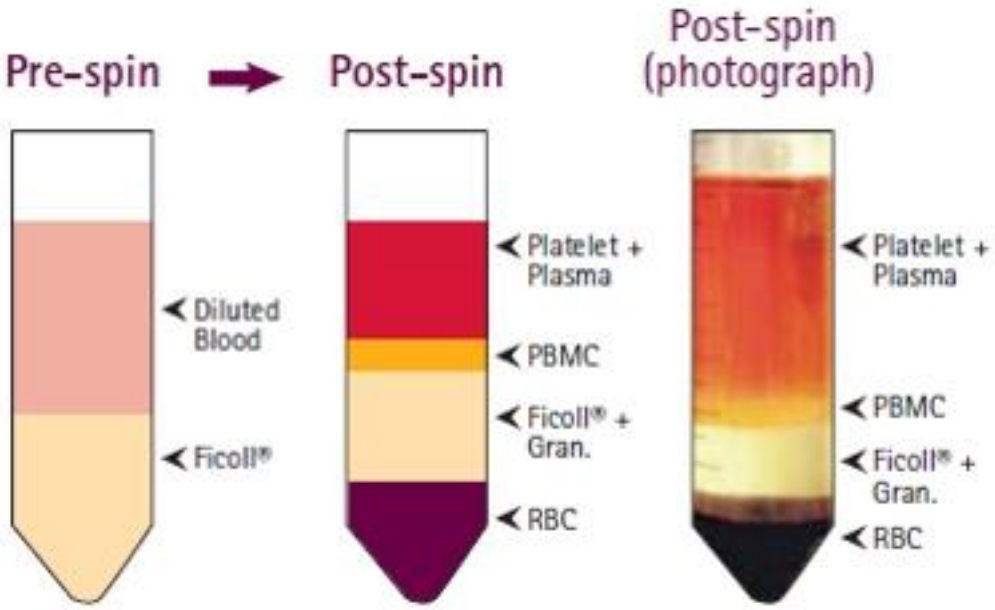

Figure 2. 1: Diagrammatic representation of blood components pre- and post Ficoll-Hypaque density centrifugation (image sourced from www.suggest-keywords.com/Zmljb2xsLWh5cGFxdWUgcHJvdG9jb2w/).

2.4. Determination of Mtb Sensitisation by Quantiferon Gold In-tube assay

The Quantiferon® Gold In-Tube (Cellestis Inc., Valencia, CA) assay was performed, according to the manufacturer's instructions, on all samples collected. One ml of whole blood was collected per tube using three tubes, labelled Tuberculosis (TB) Antigen, Nil and Mitogen, per individual and was incubated for 16-20 hours. The TB antigen labelled tube contained *Mtb* specific antigens; ESAT-6, CFP-10 and TB7.7 (peptide p38-55), while the mitogen contained phytohaemagglutinin-P, a polyclonal T cell activator, and the Nil tube served as a negative, unstimulated control. 150µL of plasma was removed to determine the concentration of Interferon gamma (IFN-γ) released from the cells, by ELISA (Cellestis Inc., Valencia CA). Prior to commencement of the assay, kit solutions were reconstituted according to the manufacturer's instructions. Working strength conjugate was prepared by adding 60µL conjugate concentration to 6mL Green Diluent for each plate. The kit standard was reconstituted with distilled water to 8.0 International Units per mL (IU/mL) and mixed until complete solubilisation. Four standard tubes and 4 dilutions were prepared by serially diluting 150µL of the reconstituted kit standard to tubes S2 and S3 respectively, containing 150µL Green Diluent. Freeze-dried conjugate was reconstituted with 0.3mL distilled water and mixed gently until complete solubilisation. Lastly, the wash buffer was prepared by adding 1 part of 20X wash buffer to 19 parts of distilled water and mixed thoroughly. Following reagent preparation, 50µL of working strength conjugate was added to each well in a 96 well plate, followed by 50µL of sample plasma from QFT tubes. 50µL of each standard, of known

concentration, was added to wells and were run in triplicate. The plate was sealed and allowed to mix for one minute using a plate shaker (Barnstead Labline). The plate was then covered in foil and incubated at room temperature for 2 hours. Following incubation, the wells were washed with 400µL wash buffer 6 times with soaking for 5 seconds between each wash. The plate was tapped faced down on a bed of absorbent paper to remove residual wash buffer. 100µL of enzyme substrate solution was added to each well and the plate was allowed to mix on the plate shaker for 1 minute, followed by incubation for 30 minutes at room temperature. After exactly 30 minutes, 50µL Enzyme Stop solution was added to all wells, thereafter the plate was read using the Bio-Rad iMark Microplate (Bio-Rad Laboratories) ELISA plate reader at 450nm with 655nm reference filter. The concentration of IFN-γ was measured in IU/ml and Ag-Nil readings of 0.35 IU/ml and above were regarded as a positive response. The remaining supernatant from the Quantiferon tubes was stored at -80°C and subsequently used for multiplex analysis.

2.5. Determination of Mtb Sensitisation by an in house Enzyme-Linked ImmunoSpot assay

The measurement of IFN-γ by ELISpot was performed as described previously [2] [3] [4] using the human Interferon-gamma ELISpot^{PRO} kit (MABTECH, Nacka Strand, Sweden). Pre-coated 96 well plates (Mabtech) were washed 4 times with sterile PBS and blocked with R10 media (RPMI supplemented with 10% FCS, 0.01mg/ml of penicillin/streptomycin and

0.25µg/ml of fungin) for 30 minutes at room temperature. Following incubation, the R10 media was removed and 250 000 PBMC were added into wells. Antigenic stimuli consisted of a pool of ESAT6 (Rv3875, 5 µg/mL) and CFP10 (Rv3874, 5 µg/mL) peptides. As positive control, anti-CD3 mAb CD3-2 at 100ng/mL final concentration was used, while unstimulated wells were the negative control. Following incubation for 18 hours at 37 °C with 5% CO_2, the plates were washed 5 times with plain PBS.

100µL of conjugated secondary antibody was added to each well and the plate was allowed to incubate at room temperature for 2 hours, after which 100µL of filtered BCIP/NBT-plus substrate solution was added to each well and developed until spots emerged, at which point the plate was washed with distilled water and allowed to dry. The number of IFN-γ producing cells were enumerated by an Immunospot Series 3B Analyser (Cellular Technology, Cleveland, OH, USA) and recorded as spot forming cells per million PBMC (SFC/million). ELISpot plates were retained for visual inspection and confirmation in the case of anomaly. Readings of 30 SFC/million and above were regarded as a positive response.

2.6. Determination of soluble markers in QFT supernatants by Multiplex assays

2.6.1. Bio-Rad Pro Human Cytokine 27-plex Panel

Frozen QFT plasma was thawed and used for multiplex analysis. 25µL of sample was used at a 1:3 dilution to determine the concentration of 27

analytes: Interleukin (IL) IL-1 beta, IL-1 Receptor Antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6 IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, EOTAXIN, basic Fibroblast Growth Factor (FGF), Granulocyte Colony-stimulating Factor (G-CSF), Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF), IFN-γ, Interferon gamma-induced Protein 10 (IP-10), Membrane Co-factor Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 alpha (MIP-1α), Macrophage Inflammatory Protein-1 beta (MIP-1β), Regulated upon Activation Normal T-cell Expressed and Presumably Secreted (RANTES), Platelet Derived Growth Factor (PDGF), Tumor Necrosis Factor alpha (TNF-α) and Vascular Endothelial Growth Factor (VEGF) by using a Bio-Plex Pro Human Cytokine 27-plex kit (Bio-Rad Laboratories, Hercules, CA).

The lyophilized standard was reconstituted with 500µL of the standard diluent provided and incubated for 30 minutes at room temperature. After the incubation, a dilution series was prepared in order to generate an eight-point standard curve with a four-fold dilution between each point. 128µL of the reconstituted standard was added to 72µL of standard diluent in tube 1 and vortexed briefly. Thereafter 50µL of the standard in tube 1 was added to a second tube containing 150µL of standard diluent, and vortexed briefly. This was repeated for six tubes. A ninth tube contained 150µL of standard diluent only and served as a blank.

25µL of each sample was diluted with 75µL of the included sample diluent and kept on ice. Beads and antibodies were vortexed briefly and re-suspended in the kit-included assay buffer and antibody diluent respectively. 50µL of magnetic beads pre-coated with antibodies to the above mentioned 27 analytes were added to all wells in a 96-well plate, and washed twice with

wash buffer using the Bio-Rad Bio-Plex Pro wash station (Bio-Rad Laboratories). Fifty µL of standards were added in duplicate and 50µL of diluted QFT supernatant was added to the remaining wells. The plate was allowed to shake, at room temperature, at ± 850 rpm for 30 minutes to facilitate sample binding to the beads. The plate was washed 3 times with wash buffer, after which 25µL of conjugated antibody was added to each well on the plate and the plate was agitated at room temperature, at ± 850 rpm, for 30 minutes. After 3 washes 50µL of Streptavidin-PE was added to each well on the plate and shaken at room temperature, at ± 850 rpm for 10 minutes. Following incubation, the plate was washed 3 times with wash buffer, the beads were re-suspended with 125µL assay buffer and shaken for five minutes.

The samples were acquired on a Bio-Plex 200 system with Luminex xMap Technology (Bio-Rad Laboratories, Hercules, CA) using Bio-Plex Manager Software (version 6.1). Concentrations that were reported to be below the limit of detection were assigned the lowest value of the corresponding analyte standard.

2.6.2. MERCK Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel – Premixed 23 Plex

Frozen QFT plasma was thawed and used for multiplex analysis. 25µL of sample was used at a 1:3 dilution to determine the concentration of 23 analytes: IL-12p40, IL-13, IL-15, IL-17, IL-22, IL-23, IL-1Ra, IL-1α, IL-9, IL-1β, lL-2, IL-4, IL-6, FGF-2, G-CSF, Interferon alpha 2 (IFN-α), IFN-γ, lP-10,

MCP-1, MIP-1α, MIP-1β, TNF-α and VEGF using a MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (Merck Millipore). The lyophilized standard was reconstituted with 250µL of distilled water. After a ten-minute incubation at room temperature, a dilution series was prepared in order to generate a six-point standard curve with a five-fold dilution between each point. 50µL of the reconstituted standard was added to 200µL of supplied assay buffer in tube 1 and vortexed briefly. Thereafter 50µL of the standard in tube 1 was added to a second tube containing 200µL of assay buffer, and vortexed briefly. This was repeated for four tubes. A seventh tube contained 200µL of assay buffer only and served as a blank. Kit-supplied quality controls 1 and 2 were reconstituted with 250µL distilled water, inverted several times to mix and allowed to sit for 10 minutes prior to use.

25µL of each sample was diluted with 75µL of the serum matrix reagent and kept on ice. Beads were vortexed briefly and re-suspended in the kit-included bead diluent. Flat bottom plates were pre-wet by adding 200µL of wash buffer into each plate well, sealing the plate, and incubating on an orbital shaker for 10 minutes at room temperature. Following the brief incubation, the wash buffer was removed from the plate, and the plate was inverted and tapped onto absorbent tissue several times to remove residual amounts of wash buffer. 25µL of each standard, control and blank was added to respective wells, while 25µL of assay buffer was added to the remaining wells allocated to samples. 25µL of kit-supplied matrix solution was added to wells containing standards, blanks, and controls and 25µL of diluted sample was added to remaining wells allocated to samples. After briefly vortexing the

beads vigorously, 15µL was added to all wells. The plate was sealed, covered in foil, and incubated with agitation (at ± 850 rpm) overnight at 4°C. Following incubation, the plate was washed twice with wash buffer using the magnetic plate washer. 15µL of detection antibody was added to all wells, and the plate was allowed to shake at room temperature, at ± 850 rpm, for an hour. 15µL of Streptavidin-PE was added to each well after incubation and the plate was allowed to shake at room temperature, at ± 850 rpm, for 30 minutes. After incubation the plate was washed twice, 150µL of sheath fluid was added to each well, and placed on the orbital shaker for five minutes. The samples were acquired on a Bio-Plex 200 system with Luminex xMap Technology (Bio-Rad Laboratories, Hercules, CA) using Bio-Plex Manager Software (version 6.1). Concentrations that were reported to be below the limit of detection were assigned the lowest value of the corresponding analyte standard.

2.6.3. Statistical analysis

HIV infected individuals were grouped based on their day 0 *Mtb* sensitisation status. Individuals were classified as *Mtb* sensitised if they had tested positive in the QFT assay and/or the ELISpot assay at at least one timepoint. Six HIV infected individuals were excluded from the analysis due to a positive TB culture test during follow up. We defined the response of the analyte as the concentration of the analyte in the nil tube plasma subtracted from the concentration of the analyte in the *Mtb* antigen (Ag) tube plasma (Ag-Nil). Resulting negative values were assigned a value of zero. Statistical

analysis was conducted using individuals that had data for all time points. The results from the HIV infected group were analysed longitudinally and a cross-sectional comparison at six months of ART was also performed with HIV uninfected individuals. Statistical analysis was performed using GraphPad Prism Version 6.0 for Mac, after consultation with a statistician. The Normality of data was determined using the D' Agostino and Pearson normality test. Normally distributed data was analysed using Analysis of Variance (ANOVA) with a Dunnett's post-test for multiple comparisons or paired t test. Paired data that was not normally distributed was analysed using the Kruskal Wallis test with a Dunnett's post-test for multiple comparisons. Unpaired data that was not normally distributed was analysed using the Mann-Whitney U test. Correlations were investigated by the non-parametric Spearman rank correlation. A p-value of less than 0.05 was considered statistically significant.

2.7. RNA Isolation from Tempus^{TM} tubes

3ml blood was collected from HIV infected individuals in Tempus^{TM} Blood RNA tubes and stored at -20°C until isolation. This procedure was performed in a Biosafety Class II Hood (BSC). Upon isolation, Tempus^{TM} tubes were left to thaw overnight at room temperature in a BSC. The following day, the thawed contents of the tube was transferred to a 50ml sterilin, 3ml of 1X PBS (Sigma Aldrich) was added and vortexed vigorously at maximum speed, for 30 seconds. Tubes were centrifuged at 4°C at 3000xg for 30 minutes after which the supernatant was discarded into biocide. The tube, containing the

pellet, was inverted on absorbent paper towels for 1-2 minutes allowing the removal of remaining supernatant. 400µL of RNA Purification Resuspension Solution was added to each tube containing the RNA pellet and vortexed briefly to resuspend the RNA. Tubes were kept on ice while the filtration membrane was being prepared. Filtration membranes were labelled and placed in a collection eppendorf tube. 100µL of RNA Purification Wash Solution 1 was added to each filtration membrane to pre-wet the filter, after which the resuspended RNA was transferred to the filter. The following steps were then performed in a designated RNA-free room to avoid RNAse contamination. RNAseZAp (Sigma Aldrich) was periodically sprayed on tubes and all surfaces prior to the following steps. The resuspended RNA was centrifuged at 16 000xg for 30 seconds, the liquid waste discarded and washed again with 500µL of RNA Purification Wash Solution 1 in the same filtration membrane containing tube. After the second wash, the waste was discarded and the filtration membrane inserted into a newly labelled collection eppendorf tube. 500µL RNA Purification Wash Solution 2 was added to each tube and centrifuged at 16 000xg for 1 minute. DNAse treatment was performed, to remove contaminating DNA, by adding 100µL AbsoluteRNA wash to each filtration membrane and incubating tubes at room temperature for 15 minutes. Following the DNAse treatment, 500µL RNA Purification Wash Solution 2 was added to each filtration membrane and incubated for 5 minutes at room temperature, after which tubes were centrifuged at 16 000xg for 30 seconds. An additional wash using RNA Purification Wash Solution 2 was performed, followed by spinning the dry filtration membrane once to remove all wash solutions. The filtration

membrane, containing the bound RNA, was then transferred to a newly labelled collection eppendorf for the elution step. 100µL Nucleic Acid Purification Elution Solution was added to each filtration membrane and incubated at 70°C in a water bath for 2 minutes, followed by a spin at 16000xg for 30 seconds. The collected RNA in the eppendorf was then added back into the filtration membrane and centrifuged at a maximum speed of 18 000xg for 2 minutes to ensure maximum elution of the bound RNA. After centrifuging, 90µL of the eluted RNA was transferred to a newly labelled eppendorf tube and subsequently stored at -80°C. An additional 2µL was transferred to a Polymerase Chain Reaction (PCR) tube for Nanodrop quantification.

2.8. Determination of RNA Quality and Quantity by Nanodrop

Isolated RNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo Scientific). The Nanodrop 2000 makes use of spectrophotometry to measure the amount of light absorbed by nucleic acids and proteins. Nucleic acids absorb light at 260nm therefore the 260/280 ratio was used to assess the purity of the RNA. A ratio of between 1.8 and 2.0 was accepted as pure RNA. The 260/230 ratio was used to assess the protein contamination since contaminants absorb light at 230nm. RNA with a 260/230 ratio lower than 2.2 was considered to be contaminated. The Nanodrop is also able to measure the number of RNA molecules within a single drop of RNA. 1µL of RNA was assessed for Nanodrop quantification. Resulting ratios and concentration of

RNA thereof were recorded and used as a guideline for Real Time PCR experiments.

2.9. Determination of RNA Quality and Quantity by Agilent BioAnalyzer

This is a more precise method for RNA quantification, as all nucleic acids absorb light at 260nm, thereby Nanodrop ratios are not specific to RNA. We had decided to further quantify the RNA samples using the Agilent BioAnalyzer (Agilent Technologies). The BioAnalyzer makes use of 1µL of RNA and generates an RNA Integrity Number (RIN). The software assigns a value to the sample based on the integrity of the sample and this is determined by the electrophoretic trace of the RNA. An Agilent RNA 6000 Nano Kit was used for sample quantification and reagents were stored at 4°C until required. 260µL of RNA 6000 Nano gel matrix was transferred to a filter membrane and centrifuged at 1 500xg for 10 minutes. 4µL of RNA 6000 Nano Dye Concentrate was added to the Nano gel matrix and centrifuged at 13 000xg for 10 minutes. 9µL of Nano gel matrix was loaded into the gel well on a RNA 6000 Nano chip while on the chip priming station. The attached plunger was used to apply pressure as the gel was evenly dispersed throughout the RNA 6000 Nano chip. 9µL was also added to two designated wells on the chip and 5µL of RNA 6000 Nano marker was added to all sample wells and the ladder well. 1µL of ladder was added to the ladder well and 1µL of sample was added to wells allocated to samples. The prepared

chip was vortexed for 1 minute in the IKA vortexer (Agilent Technologies), at 2 400rpm, after which the chip was run on the Agilent 2100 bioanalyzer.

Figure 2. 2: Agilent RNA chip making use of RNA electrophoretic trace in order to accurately quantify the concentration of RNA in 1µL of sample. (image from http://www.genomics.agilent.com/en/Bioanalyzer-DNA-RNA-Kits/Small-RNA-Analysis-Kits/?cid=AG-PT-105&tabId=AG-PR-1185).

2.10. Optimization of cDNA synthesis and RT-PCR

In order to determine which cDNA synthesis kit to use, i.e. High Capacity RNA-to-cDNA Kit or SuperScript® III First-Strand Synthesis System for RT-PCR, 24 samples of varying RNA quality were selected, based on Nanodrop readings, to be used for a trial cDNA synthesis run. Samples were diluted to 100ng using PCR grade water. A total volume of 9µL of diluted RNA was used; where RNA concentrations were too low (i.e. less than 0.00ng/µL per Nanodrop reading) 9µL of RNA was used for the reaction.

2.10.1. Reverse Transcription of RNA to cDNA using the High Capacity RNA-to-cDNA Kit

After allowing the kit components to thaw on ice 10µL 2x RT buffer and 1µL 20x enzyme mix was added together to make a mastermix per sample of RNA. 11µL RNA Mastermix was added to the 9µL RNA and centrifuged briefly to adequately mix all reagents. A total reaction volume of 20µL was used per sample. The reaction was incubated at 37°C for 60 minutes followed by a termination at 95°C for 5 minutes and a hold at 4°C in a Bio-Rad T100 Thermal cycler. Following the 4°C hold, the cDNA was stored at -20°C until use.

2.10.2. Reverse Transcription of RNA to cDNA using the SuperScript® III First-Strand Synthesis System for RT-PCR

After allowing the kit components to thaw on ice 10µL 2x RT Reaction Mix and 2µL RT Enzyme Mix was added together to make a mastermix per sample of RNA. 11µL RNA Mastermix was added to the 9µL RNA and centrifuged briefly to adequately mix all reagents. A total reaction volume of 20µL was used per sample. A Bio-Rad thermocycler was used to incubate the reaction at 50°C for 50 minutes followed by a termination at 85°C for °5 minutes and a hold at 4°C. 1µL RNAseH was added to each reaction and incubated at 37°C for 20 minutes after which the cDNA was stored at -20°C until use.

2.10.3. Real Time Polymerase Chain Reaction using SYBR Fast Green Master Mix

A SYBR Fast Green mastermix was prepared for each sample by adding 5µL SYBR, 0.2µL GNBL2 F primer (5'-CCACCACGAGGCGATTTGTG-3'), 0.2µL GNBL2 R primer (5'-TCTGAGTGGCTCTCATCCTGG-3') and 3.6µL PCR water per sample. 9µL of mastermix was aliquoted into 112 wells of a 384 well plate. 1µL cDNA sample was added to each well and samples were run in duplicate. 112 wells, containing a reaction volume of 10µL, were set up on a 384 well plate. The plate was run on a QuantStudio 7 (Life Technologies) using the default PCR thermal-cycling conditions: 95°C for 20 seconds, 95°C for 3 seconds and 60°C for 30 seconds. The reaction ran for 40 cycles.

2.10.4. Optimization conclusions

Results of the experiment showed amplification of all samples irrespective of RNA concentration. It was also determined that the SuperScript® III First-Strand Synthesis System for RT-PCR kit ought to be used as the Ct values generated for samples were lower than those generated using the High Capacity RNA-to-cDNA Kit, thereby indicating that the SuperScript® III First-Strand Synthesis System for RT-PCR kit required a smaller number of cycles in order to amplify the cDNA samples. For this reason, the SuperScript® III First-Strand Synthesis System for RT-PCR kit was selected for study sample analysis.

Table 2. 1: Ct values generated for GNBL2 transcript using High Capacity RNA-to-cDNA Kit and SuperScript® III First-Strand Synthesis System for RT-PCR for a subset of samples

Sample ID	Mastermix	Ct	RNA concentration as determined by Nanodrop
Recon022	High Capacity	31	-1.4 ng/μL
	Superscript	28	-1.4 ng/μL
Recon004	High Capacity	22	7.6 ng/μL
	Superscript	20	7.6 ng/μL
Recon009	High Capacity	23	18.3 ng/μL
	Superscript	21	18.3 ng/μL
Recon006	High Capacity	24	83.9 ng/μL
	Superscript	22	83.9 ng/μL

2.11. Real Time Polymerase Chain Reaction

2.11.1. Real Time Polymerase Chain Reaction using TaqMan Fast Advance Mastermix and Probes

TaqMan probes were used for RT-PCR as opposed to SYBR Green due to the high specificity of TaqMan probes. The following components in table 2.2. were used to make up a PCR mastermix in a 15ml Falcon tube:

Table 2. 2: RT-PCR reactions components and volumes required per reaction.

Components	Volume (µL) per reaction
TaqMan Fast Advance Master Mix (2x)	10.0
TaqMan PDAR	1.0
Nuclease-free water	7.0
Total volume per reaction	18.0

Table 2. 3: List of Taqman PDARS and amplicon sizes used in RT-PCR assays

Name of Gene	Assay ID	Amplicon Length
AXIN2	Hs00610344_m1	82
CCR2	Hs00704702_s1	61
CCR4	Hs00747615_s1	87
CCR5	Hs99999149_s1	72
CCR7	Hs01013469_m1	58
CD27	Hs00386811_m1	69
CD38	Hs01120071_m1	67
GATA3	Hs00231122_m1	80
GNLY	Hs00246266_m1	80
GZMA	Hs00989184_m1	56
GZMB	Hs01554355_m1	134
GZMK	Hs00157878_m1	75
ICOS	Hs00359999_m1	103
IL2RB	Hs01081697_m1	74
ITK	Hs00950637_m1	88
LEF1	Hs01547250_m1	98
NFKB1	Hs00765730_m1	66
PRF1	Hs00169473_m1	106
PRKCA	Hs00925193_m1	63
RORC	Hs01076122_m1	73

Name of Gene	Assay ID	Amplicon Length
SELL	Hs00174151_m1	62
TBX21	Hs00203436_m1	62
TCF7L2	Hs00175273_m1	105
TGFB1	Hs00998133_m1	57
CCL5	Hs00982282_m1	70
IGF1R	Hs00609566_m1	64
FAM129A	Hs00223000_m1	94
PRR5L	Hs01029928_m1	79
IFNG	Hs00989291_m1	73
ARHGEF18	Hs00248726_m1	106
GAPDH	Hs02758991_g1	93
Beta Actin	Hs99999903_m1	171
18S	Hs99999901_s1	187

After the PCR reagents were added to a Falcon tube, the tube was vortexed to ensure adequate mixing, and emptied into a nuclease-free reservoir for dispensing with a multichannel pipette. 18µL of mastermix was added to all wells in a 96-well optical reaction plate, with each well containing 2µL of sample cDNA, after which the plate was sealed tightly with an optical plate sealer (Applied Biosystems) and centrifuged at 1500rpm for 1 minute to spin down the contents of the plate and eliminate air bubbles that may interfere with data acquisition. The plate was run on a QuantStudio™ 7 Flex (Applied

Biosystems) in fast mode, for 40 cycles, with the conditions summarized in Table 2.4 below:

Table 2. 4: Cycling conditions for RT-PCR using Taqman Probes.

PCR Stage	Temperature (°C)	Time (sec)
Polymerase activation	95	20
Denaturation	95	1
Extension	60	20

Quantstudio™ 6 and 7 Flex Real-Time PCR software (Applied Biosystems) was used for the instrument setup. This software measures the PCR cycle at which the gene of interest begins to exponentially expand above the threshold limit and this is referred to as the Ct value.

2.11.2. Data clean up and statistical analysis

Following acquisition of patient samples, Ct values were exported to Microsoft Excel 2011 for Mac in order to normalize Ct values to housekeeping genes (18s, β-actin and GAPDH). Transcripts that failed to amplify during the 40 cycles were excluded from the analysis. Normalization, also referred to as ΔCt values, was achieved using the formula, in which correction for CD4 count was also included, for each patient sample:

$$\Delta Ct = \frac{Ct\ of\ gene\ of\ interest - Ct\ of\ housekeeping\ gene}{Log2[CD4]} \text{x } 1\,000$$

Following calculations of ΔCt, a decision was made to only include longitudinal samples with a full set of time points in the analysis to avoid missing values. Statistical analysis was performed using GraphPad Prism (version 6.0) for Mac. The Normality of data was determined using the D' Agostino and Pearson normality test. Normally distributed data was analysed using Analysis of Variance (ANOVA) with a Dunnett's post-test for multiple comparisons or paired t test. Paired data that was not normally distributed was analysed using the Kruskal Wallis test with a Dunnett's post-test for multiple comparisons. Unpaired data that was not normally distributed was analysed using the Mann-Whitney U test. A p-value of less than 0.05 was considered statistically significant.

2.12. Characterization of T cells using Flow Cytometry

Flow cytometry is a technology that makes use of a laser and fluorescently labelled antibodies in order to analyze characteristics of cells. Cells are stained using fluorescently labelled antibodies and are allowed to pass through multiple lasers, each of a different colour, thus being excited and emitting light at various wavelengths based on the colour of the laser. The light emitted is then collected and filtered using mirrors and filters (i.e. optics) and directed to photomultiplier tubes (PMT). PMTs measure the wavelengths emitted and convert the optical signals into digital values that are captured by

the flow cytometer's accompanying computer and thus allowing visualization. PMT voltages were adjusted daily, using Rainbow fluorescent particle (Spherotech) beads, to ensure that consistent MFIs for each channel were maintained.

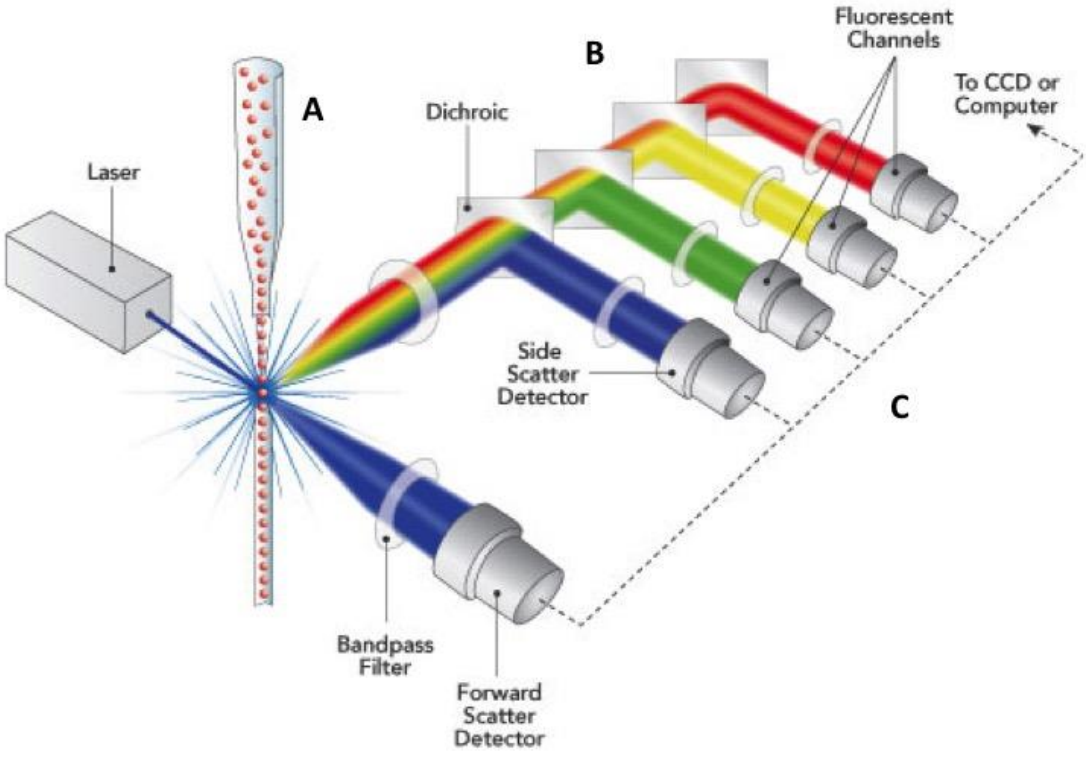

Figure 2. 3: Diagrammatic representation of a flow cytometer indicating the (A) fluidics (B) optics (C) electronics components that makes up the flow cytometer (image sourced from www.semrock.com/flow-cytometry.aspx).

2.12.1. Optimization of Flow Cytometry Experiments

2.12.1.1. Marker selection for polychromatic panel

A primary aim of the study was to characterize how *Mtb* specific T cells, change proportionally in response to ART, in cryopreserved PBMC collected from HIV infected persons. In order to ensure that live cells were characterized, and to reduce background staining, a viability marker was added to the panel. Since the study focused specifically on the characterization of T cells, antibodies against CD14 and CD19 were included in the panel in order to exclude monocytes and B cells respectively. A CD3 marker was included in order to identify all T cells. CD4 was included in order to identify CD4 positive cells since this was a focus of the study. Due to limited channels on the BD Fortessa, it was decided that CD8 responses could be analyzed by gating on CD3 positive and CD4 negative cells, thereby allowing the exclusion of a CD8 marker. It was of importance to assess T cell memory subsets and so the markers CD27 and CD45RA were included in the panel. In order to assess the functionality of the T cells of interest, chemokine markers CXCR3, CCR4 and CCR6 were included in the panel. The combination of these markers allows the distinction between T_{H1}, T_{H2}, T_{H17} and T_{H1}/T_{H17} T cells. HLA-DR and KLRG-1 were added to the panel in order to determine the activation and senescence profiles of the cells of interest, respectively. Finally the cytokines IFN-γ, TNF-α, IL-2 and IL-17A were of interest due to their roles in polyfunctional T cells: IL-2 plays an important role in the proliferation of T cells upon antigen stimulation, IFN-γ is known for its implications in disease control, and TNF-α is involved in the

recruitment of monocytes and neutrophils, IL-17A was found at the site of disease in TB infected individuals [5] and preliminary multiplex analysis indicated that measuring this cytokine and determining its source would be useful. It was also important to determine the source of the cytokines, as a follow up from the results observed in multiplex assays.

2.12.1.2. Antibody titrations

Antibody titrations were performed on all antibodies used in the panel in tables 2.5. and 2.6. Titrations were performed in order to obtain the optimal antibody titer to be used for cell staining. This further reduces background staining when antibodies bind to receptors on cells; and ensures the best separation between negatively and positively labelled cells (Lamoreaux *et al*. 2006). The optimal antibody titer was determined by measuring the MFI and standard deviation (SD) of positive and negative populations of cells in a titration series. The MFI and SD were used in the formula below in order to determine the stain index (SI).

$$SI = \frac{Positive\ MFI - Negative\ MFI}{2(Negative\ SD)}$$

The SI and corresponding antibody concentration was plotted in order to generate saturation curves (Figure 2.5. A). Graphs of the positive and negative MFI and corresponding antibody concentration was plotted in order to determine the optimal titer at which the best separation occurred (Figure

2.5. B). Lastly, the percentage of positive cells was also plotted against the corresponding antibody concentration in order to determine the antibody titer at which the optimal yield of percentage positive cells occurs (Figure 2.5. C).

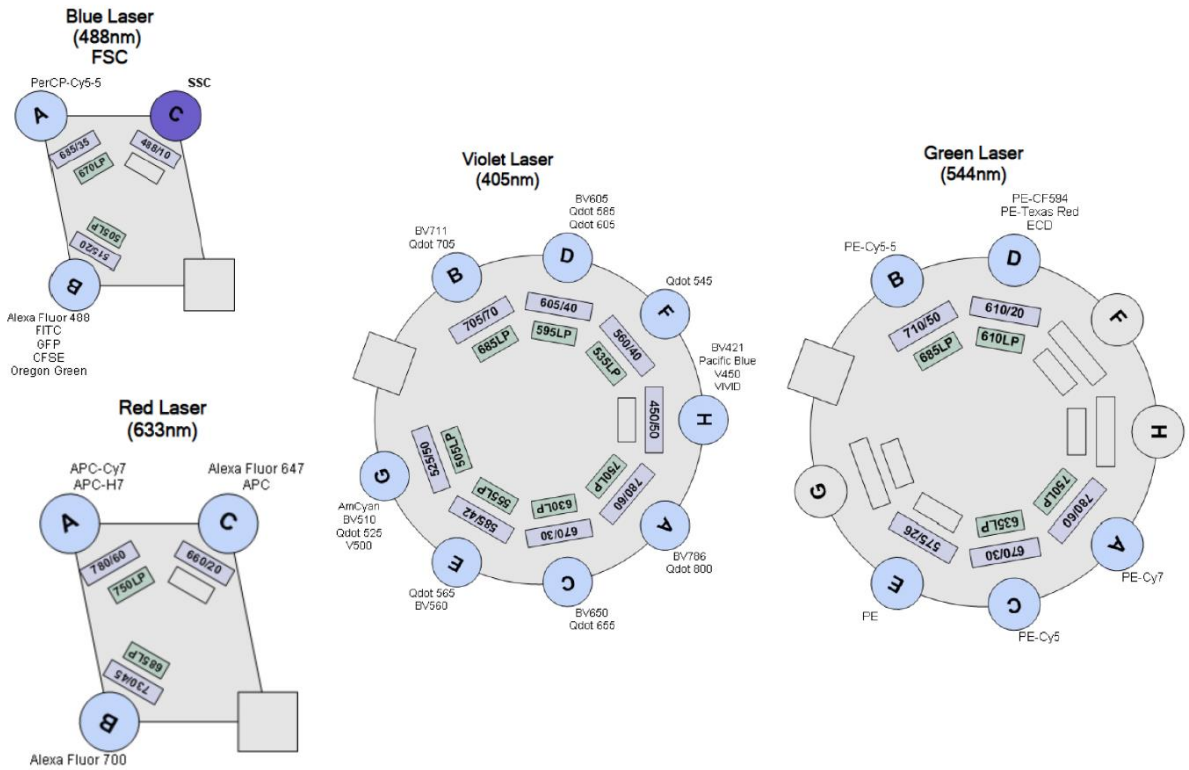

Figure 2. 4: Configuration of the BD Fortessa flow cytometer used in the study, based at the South African Tuberculosis Vaccine Initiative (SATVI). The cytometer consists of a (A) trigon Blue laser, (B) trigon Red laser, (C) an octagon Violet laser and (D) an octagon Green laser; and subsequent channels for each laser.

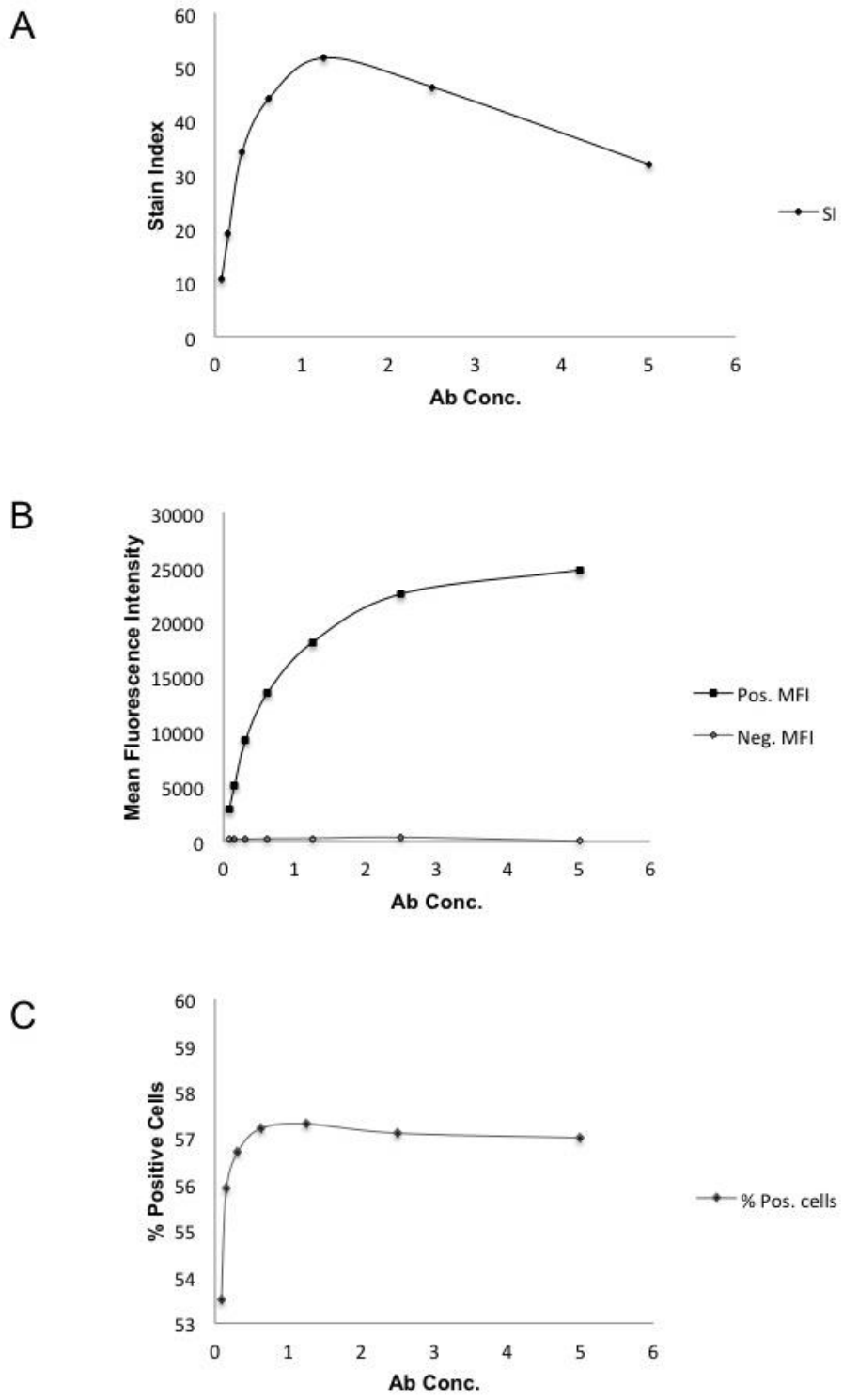

Figure 2. 5: Graph depicting the (A) stain index of HLA-DR antibody stain index (B) the MFI of a HLA-DR negatively stained cell population and MFI of a HLA-DR positively stained cell population (C) percentage of HLA-DR positively stained cells vs. the antibody volume.

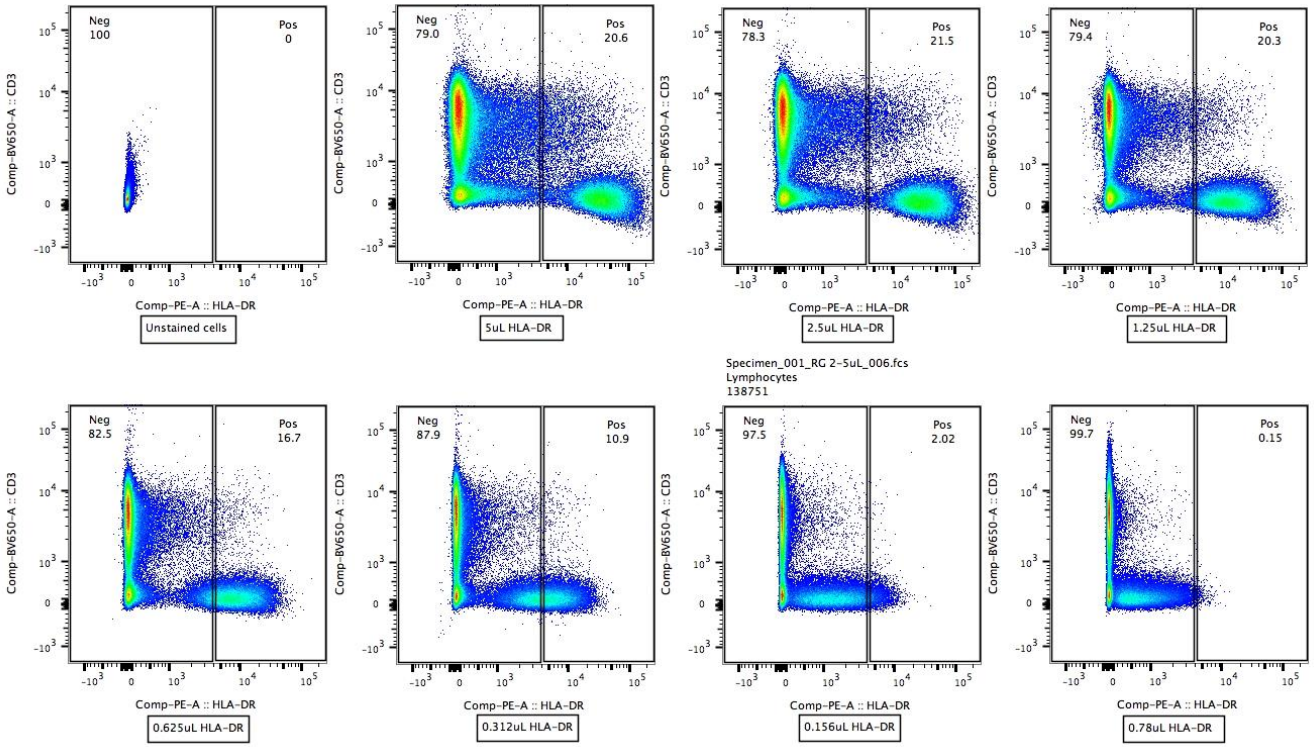

Figure 2. 6: Representative flow cytometry plots for an HLA-DR titration. The volume of HLA-DR antibody added to each tube is shown below each flow plot. Thawed PBMC were surface stained and the live, single cell and lymphocyte populations were gated on. A two-fold dilution series, using PBS, was performed in order to dilute the HLA-DR antibody.

2.12.1.3. Antigen stimulation

Antigen presenting cells (APCs), such as macrophages, dendritic cells, Langerhans cells and to an extent B-cells, present antigen via MHC molecules. T cells bind to the antigen being presented via T cell receptors. A CD receptor also binds to the MHC molecule (i.e. CD4 binds to MHC Class II molecules and CD8 binds to MHC Class I molecules), and CD28 or CD27 present on the surface of the T cell binds to B7 present on the surface of the antigen-presenting cell. These three bonds are required for T cell activation. T cells are activated upon antigen recognition, expand clonally and produce cytokines. In order to mimic this process *in vitro*, pathogen specific antigens are used to stimulate T cells thus resulting in activation, clonal expansion and cytokine production. *Mycobacterium* Whole Cell Lysate (*Mtb* WCL) from *Mtb* strain H37Rv was the antigen of choice for the polychromatic flow cytometry assay based on data from previous studies [6] *Mtb* WCL (obtained from BEI Resources) consists of proteins, lipids and carbohydrates present within the bacterial cell wall, was stored at a concentration of 20µg/mL at -20 °C and was used at a concentration of 2µL/mL in this assay. A positive control was included to ensure that the assay worked optimally and that cells were not anergic. *Staphylococcus aureus* Enterotoxin B (SEB) is produced by the bacterium species *Staphylococcus aureus* and is commonly known as the toxin responsible for food poisoning thus making SEB a good positive control. SEB was stored at a concentration of 5µg/ml at -20 °C and a concentration of 5µL/mL was used in this assay.

2.12.1.3. Fluorescence Minus One Controls

A Fluorescence Minus One (FMO) control contains all fluorochromes in a polychromatic panel in a single tube, except for one that is currently being measured. This control is set up in order to ensure that positive events of interest are real events and not present due to the spread of other fluorochromes into the channel of interest. FMO controls were conducted for all markers to ensure no spillover into channels, that all markers in the panel were compatible and that gates were drawn in the appropriate positions on flow plots.

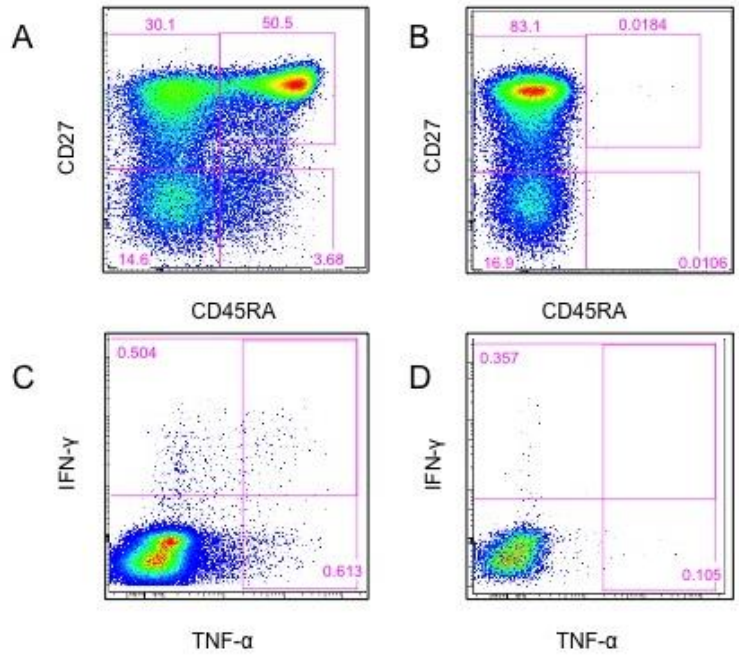

Figure 2. 7: FMOs of a surface (A) and intracellular marker (C) included in the polychromatic panel. (A) CD45RA marker included in surface and ICS staining, (B) absence of the CD45RA marker in surface and ICS staining, (C) TNF-α included in the surface and ICS staining and (D) absence of the TNF-α marker in surface and ICS staining. FMO controls were conducted for all markers to ensure no spillover into channels, to ensure that all markers in the panel were compatible and ensure that gates were placed in the appropriate positions.

2.12.1.4. Gating strategy

In the context of flow cytometry, gating refers to the selection of a specific region on the flow plot that depicts the cells of interest. Cells inside the gate are the cells that will continue to be analyzed while those cells falling out of the gate will be excluded from the analysis. It is important to establish a

gating strategy for optimal analysis of data and that this strategy be applicable when analyzing all subsequent datasets.

In this study, we used time as a parameter in order to include those cells acquired during consistent acquisition. Using this parameter, it was also possible to exclude any cell clumps or bubbles that may have been present within the flow cell at the time of acquisition. The FSC-A and FSC-H parameters were then used to gate on single cells, and SSC-A and FSC-A in order to gate on lymphocytes. Since the study focused on T cells, CD14 and CD19 markers were used to identify and exclude monocytes and B cells respectively, thus the CD14/CD19 negative, IFN-γ positive population was gated on. IFN-γ positive cells were gated on at this point in the gating strategy as gating on surface markers may underestimate cytokine responses. The IFN-γ parameter was also used to ensure that all live and IFN-γ producing cells were adequately selected, after which CD4 positive cells were gated on. Next, CD4 positive cells were characterized by the following markers; CCR4, CCR6, CXCR3, HLA-DR, KLRG-1. CD45RA and CD27 were used in order to assess the memory profiles of $CD4^+$ T cells. Lastly, the ability of CD4 cells to produce the cytokines IFN-γ, TNF-α, IL-2 and IL-17A was determined.

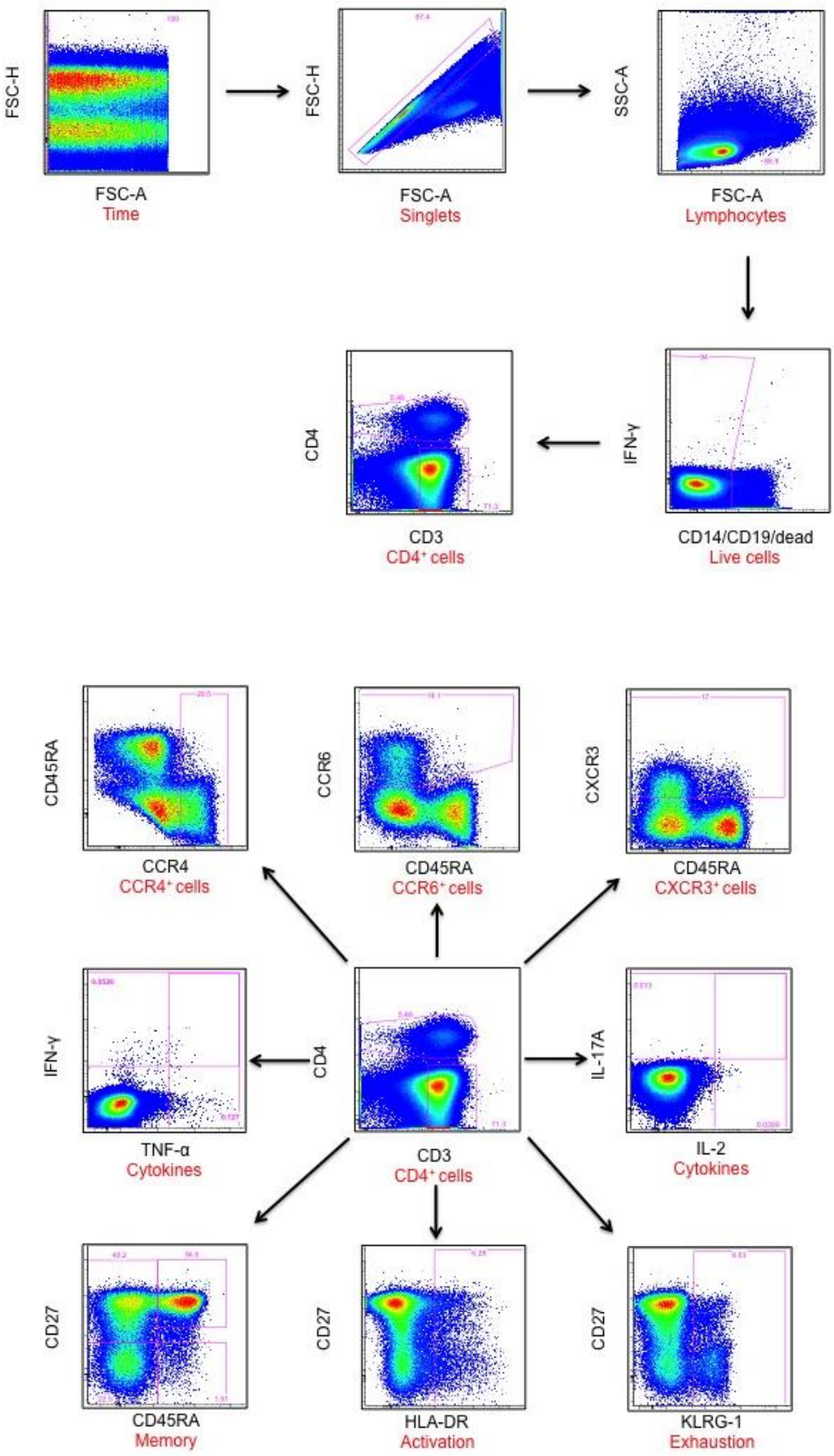

Figure 2. 8: Gating strategy used for polychromatic flow panel.

2.12.1.5. Flow Cytometry Optimization Conclusions

Markers of interest and fluorochromes to which they were conjugated, were selected based on the channels and lasers available on the SATVI BD Fortessa shown in Figure 2.4. Each antibody was titrated individually to generate SI, MFI and percentage of positive cells vs. antibody volume graphs to select the optimal antibody titer. The polychromatic panel was then tested for all surface markers to ensure that the antibodies were compatible. Once this was achieved the intracellular cytokine antibodies were titrated in order to determine optimal antibody titers, and the entire polychromatic panel was tested on fresh and frozen PBMC to ensure that all antibodies were compatible. Tables 2.5. and 2.6. contain a summary of all antibodies and corresponding final volumes used in this study. FMOs for every fluorochrome were setup to ensure that there was no spillover into adjacent channels and to ensure that the gates for cell populations were adequately placed on the flow plots. A gating strategy for optimal analysis was determined resulting in an optimized polychromatic flow cytometry panel for the characterization of T cells in HIV infected individuals sensitized by *Mtb*.

2.12.2. Thawing of frozen PBMC and stimulation for the flow cytometry experiments

Cryovials containing cryopreserved PBMC were removed from liquid nitrogen and placed into a 37-degree incubator to thaw for 10 minutes. Following

thawing cells from a single cryovial were transferred in a drop-wise fashion, using a Pasteur pipette, to 10ml of R10 fresh media in a 50ml sterilin. Cells were centrifuged for 10 minutes at 400rpm in a Heraeus Megafuge 40R centrifuge (Thermo Scientific), after which the cell supernatant was discarded and cells were resuspended by tapping the sterilin. 10ml of fresh R10 media was added to each sterilin and vortexed briefly to facilitate mixing. Cells were then counted using a counting chamber and the Bio-Rad TC20 Automated cell counter (Bio-Rad) for enumeration. 20µL of cells was added to 20µL 0.4% Trypan Blue (Sigma Life Sciences) and mixed briefly, 10µL was then pipetted into a counting chamber and inserted into the TC20. Remaining cells in the sterilin were washed and resuspended with R10 media to achieve a final concentration of $1x10^6$ cells/ml. Cells were then transferred to FACS tubes (BD Biosciences) and individual tubes were stimulated with (1) *Mtb* WCL at a concentration of 20µg per ml of cells; (2) SEB at a concentration of 5µg per ml of cells; and (3) a single tube was left unstimulated for each participant. FACS tubes were incubated at 37 °C, 5% CO_2 for 3 hours prior to the addition of Brefeldin A (BFA) at a concentration of 5µg per ml of cells. BFA is a protein transport inhibitor that blocks the transport of proteins (cytokines) from the endoplasmic reticulum to the Golgi apparatus. Cells were then incubated for 18 hours at 37 °C, 5% CO_2 prior to staining.

2.12.3. Surface and Intracellular Staining of PBMC

Staining in this context refers to the usage of an antibody that is conjugated to a fluorochrome being used to bind to its receptor on a cell of interest. Light

of a specific wavelength, depending on the fluorochrome bound to the antibody, is emitted when the cells are excited by a laser in a flow cytometer. Intracellular staining refers to the same procedure, with the exception of the receptors on the cell of interest being found on the inside of the cell thereby requiring a further step of permeablisation to make the cell penetrable by the antibody. The following procedure was performed in the dark due to light sensitive reagents. Following overnight incubation at 37 °C, 1ml PBS was added to each tube of cells and tubes were centrifuged for 5 minutes at 1500rpm. Supernatants were decanted after which cells were resuspended by briefly vortexing. 50µL Near InfraRed (NIR) live dead stain was added to each tube and vortexed lightly to mix. Tubes were covered in foil and incubated at room temperature for 10 minutes; during which time the surface stain antibody mixture was made. The volumes of antibodies in Table 2.5. were added together to make up the surface stain antibody cocktail. BD Brilliant Violet stain buffer (BD Biosciences) served as the buffer for the antibody cocktail and was used to bring the volume to 50µL antibody cocktail per sample.

Table 2. 5: Anti-human antibodies used in flow cytometry surface stain panel

Marker	Fluorochrome	Clone	Manufacturer	Catalogue no.	Titer used
CCR4	BV510	L291H4	BioLegend	359416	1.25
CD45RA	BV570	HI100	BioLegend	304131	0.25
CCR6	BV605	G034E3	BioLegend	353420	1.25
CD3	BV650	OKT3	BioLegend	317323	0.4
CD27	BV711	O323	BioLegend	302834	0.4
CD4	FITC	RPA-T4	e-Bioscience	11-0049-42	0.4
CD14	APC-Cy7	61D3	Invitrogen	MHCD1427	0.7
CD19	APC-Cy7	SJ25-C1	Invitrogen	MHCD1927	0.7
HLA-DR	PE		BD	347401	1.2
KLRG-1	PE.Cy5-5	13F12F2	e-Bioscience	46-9488-42	0.7
CXCR3	PE.Cy7	IC6/CXCR3	BD	560831	1.25
Viability	APC-Cy7	N/A	Invitrogen	L23102	0.7

Following incubation with NIR live dead dye, cells were washed using 1ml PBS containing 2% FCS at 1500rpm for 5 minutes; after which the cell supernatant was discarded and cells resuspended by briefly vortexing. 50µL of the surface stain antibody cocktail was added to each tube and mixed well. Tubes were covered in foil and left to incubate at room temperature for 25 minutes to facilitate binding of antibodies to their respective cell surface receptors. Cells were washed with PBS containing 2% FCS following incubation and cell supernatants were discarded. 250µL BD CytoFix/CytoPerm buffer (BD Biosciences) was added to each tube and

incubated for 20 minutes at 4 °C to allow permeabilization of cells. Permeabilization is a reversible process that allows the antibodies to enter the cells and bind to markers of interest. This is achieved by the use of saponin, present in the buffer that binds to cholesterol molecules on the surface of the cell. This binding results in the formation of insoluble complexes between cholesterol and saponin resulting in the rearrangement of lipids and thus a more permeable structure. During the permeabilization incubation step, the intracellular marker cocktail of antibodies was made up according to volumes in table 2.6. BD Perm/Wash (BD Biosciences) served as the buffer for the intracellular cytokine antibody cocktail and was used to bring the volume to 50µL intracellular cytokine antibody cocktail per sample.

Table 2. 6: Anti-human cytokine antibodies used in flow cytometry intracellular staining

Marker	Fluorochrome	Clone	Manufacturer	Catalogue no.	Titer used
IL-17A	AF647	B27	BioLegend	512310	1.0
IFN-γ	AF700	L243	BD	557995	0.25
IL-2	PE/Dazzle	MQI-17H12	BioLegend	500344	1.25
TNF-α	eFluor450	Mab11	e-Bioscience	57-7349-73	0.8

Following incubation, cells were washed with 1.5mL BD Perm/Wash (BD Biosciences) and 50µL of intracellular cytokine antibody cocktail was added

to each tube; after which the tubes were incubated at 4 °C for 30 minutes to allow for optimal binding of intracellular cytokine antibodies to their respective targets. For the final step following the incubation, cells were washed with 1.5mL BD Perm/Wash (BD Biosciences) and subsequently fixed with 200µL of 1% paraformaldehyde (Sigma Aldrich) following cell supernatant removal. Lastly, tubes were covered with foil and incubated at 4 °C until acquisition on the same day.

2.12.4. Compensation for Spectral Overlap

During acquisition, fluorescence from a single fluorochrome may be detected in more than one detector (i.e. the intended primary detector and additional secondary detectors). This is referred to as spectral overlap. Correction for spectral overlap is achieved by mathematically subtracting the resulting spillover (Figure 2.9) using single stained beads. It is important to note that compensation control beads are as bright as, if not brighter, than the cells of interest, therefore the same antibody titers were used for the compensation controls and the polychromatic assay. For the preparation of compensation controls 100µL PBS containing 2% FCS was added to each tube followed by 40µL of Anti-Mouse Ig, κ positive compensation beads (BD Biosciences). Antibody titers in tables 2.5 and 2.6 were then added to corresponding tubes and lightly vortexed to mix. Tubes were covered in foil and incubated at room temperature for 10 minutes to facilitate binding to compensation beads. After the incubation, the single stain tubes were washed with 1ml PBS containing 2% FCS and the supernatant was discarded. The beads were then vortexed

lightly to ensure resuspension, and 200µL 1% paraformaldehyde was added to each tube to fix beads. After fixation, tubes were covered in foil and stored in a 4 °C fridge for up to 7 days.

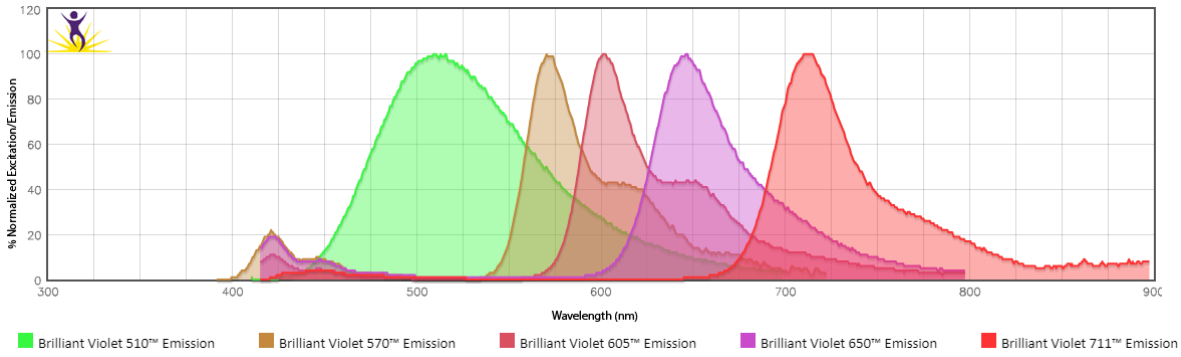

Figure 2. 9: Diagrammatic representation of expected spectral overlap in polychromatic panel generated by BioLegend Fluorescence Spectra Analyzer (www.biolegend.com/spectraanalyzer). Compensation was used to correct for the spectral overlap by mathematically subtracting the spillover in each channel.

2.12.5. Data acquisition

Samples were acquired on a BD Fortessa flow cytometer (Becton Dickinson) with FACSDiva software version 6.0. (Becton Dickinson) on the same day that antibody staining had occurred. Due to variable initial cell counts and low cell viability, all cells were acquired. An average of 1 000 000 events were acquired per tube. Compensation beads were acquired daily before the acquisition of samples, to allow for post-acquisition compensation.

2.12.6. Data clean up and statistical analysis

Flow cytometry data analysis was conducted using Flowjo v9.9.4. (Treestar), Pestle version 1.7 and SPICE version 5.3 (from Mario Roederer, NIAID, NIH) [7]. Statistical analysis was conducted using GraphPad Prism (version 6.0). *Mtb* specific responses for each sample were valid if the antigen-stimulated tube contained a value greater than twice the value detected for the unstimulated tube, as described [8, 9]. If antigen stimulated responses were less than twice the value detected for the unstimulated tube, a value of 0 was assigned to the sample. Cytokine responses were measured by subtracting the unstimulated tube from the antigen-stimulated tube. All resulting negative values were assigned a value of 0. The absolute number of cells was calculated using the following formula:

$$Absolute\ number = \frac{CD4\ count\ x\ Frequency\ of\ cell\ population}{100}$$

The Normality of data was determined using the D' Agostino and Pearson normality test. Normally distributed data was analysed using Analysis of Variance (ANOVA) with a Dunnett's post-test for multiple comparisons or paired t test. Paired data that was not normally distributed was analysed using the Kruskal Wallis test with a Dunnett's post-test for multiple comparisons. Unpaired data that was not normally distributed was analysed using the Mann-Whitney U test. Correlations were investigated by the non-parametric Spearman rank correlation. A p-value less than or equal to 0.05 was considered significant. P-values are illustrated in subsequent figures by asterixes and mean the following: ns refers to a p-value > 0.05, a single

asterisk (*) refers to a p-value ≤ 0.05, two asterix (**) refer to a p-value ≤ 0.01, three asterix (***) refer to a p-value ≤ 0.001 and four asterix (****) refer to a p-value ≤ 0.0001.

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Chapter 3: Determination of *Mycobacterium tuberculosis* antigen-specific response in persons with and without HIV infection

3.1. Introduction

According to WHO 5-10% of individuals will develop the active form of TB disease in their lifetime [1, 2] In addition, the mechanism responsible for the progression from LTBI to active TB disease has yet to be defined. For many years the tuberculin skin test (TST) was used to determine exposure to the disease but this test is known to be confounded by vaccination with the Bacillus Calmette-Guerin (BCG) in addition to sensitisation by environmental mycobacteria [3]. These factors may render false positive TST results. Recently, an incremental advance has been provided by diagnostic tests that measure the cellular immune response to *Mtb* in the form of IFN-γ production in response to *Mtb* antigen stimulation. These Interferon gamma release assays (IGRA) make use of three proteins synthesised by *Mtb*, thus allowing these tests to be highly specific for an *Mtb* response [3]. The early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP10) are encoded by the region of difference (RD1) of *Mtb*. This region, along with RD2 and RD3, were found to be deleted from the BCG vaccine strain, but RD1 has been established to be conserved in virulent *Mtb* strains [4]. The TST test on the other hand, is based on the tuberculin purified protein derivative (PPD) that is a mixture of proteins and fragments, some of which

is shared with other mycobacterial species and BCG vaccine strains, thus accounting for the poor specificity of the test [5]. The TST is also impaired by HIV infection [6] due to impaired cell mediated immunity in these individuals [7, 8].

Two such IGRAs that measure the cellular immune response to TB, are the whole blood based QuantiFERON-TB Gold (QFT) test and the T-SPOT.*TB* test, which is based on the use of peripheral blood mononuclear cells (PBMC) in an enzyme-linked immunospot assay (ELISPOT) [3]. Whole blood is used for QFT assays and IFN-γ is released when the blood sample is incubated with the *Mtb* specific antigens [3]. An ELISA is then used to measure the amount of IFN-γ released by cells in the blood. In a study that compared the TST, QFT and T-SPOT.*TB* assays Rangaka *et al*. demonstrated that the T-SPOT.*TB* yielded a significantly higher proportion of interpretable results than the QFT and TST tests [3]. Results of the study also indicated that IGRAs, such as the QFT and T-SPOT.*TB*, had a higher sensitivity for TB infection. In addition, an advantage of using IGRAs is that should a patient not return to the clinic for results, the determination will remain valid [3]. This does not hold true for the TST test as multiple visits are required.

IGRAs measure the amount of IFN-γ that is produced by cells in response to *Mtb* antigenic stimulation [9] and have been shown to produce accurate results in individuals with active TB as well as immunocompromised individuals [9]. However, there is still concern that these tests do not produce

optimal results in severely immunocompromised individuals who do not always mount a detectable IFN-γ response upon encounter with *Mtb* antigen [10]. This may be improved by measuring alternative biomarkers to IFN-γ. The quantity of such biomarkers could indicate either a normal or pathogenic response and therefore provide us with an individual's health status [11]. Biomarkers in TB patients with active disease should predict long lasting treatment success, and in LTBI patients should predict treatment success and indicate whether reactivation is possible. In uninfected individuals, the quantity of a specific biomarker should indicate protection from TB when vaccinated [11].

3.2. Characteristics of HIV infected and HIV uninfected persons included in this study

Forty-four HIV infected and 50 HIV uninfected persons were included in this analysis. Six HIV infected persons were excluded from the study due to the identification of opportunistic infections during ART follow up. The characteristics of all patients included in the study are summarized in Tables 3.1. and 3.2. HIV infected persons had a median age of 35 years while HIV uninfected persons had a median age of 27 years. Although the median age of HIV infected persons differed significantly from the median age of HIV uninfected persons, we do not believe that this difference impacted downstream statistics and subsequent significant differences in immunological markers measured. Viral suppression was achieved in HIV infected persons as the median viral load prior to ART was 62 933 viral

copies/ml, decreasing significantly (p-value < 0.0001) to levels below the detection limit after six months of therapy, with CD4 counts significantly increasing (p-value < 0.0001). The detection limit for the viral load PCR is 40 viral copies/ml with readings <40 being regarded as below the detection limit, according to the National Health Laboratory Services (NHLS).

Table 3. 1: Characteristics of all patients included in this study.

	HIV Infected Persons (n=44)	**HIV Uninfected Persons (n=50)**	**HIV Infected vs. HIV Uninfected p-value**
Median Age (years)	35 (30-40)	27 (21-36)	0.0003
Gender Male/Female	14/30	25/25	0.0742

Table 3. 2: CD4 counts and Viral loads of patients.

Median (IQR)	**HIV Infected Persons (n=44)**				**HIV Uninfected Persons (n=50)**
	D0 of ART	**1M**	**3M**	**6M**	
CD4 count	201 (134-239)	278 (179-369)	298 (167-392)	282 (187-426)	819 (645-1 070)
Viral Load	62 933 (27 491-223 011)	244 (94-554)	40 (39-108)	39 (39-39)	N/A

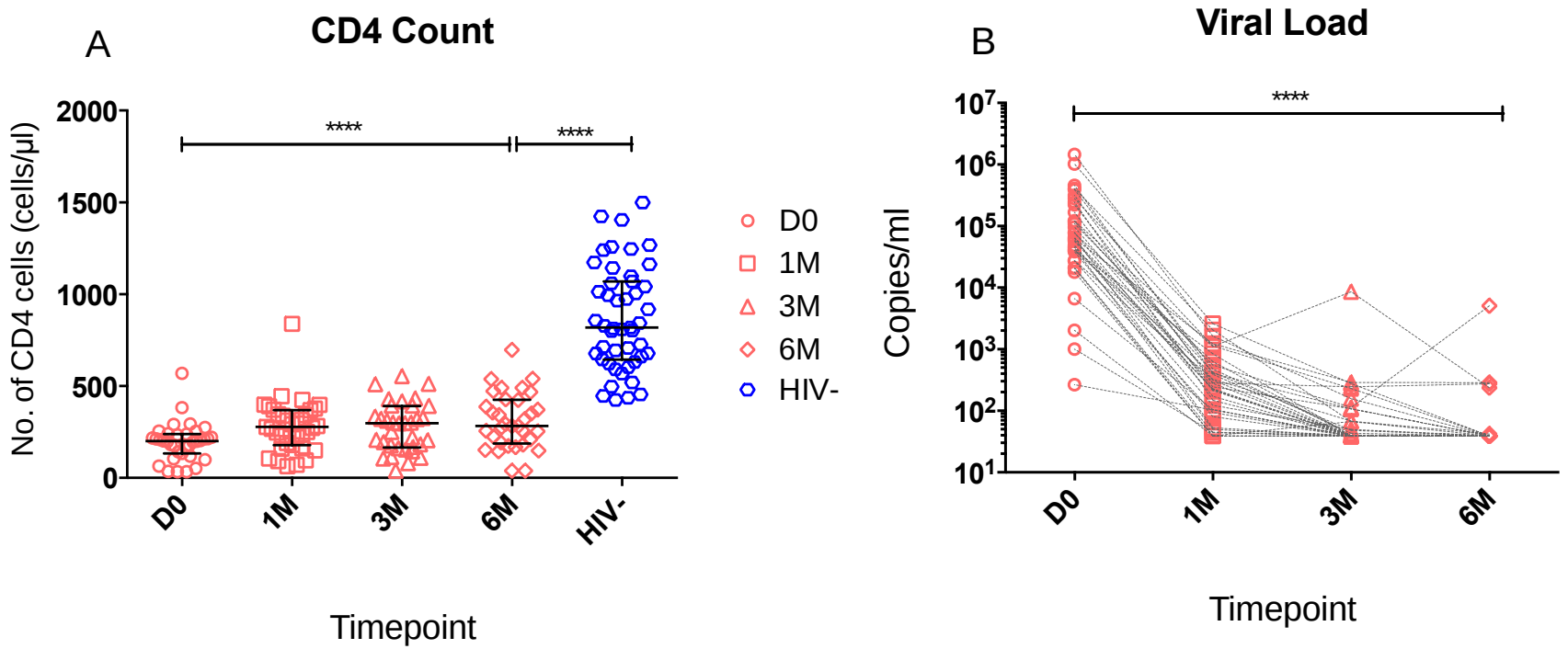

Figure 3. 1: CD4 counts and viral loads of all persons included in the study. (A) CD4 counts of HIV infected persons (red, n=44) and HIV uninfected persons (blue, n=50) and (B) longitudinal viral loads, as measured by copies/ml, of HIV infected persons during 6 months of ART.

3.3. Methods and statistical analysis

3.3.1. Quantiferon-TB® Gold In-Tube assay

The QFT (Cellestis Inc., Valencia, CA) assay was performed in order to determine Mtb sensitisation at the time of recruitment. Details are described in chapter 2, section 2.4. IFN-γ was measured in IU/ml and following subtraction of the unstimulated sample from the stimulated sample a reading of 0.35IU/ml, and a satisfactory mitogen response, was regarded as a positive response as per the manufacturer's recommendation.

3.3.2. Human Interferon-gamma ELISpot assay

Mtb sensitisation was additionally evaluated by the ELISpot assay using the human Interferon-gamma ELISpot^{PRO} kit (MABTECH, Nacka Strand, Sweden) as described previously [12] [13] [14] and in more detail in chapter 2 section 2.5. ELISpot readings were recorded as numbers of Spot Forming Cells (SFC) per million PBMC and 30 SFC/million was regarded as cut off for a positive response.

3.3.3. IFN-γ detection using Luminex

Supernatants from the QFT assays were used to measure soluble IFN-γ by multiplex analysis using the Bio-Rad Pro Human Cytokine 27-plex kit (Bio-

Rad Laboratories, Hercules, CA). Samples were acquired on the Bio-Plex 200 system using Bio-Plex Manager software version 6.1. Unstimulated samples were subtracted from stimulated samples and concentrations that were below the limit of detection were assigned the lowest value of the IFN-γ standard. Further details are described in chapter 2, section 2.6.1.

3.3.4. Surface and intracellular staining flow cytometry assay

Cryopreserved PBMC was thawed, stimulated overnight at 37°C, 5% CO_2 with *Mtb* WCL (20µg/ml) and stained for assessment using flow cytometry. A negative control consisted of PBMC without antigen stimulation while SEB SAg (5µg/ml) was used as a positive control. Cells were then washed and stained with LIVE/DEAD Red Dead Cell stain (Invitrogen) and stained for surface markers using the following antibodies: anti-CD19-APC-Cy7 and anti-CD14-APC-Cy7 from Invitrogen; anti-CD4-FITC and KLRG-1-PE.Cy5-5 from e-Bioscience; anti-HLA-DR-PE and anti-CXCR3-PE-Cy7 from BD; anti-CCR4-BV510, anti-CD45RA-BV570, anti-CCR6-BV605, anti-CD3-BV650 and anti-CD27-BV711 from BioLegend. After incubation, wash and permeabilization steps, the cells were stained intracellularly to detect of cytokines using anti-IFN-γ-Alexa Fluor 700 from BD; anti-IL-2-PE and anti-IL-17A-Alexa Fluor 647 from BioLegend; and anti-TNF-α-eFluor450 from e-Bioscience. The cells were then fixed and acquired on a BD Fortessa flow cytometer with FACSDiva software (version 6). *Mtb* specific responses for each sample were valid if the antigen-stimulated tube contained a value greater than twice the value detected for the unstimulated tube. If antigen

stimulated responses were less than twice the value detected for the unstimulated tube, a value of 0 was assigned to the sample. Cytokine responses were measured by subtracting the unstimulated from the antigen stimulated, and a value ≥0.05% IFN-γ was used as a cutoff to determine *Mtb* sensitisation. A detailed description of this assay can be found in chapter 2, section 2.12.3.

3.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6) using methods for non-parametric data. The Friedman test, with correction for multiple comparisons, was used for paired data comparison across all sampling time points in HIV infected individuals, while the Kruskal Wallis test with correction for multiple comparisons was used for unpaired data comparisons across all time points in HIV infected persons. The Wilcoxon test is the equivalent of the non-parametric student t-test and was used to compare paired data in HIV infected persons at day 0 of ART and after 6 months of ART. Additionally we also compared cross-sectional data from the HIV infected persons at 6 months of ART induced immune reconstitution with data from HIV uninfected persons using the Mann-Whitney test, used for unpaired data. A p-value less than or equal to 0.05 was considered significant. P-values are illustrated in subsequent figures by asterixes and mean the following: ns refers to a p-value > 0.05, a single asterisk (*) refers to a p-value ≤ 0.05, two asterix (**) refer to a p-value ≤ 0.01, three asterix

(***) refer to a p-value ≤ 0.001 and four asterix (****) refer to a p-value ≤ 0.0001.

3.4. Results

3.4.1. IFN-γ measured using the Quantiferon-TB® Gold In-Tube assay in HIV infected persons and HIV uninfected persons, for Mtb sensitisation

Figure 3.2. panel A and Table 3.3. show the overall IFN-γ response, determined using the QFT assay, of all HIV infected persons during 6 months of ART, and HIV uninfected persons included in this study. The cut off value for this assay was 0.35IU/ml and responses above this value were considered as a positive response. A Kruskal-Wallis test, correcting for multiple comparisons, in the HIV infected cohort revealed no significant difference in the IFN-γ response in these individuals, after 6 months of ART (p-value > 0.9999). However a Wilcoxon test comparing the median IFN-γ response at baseline with the median IFN-γ response after 6 months of ART revealed a significant decrease (p-value = 0.01) in HIV infected persons. Comparison with the HIV uninfected persons revealed that HIV uninfected persons had a significantly higher median IFN-γ response (p-value = 0.03).

3.4.2. IFN-γ detected by the human Interferon-gamma ELISpot^{PRO} assay in HIV infected persons and HIV uninfected persons

Figure 3.2. panel B and Table 3.3. show the numbers of IFN-γ positive cells determined using the human Interferon-gamma ELISpot^{PRO} kit. The cut off value for this assay was 30 SFC/million PBMC and responses above this value were considered positive. When corrected for multiple comparisons, Kruskal-Wallis testing revealed no significant change in the number of IFN-γ secreting cells in HIV infected persons receiving ART over 6 months (p-value > 0.9999). The paired comparison between baseline and 6 months of ART using Wilcoxon testing also revealed no significant difference in the IFN-γ response in the HIV infected persons (p-value = 0.9058). Additionally when compared to the HIV uninfected persons, there was no significant difference in the number of IFN-γ secreting cells per million PBMC between the two cohorts (p-value = 0.354).

3.4.3. IFN-γ measured using Luminex

Soluble IFN-γ was measured using Luminex analysis in a subset of HIV infected and HIV uninfected persons. Figure 3.2. panel C summarises the measurement of IFN-γ in these persons. Since an appropriate cut off value for a positive response has yet to be determined for this assay, this analysis was used to substantiate *Mtb* sensitisation determined by the QFT assay. The Kruskal Wallis test revealed no significant difference in the concentration of IFN-γ measured at day 0 of ART and after 6 months (p-value = 0.4029) in HIV infected persons, however a non-parametric t-test revealed a significant

decrease in the concentration of IFN-γ produced between these two time points (p-value = 0.049), similar to the QFT assay. Comparison with the HIV uninfected persons revealed that HIV uninfected persons had a higher median IFN-γ concentration (p-value = 0.409) compared to HIV infected persons, again similar to the QFT assay results. Thus the trends observed for the two assays measuring soluble IFN-γ were similar.

3.4.4. Mtb sensitisation determined by intracellular cytokine staining (ICS) assays using flow cytometry

Figure 3.2. panel D and Table 3.3. show the overall *Mtb*-specific cytokine response determined using flow cytometry. A positive response in the stimulated PMBC was defined if the frequency of IFN-γ^+ cells was greater than or equal to 0.05% of CD4 positive T cells in the background subtracted condition. Kruskal Wallis testing revealed no significant difference in the IFN-γ^+ response in HIV infected persons receiving ART (p-value > 0.9999). Additionally, a Wilcoxon test that compared the medians at baseline and after 6 months of ART in HIV infected persons also revealed no significant difference in the longitudinal IFN-γ^+ response (p-value = 0.518). When the HIV infected persons at 6 months of ART were compared with the HIV uninfected persons, no significant difference between the IFN-γ^+ responses (p-value = 0.981) was observed.

We also examined at the numbers of $CD4^+$ IFN-γ^+ cells longitudinally in order to determine *Mtb* sensitisation in our cohort. This data is represented based

on the cut-off used above. Kruskal-Wallis testing revealed a significant increase in the numbers of $CD4^+$ IFN-γ^+ cells in HIV infected persons receiving ART (p-value = 0.028, Figure 3.2. panel E), and this was further supported by a Wilcoxon test comparing the numbers of $CD4^+$ IFN-γ^+ cells at the start of ART and after 6 months also indicating a significant increase (p-value = 0.009). Cross sectional comparison with the HIV uninfected persons revealed HIV infected persons to have a significantly higher number of $CD4^+$ IFN-γ^+ cells compared to HIV uninfected persons (p-value = 0.013).

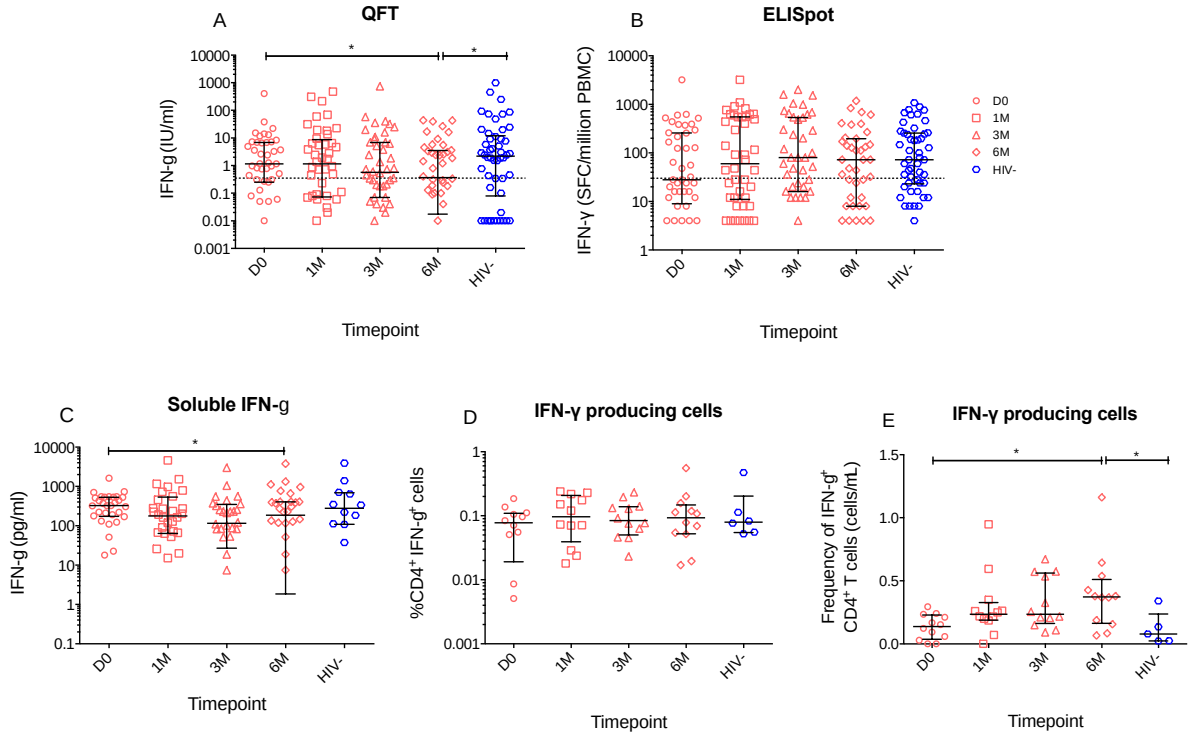

Figure 3. 2: Graphs indicating *Mtb* sensitisation, determined by various methods, in the two groups of persons included in this study. *Mtb* sensitisation determined by (A) the QFT IGRA in HIV infected (red, n=44) and HIV uninfected (blue, n=50) persons, with a positive response being ≥0.35 IU/ml, (B) the ELISpot IGRA in HIV infected (red, n=44) and HIV uninfected persons (blue, n=50), with a positive response being ≥30 SFC/million, (C) Soluble IFN-γ measured by Luminex in HIV infected (red, n=32) and HIV uninfected (blue, n=12) persons, (D) the proportion of *Mtb*-specific $CD4^+$ $IFN\text{-}\gamma^+$ T cells in HIV infected (red, n=12) and HIV uninfected (blue, n=6) persons determined by flow cytometry and (E) the number of $IFN\text{-}\gamma^+$ $CD4^+$ T cells in HIV infected (red, n=12) and HIV uninfected (blue, n=5) persons determined by flow cytometry.

Table 3. 3: Median and (IQR) longitudinal QFT, ELISpot, Luminex IFN-γ and cytokine^{+} responses determined by flow cytometry of all patients with corresponding p-values generated using the Kruskal Wallis test, non-parametric paired t-test and non-parametric unpaired t-test respectively.

Median (IQR)	HIV Infected Persons				HIV Uninfected Persons	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	1M	3M	6M				
Quantiferon (IU/ml)	1.2 (0.25-6.9)	1.2 (0.07-8.7)	0.57 (0.07-6.9)	0.37 (0.01-3.5)	2.2 (0.08-12)	>0.9999	0.0174	0.0343
Soluble IFN-γ $^{+}$ (pg/ml)	326 (172-534)	178 (64-535)	115 (27-349)	185 (1.9-404)	278 (109-688)	0.4029	0.0498	0.4095

Median (IQR)	HIV Infected Persons				HIV Uninfected Persons	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
ELISpot (SFC/million)	28 (9-259)	60 (11-549)	80 (16-536)	72 (8-196)	72 (23-257)	>0.9999	0.9058	0.3547
%CD4$^+$ IFN-γ$^+$ cells	0.078 (0.019-0.11)	0.096 (0.039-0.21)	0.084 (0.05-0.14)	0.093 (0.052-0.15)	0.08 (0.055-0.2)	>0.9999	0.518	0.981
Nr. CD4$^+$ IFN-γ$^+$ cells	0.14 (0.036-0.23)	0.23 (0.19-0.33)	0.24 (0.16-0.56)	0.37 (0.16-0.51)	0.079 (0.022-0.24)	0.028	0.0098	0.0136

Table 3. 4: Summary of *Mtb* sensitisation in all recruited persons in the HIV infected cohort illustrated by measurement of IFN-γ by QFT (IU/ml), ELISpot (SFC/million) and Luminex (pg/ml).

Patient ID:	QFT IFN-γ (IU/ml)				ELISpot IFN-γ (SFC/million)				Luminex IFN-γ (pg/ml)			
	D0	1M	3M	6M	D0	1M	3M	6M	D0	1M	3M	6M
1	0.25	0.49	0.6	0.24	16	72	80	32	223.14	0	228.29	127.14
2	0.82	5.56	0	0	164	440						
3	13.08	2.8	3.75	3.07	292	548	236	196	533.87	731.19	0	404.02
4	15.6	31.5	19.74	5.77	372	764	648	396	548.37	1156.93	1039.62	662.74
5	0.05	-0.03	-0.05	0	0	44	48	4	320.68	0	251.4	0
6	0.44	0.11	0.07	0.09	0	4	12	12	178.03	52.65	109.97	118.97
7	0.52	2.31	0.22	0	8	32	20					
8	11.85	6.48	10.92	16.77	124	636	984	688	511.49	192.01	357.88	959.31
9	22.2	59.5	6.9	3.6	4	296	540	172				
10	0.2	-9.76	41.2	7.67	16	136	1236	452				
11	3.57	0.88	0.73	0.31	124	12	60	44	736.05	226.41	0	115.03
12	0.75	0.03	0.04	0.1	0	44	16	8	110.08	273.51	0	0
13	7.31	9.45	0.3	1.2	16	400	120	72	703.77	458.73	211.02	0
14	38.2	6.3	5.12	3.3	112	140	200	132	1620.13	560.1	438.12	394.56
15	0.98	1.93	0.34	1.86	424	548	684	380	131.47	278.33	88.07	404.59
16	8.37	69	32	39.1	508	1096	1580	268				
17	3.7	18.24	24.6	0	580	624	1532					
18	3.25	1.37	0.8	1.43	28	16	80	112	367.77	202.33	231.24	227.11
19	0.01	0	57.7	26.4	-4		1040	408				
20	1.25	0.23	0.07	0.29	20	616	12	0	327.21	72.07	53.26	52.01
21	0.51	0.71	0.21	0	4	8	12					
22	6.55	1.74	6.89	2.89	52	24	80	72	534.58	66.27	370.99	771.25
23	0.27	0	0.35	0.4	12	28		4				
24	0.77	0.443	0.3	0.33	20	20	20	48	306.49	19.89	120.27	0
25	0.97	0.08	15.9	0.19	24	48	28	28	191.83	128.86	0	0

Patient ID:	QFT IFN-γ (IU/ml)				ELISpot IFN-γ (SFC/million)				Luminex IFN-γ (pg/ml)			
	D0	1M	3M	6M	D0	1M	3M	6M	D0	1M	3M	6M
26	0.13	0.09	0.05	0.18	4	4	0	8	384.21	0	101.22	272.16
27	405	478	743	42.6	3168	3200	1992	1176	10231.68	15764.73	11581.36	1317.66
28	6.86	3.85	1.85	5.55	64	92	100	112	209.39	235.28	0	224.52
29	0	0.06	0.54	0.13	0	4	0	72	51.28	162.05	7.41	145.95
30	1.15	1.02	0	0	104	48						
31	0.26	0.19	0.82	0	0	16	40					
32	15.18	22.56	2.43	4.53	392	908	532	128	553.61	978.93	255.23	372.19
33	4.96	0	0	0	124							
34	1.16	2.43	1.35	0.8	0	92	52	8	170.37	119.62	81.96	383.77
35	0.05	0.07	0.04	0.1	4	4	34	8	324.13	15.14	0	0
36	0.31	0.01	0.02	0.01	4	4	12	24	122.86	91.06	51.34	7.42
37	6.9	5.8	0.73	2.35	368	592	288	204	527.63	790.4	321.81	403.86
38	0.06	307	35.2	0	0	116	736					
39	13.98	19.29	6.19	9.18	608	824	484	140	219.91	163.36	82.63	133.84
40	2.42	0.73	0.45	2.4	212	4	20	148	22.68	67.05	18.66	18.76
41	0.31	215	0.18	-0.1	0	4	36	28	547.01	4597.97	562.06	0
42	7.22	4.65	9.03	16.2	520	552	300	836				
43	0	0.08	0.01	0	0	12	16	4				
44	2.6	13.8	40	43.5	248	492	616	612	0	1522.23	2991.08	3765.32
45	3.02	21.06	0	0	84	200						
46	0	-1.03	0.2	0	0	8	0	4	445.41	63.27	553.67	305.23
47	0.08	0.06	0.18	0.04	28	8	44	12	17.98	25.79	83.75	0
48	0	0.02	0.03	-0.16	0	12	0	4	362.45	73.29	251.85	294.35
49	1.17	0.95	-0.04	2.53	40	28	4	36	435.46	269.73	228.12	1119.92
50	0.87	0.87	0	0	72	136						

Shaded bars represent persons having IFN-γ measurements greater than the pre-determined cut-off values for *Mtb* sensitisation.

Cut off values were 0.35 IU/ml for the QFT, 30 SFC/million for the ELISpot and 100 pg/ml for the Luminex assays.

Table 3. 5: Summary of *Mtb* sensitisation in recruited persons in the HIV uninfected cohort illustrated by measurement of IFN-γ by QFT (IU/ml) and ELISpot (SFC/million).

Patient ID	QFT IFN-γ (IU/ml)	ELISpot IFN-γ (SFC/million)	Patient ID	QFT IFN-γ (IU/ml)	ELISpot IFN-γ (SFC/million)
156	48.5	48	184	997	884
157	5.9	244	185	451	324
158	2.9	232	186	0	12
159	0	8	187	73.7	760
160	92.8	672	188	250	776
161	3.7	188	189	9.2	460
162	1.9	280	190	0	24
163	0.1	24	191	0.3	8
164	2	100	192	0	0
165	0	8	193	7.8	188
166	0	0	194	1.8	44
167	Not done	Not done	195	0	16
168	24.5	108	196	0.3	36
169	1.9	180	197	0	56
170	2.4	184	198	11.3	148
171	24.3	1064	199	10.1	512
172	6.1	212	200	1.9	32
173	0.2	8	201	0.5	16
174	20.5	256	202	0	8
175	0	0	203	0	4
176	0.8	60	204	14.6	256
177	5	608	205	73	624
178	0.4	40	206	2.4	28
179	1.5	72	207	20	32
180	1.5	116	208	3	76
181	86.5	1068	209	3	28
182	0	8	210	9.2	424
183	2.7	44	211	4.1	60

Shaded bars represent persons having IFN-γ measurements greater than the pre-determined cut-off values for *Mtb* sensitisation. Cut off values were 0.35 IU/ml for the QFT and 30 SFC/million for the ELISpot assays.

Since *Mtb* sensitisation was a requirement for the aims of this study, a subset of *Mtb* sensitised HIV uninfected persons was selected as the control cohort for each experimental aim.

3.4.5. Baseline correlation data of Mtb sensitised HIV infected persons

The correlation between IFN-γ measured by Luminex and IFN-γ measured by ELISpot and Flow Cytometry intracellular staining at baseline, in HIV infected persons is summarised in Figure 3.3. and Table 3.6. The Spearman r correlation co-efficient determined for the QFT vs. the Luminex indicates no linear relationship between the variables. Additionally, a negative co-efficient also indicates an inverse relationship between the two variables tested. Similarly, the Spearman r correlation co-efficient determined for the ELISpot vs. Flow Cytometry also indicates no linear relationship between the variables, with the negative co-efficient also indicating an inverse relationship between the ELISpot and Flow Cytometry IFN-γ.

Table 3. 6: Correlation statistics between IFN-γ determined at D0 by QFT and Luminex and ELISpot and Flow Cytometry intracellular staining in HIV infected persons.

	QFT vs Luminex IFN-γ	ELISpot vs. Flow Cytometry ICS
Spearman r	-0.1364	-0.1434
P-value	0.4889	0.4846

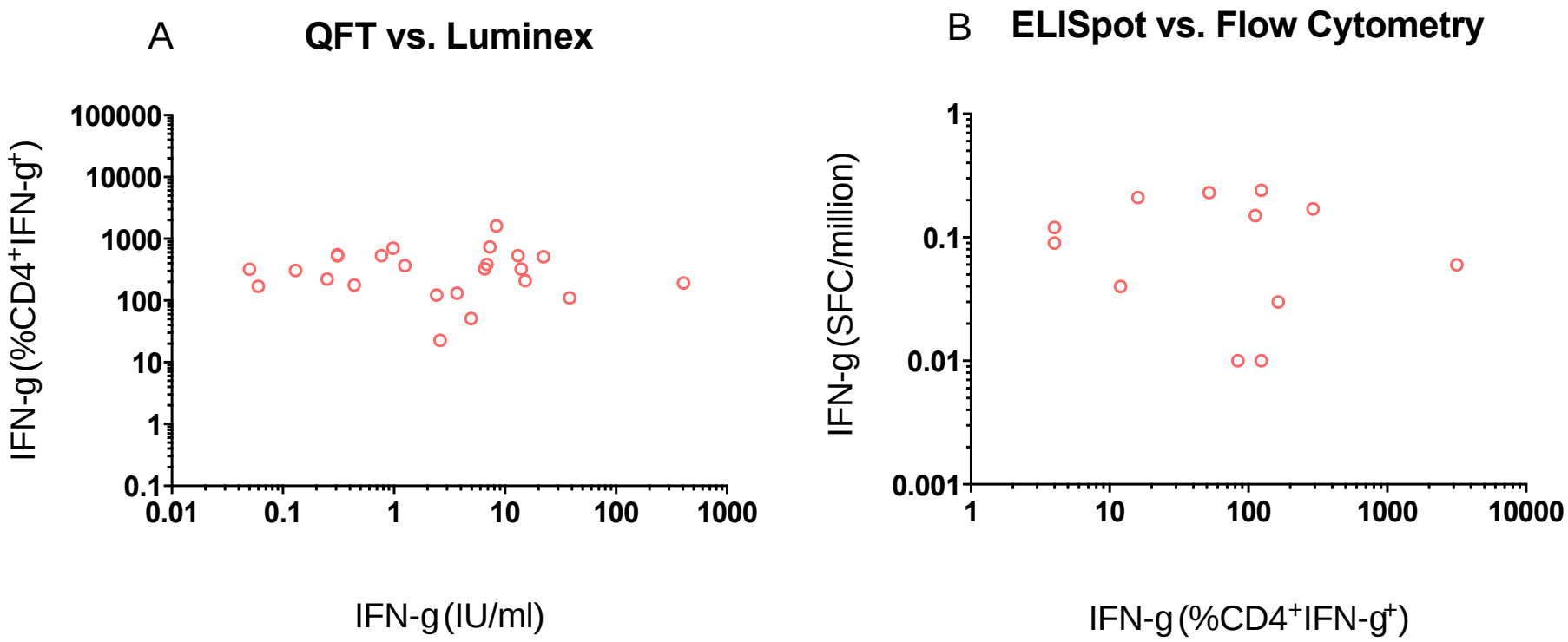

Figure 3. 3: Correlation between (A) IFN-γ determined at D0 by QFT (n=44) and Luminex (n=32) in HIV infected persons and (B) IFN-γ determined at D0 by ELISpot (n=44) and Flow Cytometry intracellular staining (n=26) in HIV infected persons.

3.5. Discussion

In order to determine *Mtb* sensitisation in our cohort of HIV infected persons receiving 6 months of ART, and our cohort of HIV uninfected persons, we used the QFT and ELISpot IGRAs. The cut-off values for a positive response to *Mtb* antigens were 0.35 IU/mL and 30 spot forming cells for the QFT and ELISpot assays respectively. Persons were regarded as *Mtb* sensitised if they registered a positive IFN-γ response (i.e. a reading higher than the above-mentioned cut-off values) for at least one timepoint, determined by either the QFT, ELISpot or Luminex assays. Discordant results between the QFT and ELISpot (observed in Tables 3.4 and 3.5) are expected due to within-subject variability and assay reproducibility [24]. The change in IFN-γ response is also expected and discordance between the two assays, per person, at one timepoint and not at another timepoint does not impact the sensitisation status of said person [24]. While we did not have a cut off value for the flow cytometry numbers of $CD4^+$ IFN-γ^+ cells analysis, *Mtb* sensitisation was also assessed by the measurement of CD4 T cell-specific soluble IFN-γ as well as the numbers and proportions of IFN-γ producing cells.

Our results indicated a significant decrease in the IFN-γ production determined by QFT in HIV infected persons during ART. Additionally, the IFN-γ levels in HIV infected persons were significantly lower than that observed for HIV uninfected persons. At D0 34/50 HIV infected persons were regarded as *Mtb* sensitised based on QFT readings. After 1 month of ART

32/50 HIV infected persons were *Mtb* sensitised based on positive QFT responses, with 27/50 after 3 months of ART and 23/50 after 6 months of ART, based on positive QFT readings. This phenomenon may be due to ART-mediated immune reconstitution resulting in the redistribution of memory T cells back into the blood compartment. Mthiyane *et al*. demonstrated a significant increase in the proportion of persons with positive IFN-γ responses during the first few months of receiving ART [15]. A possible reason for the discordance between our results and that of Mthiyane *et al.* may be due to the release of *Mtb* antigen into the bloodstream thus allowing increased detection.

Conversely there appears to be more cells secreting IFN-γ in HIV infected persons over time, as determined by the ELISpot assay, but this trend is not statistically significant. However, this was also observed in the number of $CD4^+$ IFN-γ^+ T cells flow cytometry data thus further supporting the ELISpot data. A significant expansion in the numbers of $CD4^+$ IFN-γ^+ T cells was observed during 6 months of ART in our cohort of HIV infected persons therefore also supporting the increasing trend of the number of IFN-γ^+ cells increasing determined using the ELISpot assay.

A possible reason for the discrepancy between the QFT and the ELISpot assays is because IFN-γ production is determined from a consistent number of cells (250 000 PBMC) using the ELISpot assay, while 1ml of blood is used for the QFT assay. Thus the total number of cells in the 1ml of blood is unknown at the time of sampling, and is therefore not consistent for every

person, and may possibly dilute the final number of cells able to respond to the antigenic stimuli and produce IFN-γ. Also worth mentioning is the point that different proportions of cells, e.g. memory T cells, may be present within the blood at the time of sampling and would therefore influence the IFN-γ production thus also accounting for differences between person to person. Interestingly, HIV uninfected persons at 6 months of ART had the same median number of IFN-γ-producing cells as HIV uninfected persons. Additionally, the antigens used in both assays differ with the QFT utilizing the TB7.7 antigen in addition to the RD-1 antigens, while the ELISpot uses only ESAT-6 and CFP-10 antigens [16-18].

In keeping with our QFT data the levels of soluble IFN-γ, determined by Luminex, decreased significantly in HIV infected persons on ART. HIV uninfected persons had higher levels of soluble IFN-γ compared to HIV infected persons at 6 months of ART. This is expected as QFT supernatants were collected post assay and stored for Luminex experiments.

Lastly the percentage of $CD4^+$ IFN-γ^+ cells determined by flow cytometry tended to increase in HIV infected persons after 6 months of ART, while HIV uninfected persons had a lower percentage of $CD4^+$ IFN-γ cells compared to HIV infected persons at 6 months of ART. The results of this assay are further supported by the ELISpot results, as both assays measure cell-specific IFN-γ. It is also worth noting that flow cytometry was only carried out for n=26 persons, while QFT and ELISpot assays were determined for n=32 persons. This was due to the availability of samples.

TST was once considered the gold standard in determining LTBI but this test has been shown to produce confounding results in persons that have received BCG vaccination, due to the presence of proteins that are common between *Mtb*, BCG substrains and other Non-Tuberculosis *Mycobacteria* (NTM), thus rendering a false positive response [19-21]. Given that South Africa is a country endemic to TB infection, and that infants receive the BCG vaccine at birth, the TST is confounded and may not be able to mount a response as sensitive as an IFN-γ-based technique [22, 23]. For this reason, the measurement of IFN-γ, in response to *Mtb*-specific antigen, may be useful in determining sensitisation primarily due to *Mtb*-specific antigen not being affected by BCG vaccination.

In conclusion we have demonstrated HIV infected and HIV uninfected persons included in our cohorts were sensitised to *Mtb* by at least one assay, namely the ELISpot, QFT, Luminex or intracellular flow cytometry (Figure 3.4 and Tables 3.4 and 3.5). Overall, these results indicate that *Mtb* sensitisation remains relatively consistent in our cohort of HIV infected persons throughout the duration of ART.

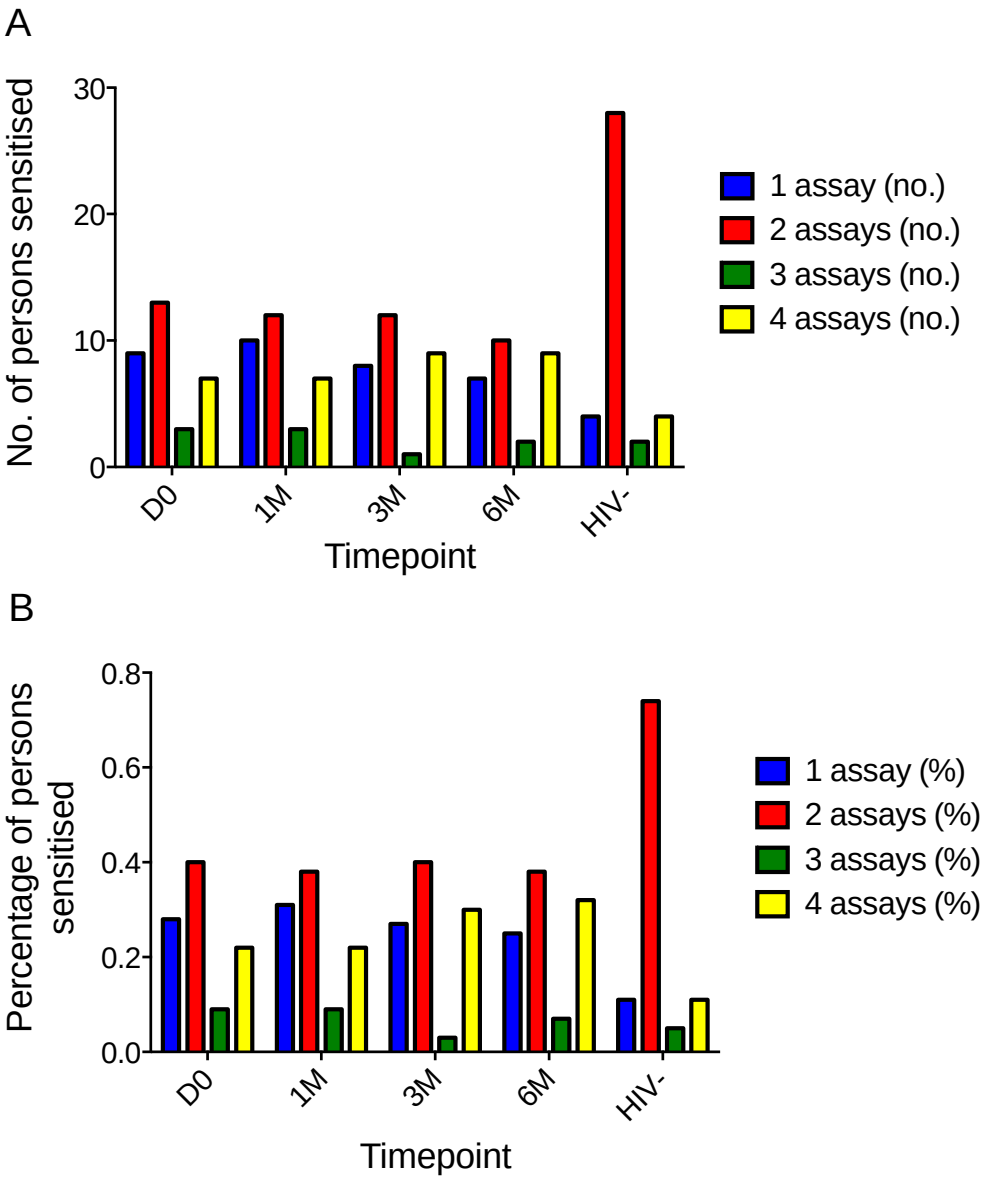

Figure 3. 4: The (A) number and (B) percentage of persons sensitised by 1, 2, 3 or 4 assays used to determine *Mtb* sensitisation stratified by time of ART. The 4 assays included are the QFT, ELISpot, $CD4^+$ $Cytokine^+$ positive T cells determined by flow cytometry and $CD4^+$ IFN-γ^+ positive T cells determined by flow cytometry.

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Chapter 4: Flow cytometric characterisation of *Mycobacterium tuberculosis* specific CD4$^+$ T cell reconstitution in HIV Infected persons starting antiretroviral therapy

4.1. Introduction

Mtb remains a global health concern, with 10.4 million people contracting TB in 2016 [1]. TB infection may persist lifelong and may lay dormant in >90% of individuals with the remaining 10% of individuals developing active TB disease. Although there are many potential vaccines that are currently in various phases of development, the only available vaccine against *Mtb* is BCG. BCG is an attenuated version of the *M. bovis* strain and is able to provide protection against disseminated TB and TB meningitis in young children, however, the effects of BCG remain inconsistent against pulmonary TB in adolescents and adults [2, 3]. BCG induces T_H1 immunity, and while this immunity is thought to last 10-15 years from vaccination [4] it may also provide immunity that exceeds several decades [5].

Upon exposure to antigen, be it via vaccination or upon infection, antigen specific T cells are activated, begin to proliferate and are able to differentiate into effector T cells. The cells that have gained an effector function act to clear the infection with a large proportion of cells dying and the remaining forming memory cells [6]. Memory forms the hallmark of the acquired

immune system with memory T cells conferring immediate protection in peripheral tissue and mounting recall responses to antigens in secondary lymphoid organs [7]. Effector memory T cells (T_{EM}) mediate protective memory while central memory T cells (T_{CM}) mediate reactive memory that home to T cell areas of secondary lymphoid organs, proliferate and differentiate to effector cells in response to antigenic stimulation [7]. T_{CM} express CD62L and CCR7 and produce the IL-2 cytokine while T_{EM} cells favour the production of effector cytokines such as IFN-γ and IL-4 [8]. Both CD4 and CD8 T cells differentiate into T_{CM} and T_{EM} cells respectively, with both capable of producing IFNγ following antigenic stimulation [7]. The presence of T_H1 CD4 T_{EM} secreting IFN-γ/TNF-α have been considered crucial in the composition of protective memory in the infected lung [9].

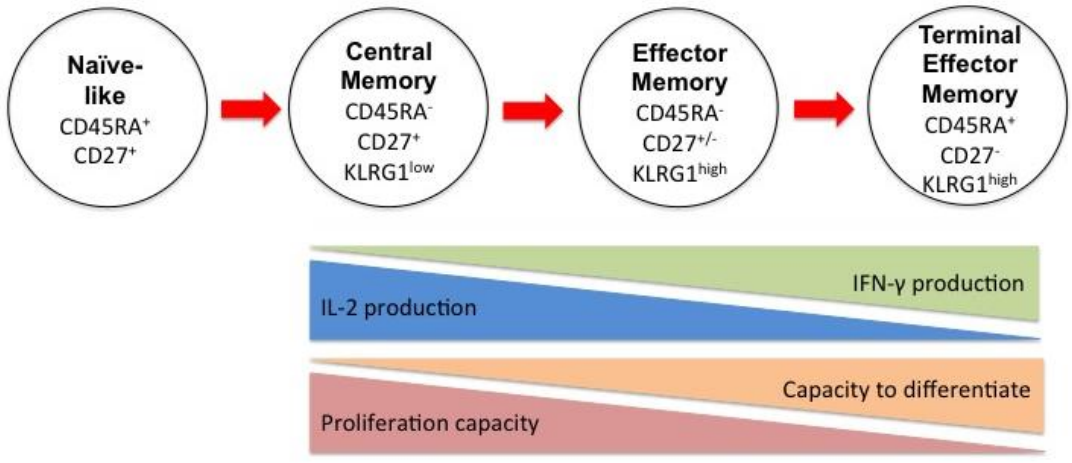

Figure 4. 1: Representation of the linear module demonstrating CD4 cell central memory and effector memory generation in humans. Adapted from Amyes *et al.* and Mahnke *et al.* [10, 11].

A previous study conducted in our lab demonstrated a significant expansion in the proportion of central memory CD4 T cells, characterised as $CD4^+$ $CD45RA^-CD27^+$, in HIV infected persons after receiving 12 weeks of reconstituting cART [12]. Additionally a corresponding significant decrease was found in the proportion of effector memory CD4 T cells, characterized as $CD4^+$ $CD27^-$ $CCR7^-$, in the same group of individuals after 12 weeks of ART. Wilkinson *et al.* thus illustrated that the strongest correlated of immunity was in fact central memory CD4 T cell driven, despite the effector function being associated with cART reconstitution.

Three studies further demonstrated the importance of protection driven by central memory T cells in animal models. Lindenstrøm and colleagues showed that BCG-primed mice boosted with the Ag85B and ESAT-6 subunit vaccine were able to control *Mtb* infection as a result of the expansion of CD4 central memory T cells, characterised as $CD4^+$ $KLRG1^-$ $IL\text{-}2^+$ [13]. Vogelzang *et al.* also used a mouse model and demonstrated that the recombinant BCG (rBCG) vaccine secreting the pore forming lysteriolysin (BCG Δ*ureC::hly)*-mediated protection was based on the expansion of central memory CD4 T cells that are $CXCR5^+$ $CCR7^+$ and express low levels of the transcription factors T-bet and Bcl-6 [14]. Vogelzang and colleagues further validated, using adoptive transfer techniques, that it was the $CD4^+$ central memory T cells rather than the $CD4^+$ effector memory T cells that mediated this protection against *Mtb* infection [14]. Lastly a recent study in which MtbΔ*sigH*-primed macaques were challenged with aerosolised *Mtb* CDC1551 revealed vaccinated macaques to elicit a strong $CD4^+$ and $CD8^+$

central memory driven response, in which T cells were characterized as being $CD4^+$ $CCR5^+$ [15].

The phenotype of memory T cells that reconstitute during 6 months of ART, in HIV infected persons sensitised by *Mtb*, is not well characterised. This study therefore aimed to analyse these T cells and determine their functionality. For the different T cell subsets, we compared the numbers and proportions at baseline with the numbers and proportions after 6 months of ART in HIV infected persons. Additionally, the same comparison was performed between HIV infected persons at 6 months of ART and HIV uninfected controls, in order to determine how cell numbers and proportions compare to that observed in the HIV uninfected cohort.

4.2. Patient Characteristics of individuals included in analysis

Twenty-six HIV infected persons had cryopreserved PBMC available for all longitudinal follow up timepoints. Additionally, stored PBMC was available from 28 HIV uninfected persons, sampled cross-sectionally. All HIV infected persons and HIV uninfected persons included in this aim were determined to be *Mtb* sensitised based on a positive IFN-γ response determined by either the QFT, ELISpot or the Luminex assay at at least one timepoint. Therefore all persons were considered to be sensitised to *Mtb*. The characteristics of all patients included in this arm of the study are summarized in Table 4.1. Although the median age of HIV infected persons differed significantly from the median age of HIV uninfected persons, we do not believe that this

difference impacted downstream statistics and subsequent significant differences in immunological markers measured. Longitudinal analysis of CD4 counts and viral loads revealed that the viral loads in HIV infected persons were suppressed to levels below the detection limit after 6 months of ART (p-value <0.0001) while CD4 counts increased significantly (p-value = 0.002) to a median of 343 cells/μL. HIV uninfected persons had a median CD4 count of 842 cells/μL.

Table 4. 1: Characteristics of patients included in the flow cytometry analysis (median with IQR)

	HIV Infected Persons (n=26)	HIV Uninfected Persons (n=28)	p-value
Median Age (years)	34 (29-38)	27 (21-34)	0.0028
Gender Male/Female	7/19	14/14	0.532

Table 4. 2: Change in CD4 counts and Viral loads during ART (median with IQR)

	HIV Infected Persons (n=26)				HIV Uninfected Persons (n=28)
	Day 0 of ART	**1M**	**3M**	**6M**	
CD4 count (cells/µL)	211 (160-254)	281 (178-364)	299 (182-394)	343 (210-424)	842 (673-1077)
Viral Load (copies/ml)	52 673 (25 895-119 799)	300 (110-613)	40 (39- 88)	39 (39-40)	N/A

4.3. Methods

4.3.1. Thawing cryopreserved PBMC

Cryopreserved PBMC were removed from liquid nitrogen and allowed to thaw in a 37-degree, 5% CO_2 incubator for 10 minutes. Thawed cells were transferred to fresh medium and washed, followed by counting using Trypan Blue (Sigma Life Sciences). 20µL of cells and 20µL 0.4% Trypan Blue (Sigma Life Sciences) were mixed briefly, 10µL was pipetted into a counting chamber and inserted into the Bio-Rad TC20 Automated cell counter (Bio-Rad). Cells were washed again, resuspended to a final concentration of $1x10^6$ cells /ml and aliquoted in FACS tubes (BD Biosciences). Cells were stimulated with (1) *Mtb* WCL at 20µg /ml; (2) SEB at 5µg /ml; and (3) left unstimulated as negative control. The tubes were incubated at 37 °C, 5% CO_2 for 3 hours prior to adding Brefeldin A (BFA) at 5µg /ml, followed by incubation overnight.

4.3.2. Surface and intracellular staining for flow cytometry

After overnight stimulation, cells were washed and first stained with LIVE/DEAD Red Dead Cell stain (Invitrogen) followed by surface markers using anti-CD19-APC-Cy7 and anti-CD14-APC-Cy7 (Invitrogen); anti-CD4-FITC and KLRG-1-PE.Cy5-5 (e-Bioscience); anti-HLA-DR-PE and anti-CXCR3-PE-Cy7 (BD Biosciences); anti-CCR4-BV510, anti-CD45RA-BV570, anti-CCR6-BV605, anti-CD3-BV650 and anti-CD27-BV711 (BioLegend). After incubation, cells were washed, permeabilized using the Cytofix/Cytoperm kit from BD Biosciences, and stained intracellularly for the

detection of cytokines using the following antibodies: anti-IFN-γ-Alexa Fluor 700 (BD Biosciences); anti-IL-2-PE and anti-IL-17A-Alexa Fluor 647 (BioLegend); and anti-TNF-α-eFluor450 (e-Bioscience). The cells were then fixed and acquired on a BD Fortessa flow cytometer with FACSDiva software (version 6).

Definition of a positive antigen specific response: *Mtb* specific responses for each sample were valid if the proportion of cells with positive intracellular cytokine staining in the antigen-stimulated tube was greater than twice that detected in the unstimulated tube. This is to ensure at least 50% of the cytokines being produced are as a result of the stimulation and not as a result of non-specific or endogenous stimuli. If antigen stimulated responses were less than twice of that detected in the unstimulated tube, a value of 0 was assigned to the sample. Cytokine responses were measured by subtracting the unstimulated from the antigen stimulated, and a value ≥0.05% for any cytokine was used as a cutoff to determine *Mtb* sensitization. This value is sum of the median and twice the standard deviation of the unstimulated/background (i.e. 0.05%) and serves to ensure that samples with a high background are not falsely reported as a positive response [21, 39]. Only positive cytokine responses were utelised for MFI and polyfunctional analyses. A detailed description of this assay can be found in chapter 2, section 2.12.3.

4.3.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6) using methods for non-parametric data. The Friedman test, with correction for multiple comparisons, was used for paired data comparison across all sampling time points in HIV infected individuals. There were four sampling timepoints (k=4) thus the Friedman test was performed with d.f. = 3 ('degrees of freedom' = k-1 and alpha = 0.05) in order to determine p-values. The Kruskal Wallis test with correction for multiple comparisons was used for unpaired data comparisons across all time points in HIV infected persons. The Wilcoxon test (equivalent of the non-parametric student t-test) was used to compare paired data in HIV infected persons at day 0 and 6 months of ART. Additionally we also compared cross-sectional data from the HIV infected persons at 6 months of ART with HIV uninfected persons using the Mann-Whitney test, used for unpaired data comparisons. A p-value less than or equal to 0.05 was considered significant. P-values are illustrated in subsequent figures by asterixes and mean the following: ns refers to a p-value > 0.05, a single asterisk (*) refers to a p-value ≤ 0.05, two asterix (**) refer to a p-value ≤ 0.01, three asterix (***) refer to a p-value ≤ 0.001 and four asterix (****) refer to a p-value ≤ 0.0001.

4.4. Results

4.4.1. Total CD4⁺ T cell analysis

4.4.1.1. Memory phenotype of CD4⁺ T cells during 6 months of ART

A summary of the T cell memory phenotypes is shown in Figure 4.2. and Table 4.3. Analysis of naïve-like T cells, characterized as $CD4^+CD27^+CD45RA^+$, in HIV infected persons revealed no significant overall change in proportions and numbers over 6 months of ART after correction for multiple comparisons between the four timepoints (Figure 4.2. panel A and E, p-values 0.1445 and 0.3673 respectively). However comparing paired data between day 0 and 6 months of ART using non-parametric paired t-test revealed a significant increase in the proportions and numbers of naïve-like T cells in HIV infected persons (p-values = 0.0176 and 0.0067 respectively). A cross-sectional comparison between the HIV uninfected and HIV infected persons at 6 months of ART, revealed that the numbers of naïve-like T cells were significantly lower in the HIV infected persons (p-values <0.0001), while the proportions were comparable.

Central memory T cells, characterized as $CD4^+CD27^+CD45RA^-$, also showed no significant change in the proportions and numbers over 6 months of ART (p-values = 0.5140 and 0.0633). However, paired comparison between baseline and 6 months of ART revealed a significant expansion both in proportion and numbers of central memory T cells (Figure 4.2. panel B and F, p-values 0.0081 and <0.0001 respectively). When comparing to HIV

uninfected persons, the numbers of central memory T cells were significantly lower in HIV infected persons at 6 months of ART (p-value <0.0001), while the proportions were comparable (p-value = 0.5220).

Contrary to the early differentiated cells described above, the proportion of effector memory T cells, characterized as $CD4^{+}CD27^{-}CD45RA^{-}$, significantly decreased (Figure 4.2. panel C, p-value = 0.001) after correction for multiple comparisons, in HIV infected persons during 6 months of ART. No significant change was observed for the numbers however (Figure 4.2. panel G, p-value >0.9999), after correction for multiple comparisons. Similar results were found when comparing proportions and numbers of effector memory T cells at baseline and 6 months of ART using non-parametric t-tests (p-values 0.0002 and 0.1730). Cross-sectional comparison between the HIV infected persons at 6 months of ART and the HIV uninfected persons revealed no significant difference in the proportion of effector memory T cells (p-value = 0.4365), however HIV uninfected persons had significantly higher numbers of effector memory T cells compared to HIV infected persons (p-value <0.0001).

Lastly terminal effector memory T cells, characterised as $CD4^{+}CD27^{-}CD45RA^{+}$, showed no significant difference in proportion and numbers in HIV infected persons over 6 months of ART using multiple comparison testing (Figure 4.2. panel D and H respectively, p-values 0.3608 and >0.9999 respectively). However non-parametric t-tests between baseline and 6 months of ART, revealed a significant decline in the proportion (p-value

=0.0407) but not numbers (p-value = 0.1014) of terminal effector memory T cells. Comparison with the HIV uninfected persons revealed no significant proportional difference between the two cohorts (p-value = 0.0891) but HIV uninfected persons had significantly higher numbers of terminal effector memory T cells (p-value = 0.0061) compared to HIV infected persons at 6 months of ART.

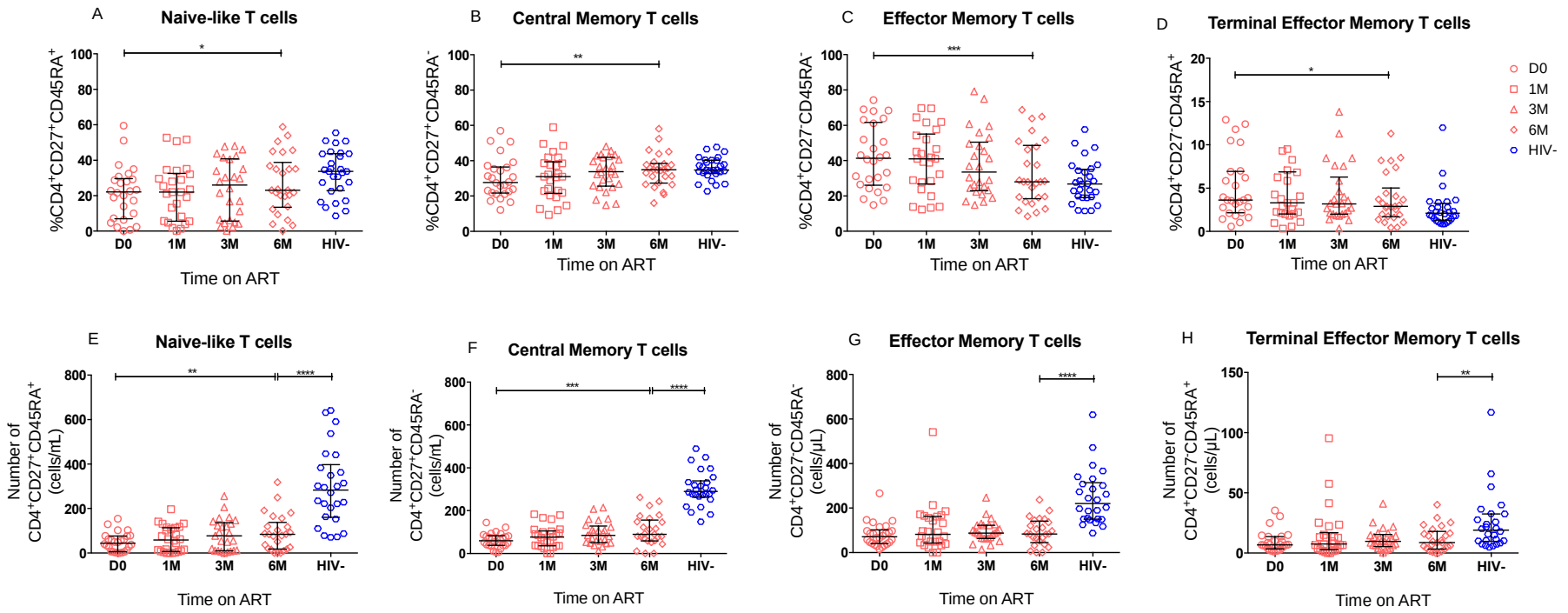

Figure 4. 2: Memory phenotype of CD4⁺ T cells based on CD27 and CD45RA surface expression determined by flow cytometry. A – D: Proportion of CD4+ T cells expressing, or not expressing, CD27 and CD45RA in HIV infected persons (red, n=26) during 6 months of ART, and HIV uninfected persons (blue, n=28). E – H: Number of CD4+ T cells expressing CD27 and CD45RA per 106 cells in HIV infected persons (red, n=26) during 6 months of ART, and HIV uninfected persons (blue, n=28). Graphs represent medians and IQR data for each cell subset and p-values were determined using the paired t-test.

Table 4. 3: Median and (IQR) frequency and numbers of $CD4^+$ T cells based on CD27 and CD45RA expression with corresponding p-values.

Median (IQR)	HIV Infected Persons (n=26)				HIV Uninfected Persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	**1M**	**3M**	**6M**				
$\%CD4^+$ $CD27^+CD45RA^+$	22 (7-30)	22 (5.6-34)	26 (5.7-41)	23 (13-43)	34 (23-44)	0.1445	0.0176	0.1913
$\%CD4^+$ $CD27^+CD45RA^-$	28 (22-36)	31 (21-41)	34 (26-42)	35 (27-39)	35 (33-40)	0.5140	0.0081	0.5220
$\%CD4^+$ $CD27^-CD45RA^-$	41 (26-62)	41 (25-57)	34 (23-50)	28 (17-50)	27 (19-35)	0.001	0.0002	0.4365
$\%CD4^+$ $CD27^-CD45RA^+$	3.6 (2.2-6.9)	3.3 (2-7)	3.2 (2-6.3)	2.9 (1.6-6.2)	2.1 (1.3-3.2)	0.3608	0.0407	0.0891

Median (IQR)	HIV Infected Persons (n=26)				HIV Uninfected Persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
Nrs/10^6 $CD4^+$ $CD27^+CD45RA^+$	44 (6.2-76)	59 (0-114)	77 (0.15-136)	84 (0-138)	283 (161-398)	0.3673	0.0067	<0.0001
Nrs/10^6 $CD4^+$ $CD27^+CD45RA^-$	59 (38-83)	76 (36-106)	84 (51-128)	89 (30-120)	290 (262-339)	0.0633	0.0008	<0.0001
Nrs/10^6 $CD4^+$ $CD27^-CD45RA^-$	71 (41-102)	82 (42-162)	88 (64-123)	84 (45-141)	220 (151-314)	>0.9999	0.1730	<0.0001
Nrs/10^6 $CD4^+$ $CD27^-CD45RA^+$	6.8 (3.4-14)	7.6 (12.8-17)	9.7 (5.3-15)	8.7 (3.4-18)	19 (9.6-32)	>0.9999	0.1014	0.0061

4.4.1.2. Activation and Senescence markers on CD4⁺ T cells during 6 months of ART

The expression of HLA-DR was used to determine activation in $CD4^+$ T cells, while KLRG-1 expression was used to determine senescence in $CD4^+$ T cells. A summary of the results is shown in Figure 4.3. and Table 4.4. After correction for multiple comparisons, the proportion (p-value < 0.0001) but not numbers (p-value >0.9999) of activated T cells, characterized as $CD4^+HLA\text{-}DR^+$, significantly decreased in HIV infected persons during 6 months of ART (Figure 4.3. panels A and C respectively). Non-parametric t-test similarly revealed a significant decrease in the proportion (p-value <0.0001) but not numbers (p-value = 0.5424) of activated T cells in HIV infected persons between day 0 and 6 months of ART. We next compared the proportions and numbers of activated T cells in HIV infected persons to that of HIV uninfected persons and found that HIV infected persons had a significantly higher proportion (p-value = 0.0005), but lower numbers (p-value = 0.0073), of activated T cells at 6 months of ART.

Correction for multiple comparisons revealed no significant change in the proportion and numbers of senescent T cells in HIV infected persons over 6 months of ART (Figure 4.3. panels B and D, p-values 0.3190 and <0.9999 respectively). However non-parametric t-test between baseline and 6 months of ART revealed a significant decrease in the proportion of KLRG-1+ T cells (p-value =0.0493). The numbers of KLRG-1+ T cells similarly tended to decline in HIV infected persons during ART (p-value = 0.0516). Comparing to

HIV uninfected persons, there was no significant difference in the proportion of KLRG-1^{+} T cells in HIV infected persons at 6 months of ART (p-value=0.3686), but the numbers were significantly higher compared to HIV infected persons at 6 months of ART (p-value = 0.0001).

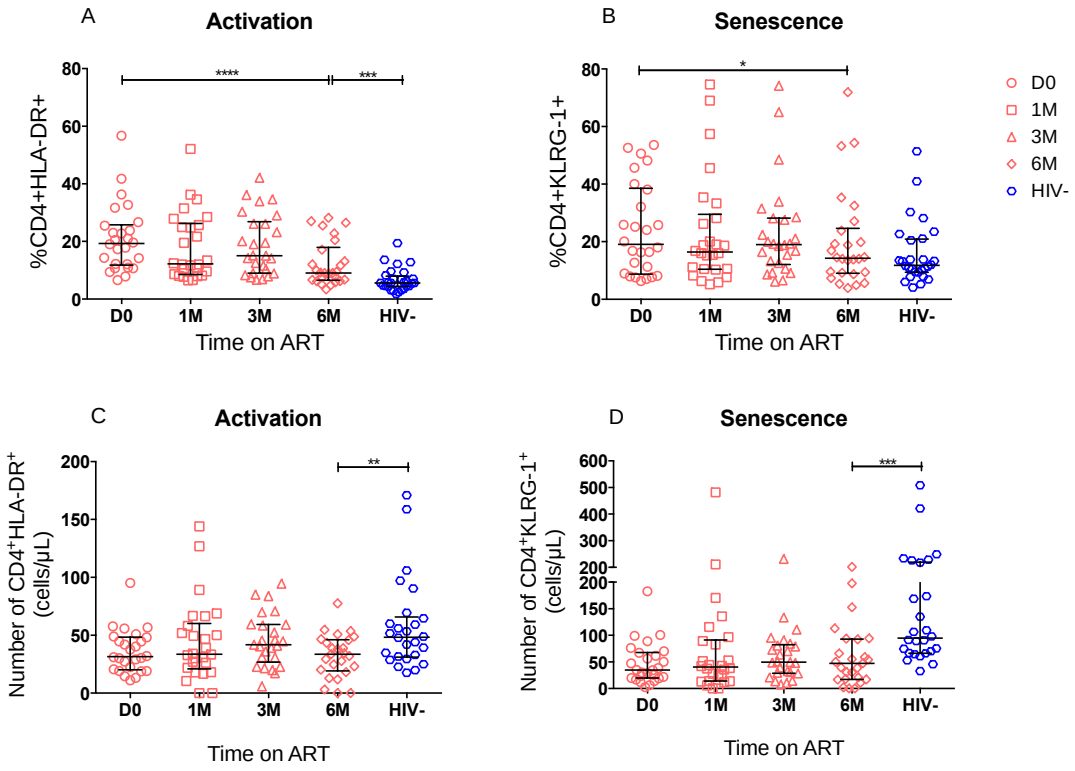

Figure 4. 3: Activation and Senescence of CD4^{+} T cells based on HLA-DR and KLRG-1 expression determined by flow cytometry.

Proportion of CD4^{+} T cells expressing HLA-DR (A) and KLRG-1 (B) in HIV infected persons (red, n=26) over 6 months of ART, and HIV uninfected persons (blue, n=28). Numbers of CD4^{+} T cells expressing HLA-DR (C) and KLRG-1 (D) in HIV infected persons (red, n=26) after 6 months of ART, and HIV uninfected persons (blue, n=28). Graphs represent medians and IQR data.

Table 4. 4: Median and (IQR) frequency and number of $CD4^+$ T cells expressing HLA-DR and KLRG-1 with corresponding p-values.

Median (IQR)	HIV Infected persons (n=26)				HIV Uninfected persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
$\%CD4^+$ $HLA\text{-}DR^+$	19 (12-26)	12 (8-26)	15 (9-27)	9 (6.1-18)	5.6 (4.4-7.9)	<0.0001	<0.0001	0.0005
$\%CD4^+$ $KLRG\text{-}1^+$	19 (8.7-39)	16 (8.1-29)	19 (12-28)	14 (6.9-25)	12 (9.4-21)	0.3190	0.0493	0.3686
$Nrs/10^6$ $CD4^+HLA\text{-}DR^+$	32 (20-48)	34 (21-60)	42 (27-59)	34 (19-46)	48 (31-66)	>0.9999	0.5424	0.0073
$Nrs/10^6$ $CD4^+KLRG\text{-}1^+$	35 (20-68)	40 (14-91)	50 (29-82)	47 (17-93)	95 (66-219)	>0.9999	0.0516	0.0001

4.4.1.3. Chemokine receptor expression on CD4⁺ T cells during 6 months of ART

A summary of the expression of the chemokine receptors, CXCR3, CCR4 and CCR6, on CD4⁺ T cells is shown in Figure 4.4. and Table 4.5. Non-parametric tests correcting for multiple comparisons revealed no significant change in the proportion of T cells expressing CXCR3 (p-value = 0.9755), CCR4 (p-value = 0.7957) or CCR6 (p-value = 0.1901) in HIV infected persons during 6 months of ART (Figure 4.4. panels A, B and C respectively). With the exception of CCR4, non-parametric t-testing mirrored the above mentioned trends with p-values = 0.4227 and 0.0993 for CXCR3 and CCR6 respectively. Additionally, non-parametric testing revealed a small but significant increase (p-value = 0.0414) in the proportion of T cells expressing the CCR4 chemokine receptor. Interestingly while the proportion of T cells expressing CXCR3 and CCR6 chemokine receptors was significantly higher in HIV uninfected persons compared to HIV infected persons at 6 months (p-values = 0.0019 and 0.0233), there was no significant difference in the proportion of T cells expressing CCR4 receptors between the groups (p-value = 0.3460).

Similarly, no significant change was observed in the numbers of T cells expressing CXCR3 (p-value > 0.999) or CCR6 (p-value = 0.9117) in HIV infected persons over 6 months of ART, after correction for multiple comparisons. However the numbers of T cells expressing CCR4 chemokine receptors significantly expanded (p-value = 0.0085). Non-parametric testing

in HIV infected persons between day 0 and 6 months of ART revealed a significant increase in T cell numbers expressing CXCR3 (p-value = 0.0096), CCR4 (p-value = 0.0001) or CCR6 (p-value = 0.0451) (Figure 4.4. panels D, E and F respectively). Lastly, cross-sectional comparisons revealed that HIV uninfected persons had significantly higher numbers of T cells expressing CXCR3 (p-value < 0.0001), CCR4 (p-value < 0.0001) or CCR6 (p-value < 0.0001) compared to HIV infected persons at 6 months of ART.

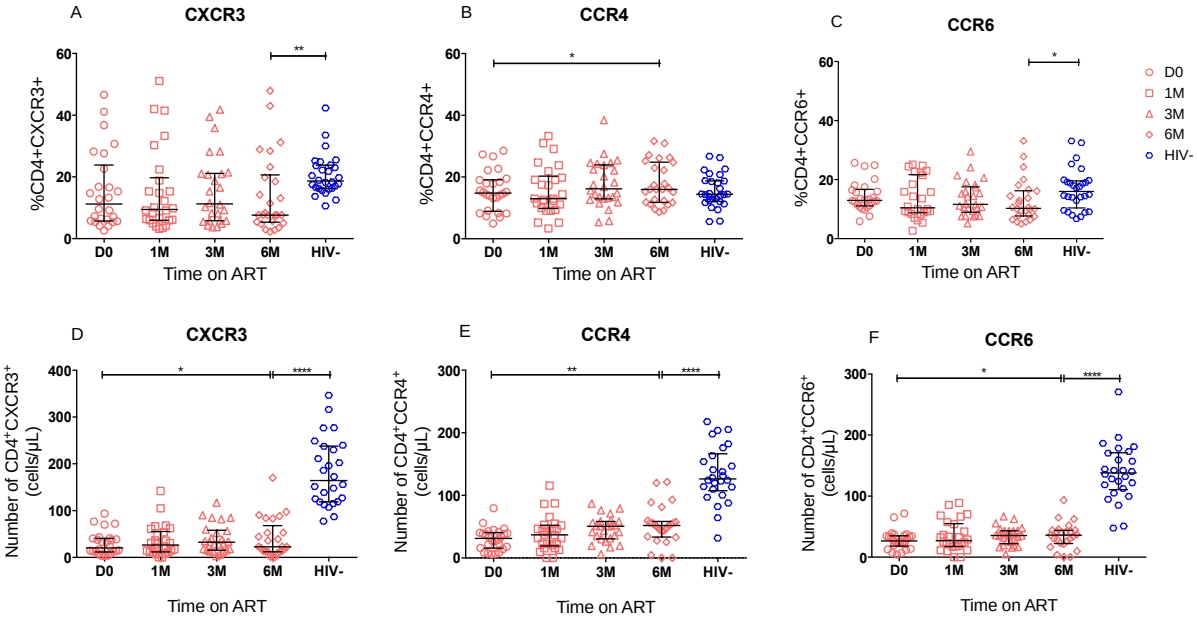

Figure 4. 4: Chemokine receptor expression on CD4⁺ T cells defined by flow cytometry analysis.

A – C: Proportion of $CD4^+$ T cells expressing CXCR3, CCR4 or CCR6 in HIV infected persons (red, n=26) after 6 months of ART, and HIV uninfected persons (blue, n=28). D – F: numbers of $CD4^+$ T cells expressing CXCR3, CCR4 or CCR6 per 10^6 cells in HIV infected persons (red, n=26) after 6 months of ART, and HIV uninfected persons (blue, n=28). Graphs represent medians and IQR data.

Table 4. 5: Median and (IQR) frequency and number of $CD4^+$ T cells expressing CXCR3, CCR4 or CCR6 with corresponding p-values.

Median (IQR)	HIV Infected persons (n=26)				HIV Uninfected persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	**1M**	**3M**	**6M**				
$\%CD4^+$ $CXCR3^+$	11 (5.7-24)	9.1 (4.8-20)	11 (5.8-21)	7.6 (4.4-21)	19 (16-24)	0.9755	0.4227	0.0019
$\%CD4^+$ $CCR4^+$	15 (8.9-19)	13 (9.1-20)	16 (13-24)	16 (11-25)	14 (12-19)	0.7957	0.0414	0.3460
$\%CD4^+$ $CCR6^+$	13 (11-17)	10 (7.9-22)	12 (8.9-18)	10 (6.4-16)	16 (10-20)	0.1901	0.0993	0.0233

Median (IQR)	HIV Infected persons (n=26)				HIV Uninfected persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	**1M**	**3M**	**6M**				
Nrs/10^6 $CD4^+CXCR3^+$	20 (12-41)	26 (12-55)	33 (15-58)	23 (12-68)	164 (119-238)	>0.9999	0.0096	<0.0001
Nrs/10^6 $CD4^+CCR4^+$	31 (15-40)	37 (19-52)	50 (30-58)	52 (33-58)	126 (107-166)	0.0085	0.0001	<0.0001
Nrs/10^6 $CD4^+CCR6^+$	27 (18-35)	27 (17-55)	36 (22-43)	36 (22-44)	128 (111-171)	0.9117	0.0451	<0.0001

4.4.1.4. Cytokine production by CD4⁺ T cells during 6 months of ART

A summary of the cytokines, IFN-γ, TNF-α, IL-2 and IL-17A, produced by *Mtb* WCL stimulated CD4⁺ T cells, is shown in Figure 4.5 and Table 4.6. Figure 4.6. indicating the baseline levels of cytokines is also included, in which unstimulated PBMC is used to determine basal cytokine production.

Using non-parametric tests correcting for multiple comparisons, we noted that the proportion and numbers of T cells producing IFN-γ, defined as CD4⁺ IFN-γ⁺, remained unchanged in HIV infected persons during 6 months of ART (Figure 4.5. panels A and E, p-values >0.9999 and 0.7453 respectively). Comparing paired data between day 0 and 6 months of ART also showed no significant change in the proportion of CD4⁺ IFN-γ⁺ cells (p-value = 0.7660), however the numbers of CD4⁺ IFN-γ⁺ cells showed a significant increase (p-value = 0.0127) in these persons. Cross-sectional comparison with HIV uninfected persons revealed no significant difference in the frequency or numbers of CD4⁺ IFN-γ⁺ cells at 6 months of ART (p-values = 0.4264 and 0.3222 respectively).

The proportion of cells producing TNF-α in response to *Mtb* WCL stimulation, defined as CD4⁺TNF-α⁺, significantly decreased in HIV infected persons over 6 months of ART (Figure 4.5. panel B, p-value = 0.003) after correction for multiple comparisons. A similar observation was made when comparing day 0 with 6 months of ART (p-value = 0.0130), however the numbers of CD4⁺TNF-α⁺ cells remains unchanged in those with a measurable response

(Figure 4.5. panel F, p-value = 0.1909). HIV uninfected persons had a significantly lower proportion (p-value <0.0001) and numbers (p-value = 0.0053) of $CD4^+TNF\text{-}\alpha^+$ cells, compared to HIV infected persons at 6 months of ART.

Similar to TNF-α, the proportion of IL-2 producing T cells in response to *Mtb* WCL stimulation, defined as $CD4^+IL\text{-}2^+$ T cells, significantly decreased in HIV infected persons over 6 months of ART (Figure 4.5. panel C, p-value = 0.0005) following correction for multiple comparisons. Paired comparison between day 0 and 6 months data also showed a significant decrease (p=0.002). Interestingly however, the numbers $CD4^+IL\text{-}2^+$ T cells in HIV infected persons during 6 months of ART tended to increase, although not reaching significance (Figure 4.5. panel G, p-value = 0.0826). Cross-sectional comparison showed HIV infected persons at 6 months of ART to have significantly higher proportion (p-value < 0.0001), but not numbers (p-value = 0.1932) of $CD4^+IL\text{-}2^+$ T cells compared to the HIV uninfected persons.

The proportion of IL-17A producing T cells, defined as $CD4^+IL\text{-}17A^+$, significantly decreased in HIV infected persons during 6 months of ART (Figure 4.5. panel D), p= 0.0005 using non-parametric t-tests and p= 0.0035 with correction for multiple comparisons. The numbers of $CD4^+IL\text{-}17A^+$ T showed no change in HIV infected persons during 6 months of ART (p-value = 0.1909 with a p-value > 0.999 after correction for multiple comparisons). HIV uninfected persons had significantly higher proportions (p-value <

0.0001) but not numbers (p-value = 0.5832) of $CD4^{+}IL\text{-}17A^{+}$ T cells, compared to HIV infected persons at 6 months of ART. However this was only observed in n=4 patients for the number of IL-17 producing cells.

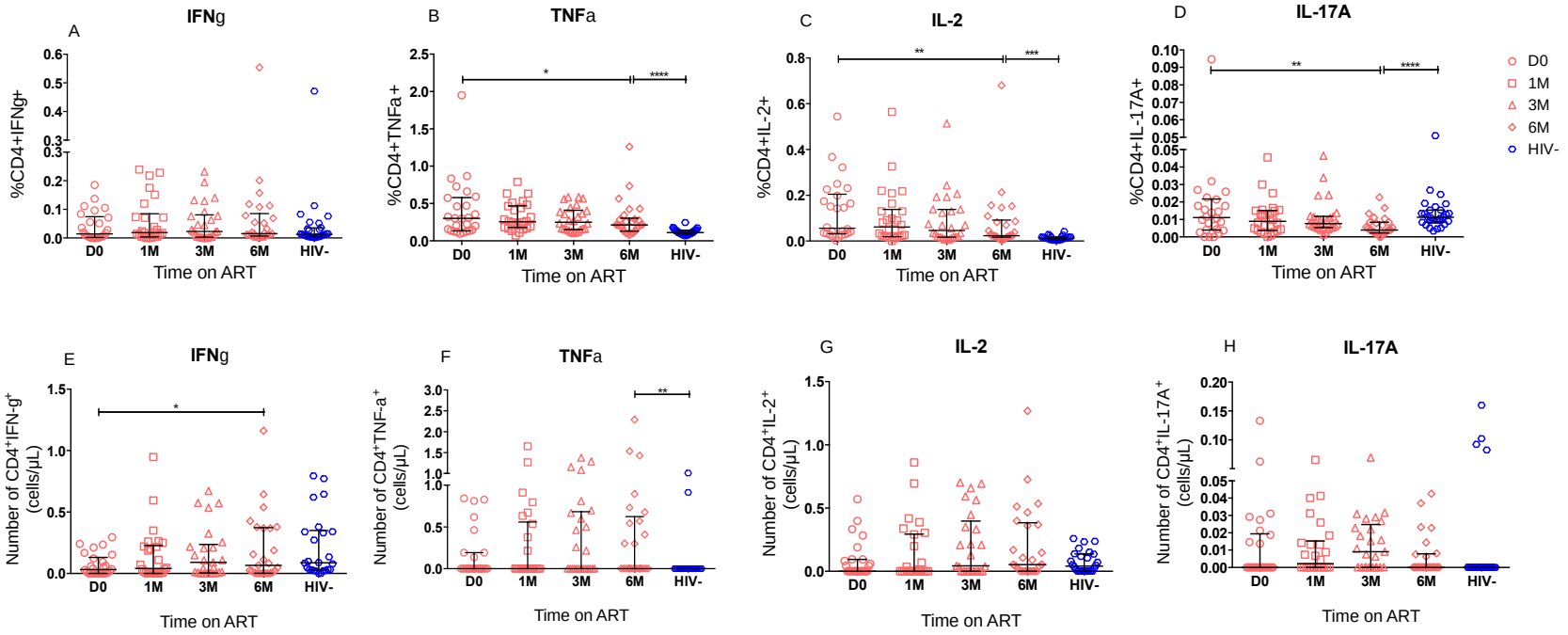

Figure 4. 5: Cytokine production of CD4⁺ T cells determined by flow cytometry.

A – D: Proportion of CD4⁺ T cells producing IFN-γ, TNF-α, IL-2 or IL-17A in HIV infected persons (red, n=26) during 6 months of ART, and HIV uninfected persons (blue, n=28). E – H: Numbers of CD4⁺ T cells producing IFN-γ, TNF-α, IL-2 or IL-17A in HIV infected persons (red, n=26) during 6 months of ART, and HIV uninfected persons (blue, n=28). Graphs represent medians and IQR of data.

Table 4. 6: Median and (IQR) frequency and number of $CD4^+$ T cells producing IFN-γ, TNF-α, IL-2 or IL-17A with corresponding p-values.

Median (IQR)	HIV Infected Persons (n=26)				HIV Uninfected Persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
$\%CD4^+$ IFN-γ^+	0.014 (0.0018-0.074)	0.019 (0.0039-0.085)	0.023 (0.0032-0.081)	0.015 (0.0065-0.086)	0.012 (0.0042-0.035)	>0.9999	0.7660	0.4264
$\%CD4^+$TNF-α^+	0.30 (0.14-0.58)	0.26 (0.18-0.47)	0.25 (0.15-0.41)	0.21 (0.13-0.3)	0.11 (0.088-0.14)	0.0158	0.0130	<0.0001
$\%CD4^+$IL-2^+	0.056 (0.032-0.21)	0.061 (0.019-0.14)	0.046 (0.017-0.14)	0.023 (0.017-0.091)	0.012 (0.0055-0.018)	0.0005	0.0020	<0.0001
$\%CD4^+$IL-17A^+	0.011 (0.0039-0.022)	0.0090 (0.0037-0.015)	0.0077 (0.0054-0.012)	0.0039 (0.0023-0.0084)	0.011 (0.0085-0.015)	0.0035	0.0005	<0.0001

Median (IQR)	HIV Infected Persons (n=26)				HIV Uninfected Persons (n=28)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	**1M**	**3M**	**6M**				
Nrs/10^6 $CD4^+$ $IFN\text{-}\gamma^+$	0.032 (0-0.13)	0.041 (0-0.23)	0.091 (0-0.24)	0.066 (0-0.37)	0.087 (0.02-0.35)	0.7453	0.0127	0.3222
Nrs/10^6 $CD4^+TNF\text{-}\alpha^+$	0.0 (0-0.19)	0.0 (0-0.56)	0.0 (0-0.68)	0.0 (0-0.62)	0.0 (0-0)	>0.9999	0.1909	0.0053
Nrs/10^6 $CD4^+IL\text{-}2^+$	0.0 (0-0.094)	0.0 (0-0.29)	0.043 (0-0.4)	0.053 (0-0.38)	0.041 (0-0.14)	0.3490	0.0826	0.1932
Nrs/10^6 $CD4^+IL\text{-}17A^+$	0.0 (0-0.019)	0.0022 (0-0.015)	0.0091 (0-0.025)	0.0 (0-0.0078)	0.0 (0-0)	>0.9999	0.1909	0.5832

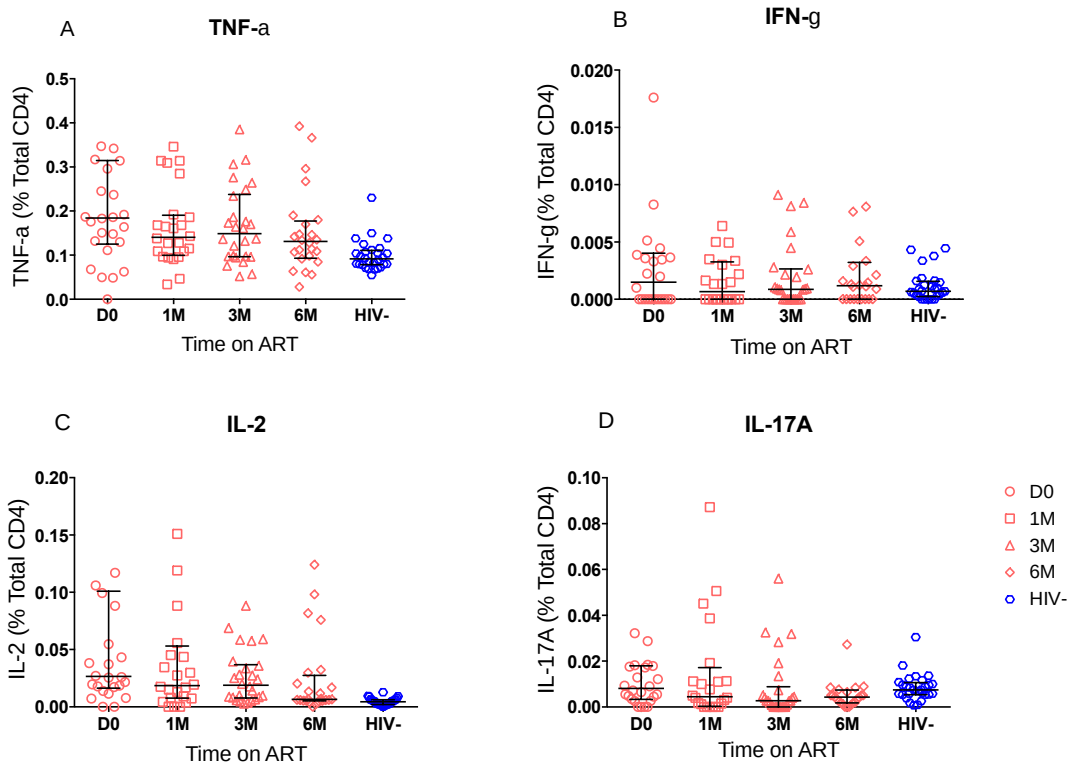

Figure 4. 6: Measurement of cytokines from unstimulated PBMC determined by flow cytometry.

Unstimulated PBMCs were isolated from HIV infected persons receiving 6 months of ART (n=26, red), and HIV uninfected persons (n=28, blue) and were intracellularly stained for (A) TNF-α, (B) IFN-γ, (C) IL-2 and (d) IL-17A.

4.4.1.5. Assessment of Mtb-specific CD4⁺ T cell functionality based on cytokine production

This section focuses on the qualitative cytokine data from *Mtb*-specific CD4⁺ T cells. Boolean gating was used in order to determine the functionality of *Mtb*-specific CD4⁺ T cells and this analysis was conducted in order to assess

the functional capacity *of* *Mtb*-specific $CD4^+$ T cells from HIV infected persons regained following 6 months of ART.

Our findings identified eight distinct cytokine-producing antigen-specific $CD4^+$ T cell populations in HIV infected and uninfected persons, summarised in Figure 4.7. and Table 4.7. These populations were further grouped based on the number of cytokines each population was able to produce (i.e. 1, 2 or 3 cytokines), summarised in Figures 4.7. and 4.8. and Table 4.7.

Our *Mtb*-specific qualitative cytokine data showed that HIV infected persons at day 0 of ART had a single cytokine positive (IFN-γ^+ or TNF-α^+ or IL-2^+) cell dominant response (64%), followed by the double positive cytokine producing cells (26%) and lastly the triple positive cytokine producing cells (10%). The proportion of triple positive cytokine producing cells significantly increased in HIV infected persons after 6 months of ART (p-value = 0.0121) and was significantly higher than proportions observed in the HIV uninfected persons (Figure 4.8. panel A, p-value = 0.0008).

Interestingly, the cytokine response at 6 months of ART in HIV infected persons was dominated by the *Mtb*-specific double positive cytokine producing cells (55%) followed by the *Mtb*-specific single positive cytokine producing cells (25%) and the *Mtb*-specific triple positive cytokine producing cells (20%). When compared with the HIV uninfected persons, higher proportions of the double positive cytokine producing cells were observed in the HIV infected persons at 6 months of ART. Further analysis revealed that

the IL-2$^+$TNF-α$^+$ and the TNF-α$^+$IL-17A$^+$ *Mtb*-specific cytokine subsets (Figure 4.8. panels D and E respectively) may have contributed to the increased proportion of double positive cytokine producing cells as the proportion of these cells appear to increase, although not significantly (p-values = 0.3905 and 0.1421 respectively), in HIV infected persons throughout 6 months of ART. Proportions of *Mtb*-specific IL-2$^+$TNF-α$^+$ and the TNF-α$^+$IL-17A$^+$ double positive cytokine secreting cells were higher in HIV infected persons at 6 months of ART (p-values <0.0001 and 0.2040 respectively) than proportions observed in HIV uninfected persons.

The single positive (IFN-γ$^+$ or TNF-α$^+$ or IL-2$^+$) cytokine producing cells dominated the response (78%) in HIV uninfected persons (Figure 4.7. panel C), followed by the double positive cytokine producing cells (19%) and lastly the triple cytokine producing cells (3%). When we assessed the *Mtb*-specific CD4$^+$ single positive cytokine producing T cells individually, we noted that HIV uninfected persons had significantly higher proportions of *Mtb*-specific IFN-γ$^+$ single positive cells, and *Mtb*-specific IL-17A$^+$ single positive cells compared to HIV infected persons during 6 months of ART (Figure 4.8. panel F and H, p-values < 0.0001 respectively). The higher proportions of these single cytokine producing cells thus contribute to the dominance observed in HIV uninfected persons. Although not significant, HIV uninfected persons also had higher proportions of *Mtb*-specific IL-2$^+$ single positive cells compared to HIV infected persons after 6 months of ART (Figure 4.8. panel G, p-value = 0.4066).

Longitudinal analysis revealed that the proportions of *Mtb*-specific IL-17 single positive cells did not differ in HIV infected persons during 6 months of ART (p-value = 0.2643) however compared to HIV uninfected persons, significantly higher proportions of *Mtb*-specific IL-17 single positive cells were observed in HIV uninfected persons (Figure 4.8. panel H, p-value <0.0001). The proportions of *Mtb*-specific IL-2 single positive cells showed a significant decrease in the proportion of cells in HIV infected persons during ART (Figure 4.8. panel C, p-value <0.0001), while *Mtb*-specific single positive IFN-γ secreting cells remained unchanged in HIV infected persons during ART (Figure 4.8. panel F, p-value 0.2437).

Additionally, the longitudinal analysis of *Mtb*-specific IFN-γ^+TNF-α^+ double positive cells increased in HIV infected persons during 6 months of ART (Figure 4.8. panel B, p-value = 0.2437) although not significantly, and proportions of *Mtb*-specific IFN-γ^+TNF-α^+ double positive cells were similar in HIV infected persons (p-value = 0.0520). Lastly, the proportion of *Mtb*-specific IL-2^+IL-$17A^+$ double positive cells significantly decreased in HIV infected persons during ART (Figure 4.8. panel C, p-value = 0.0494) and significantly higher proportions of this cell subset were observed in HIV uninfected persons (p-value = 0.0219).

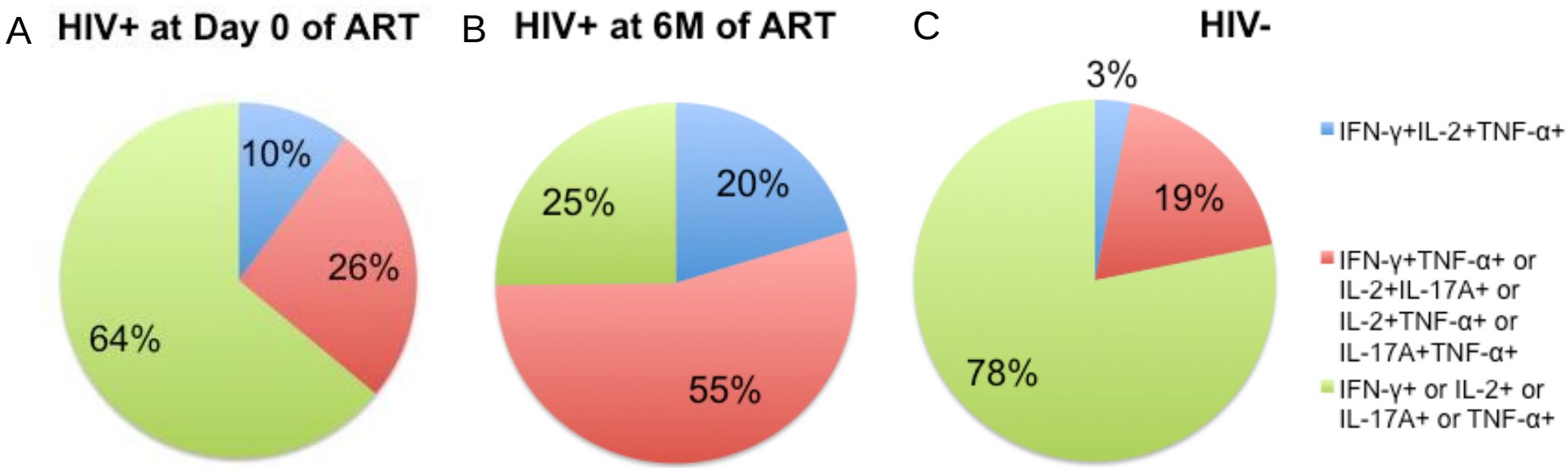

Figure 4. 7: Pie charts showing co-production of cytokines from *Mtb*-specific CD4⁺ T cells determined by flow cytometry analysis. Pie chart representing the polyfunctional capacity of *Mtb*-specific CD4⁺ T cells in (A) HIV infected persons at day 0 of ART, (B) HIV infected persons at 6 months of ART and (C) HIV uninfected persons. Blue slices represent the proportion of cells producing three of the cytokines measured, in this case IFN-γ⁺TNF-α⁺IL-2⁺ producing cells, while the red slices represent the proportion of cells producing a combination of any two cytokines and the green slices represent cells producing only one cytokine.

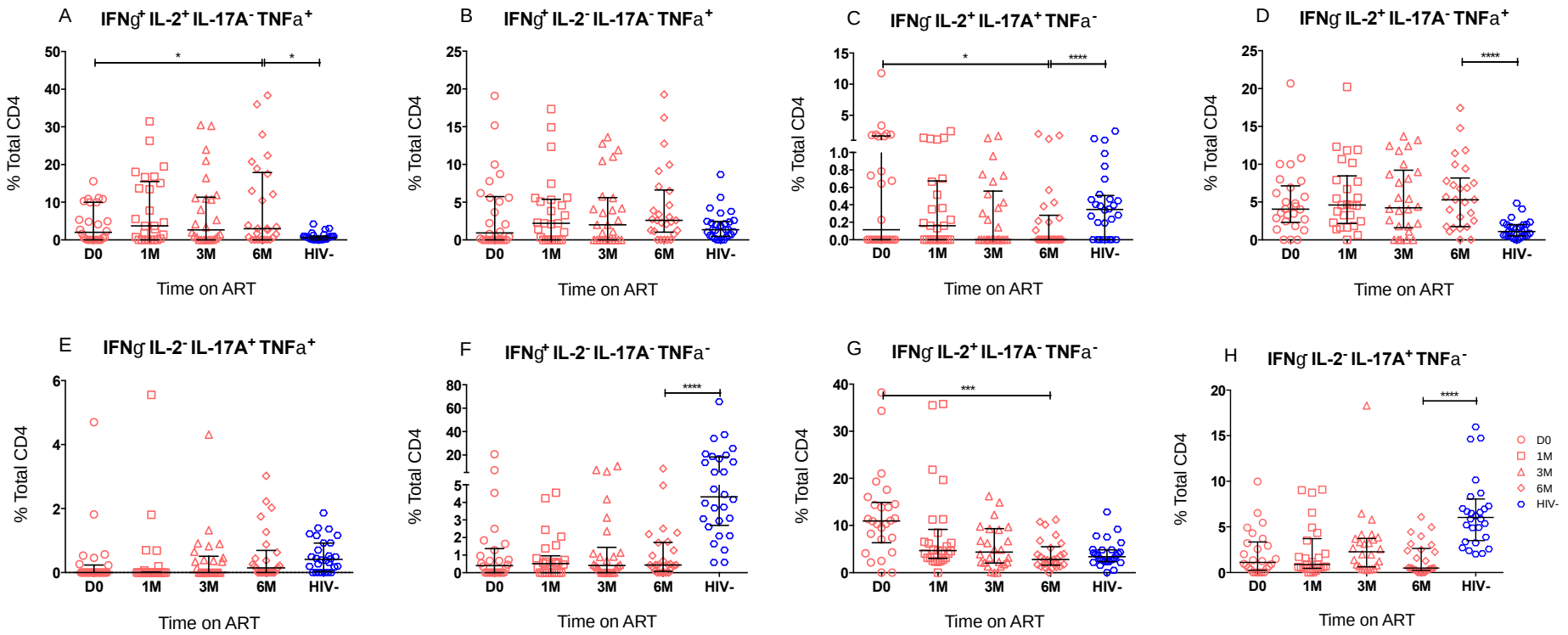

Figure 4. 8: Frequency of *Mtb* specific CD4⁺ T cells expressing IFN-γ, TNF-α, IL-2 and IL-17A cytokines as measured by flow cytometry.

Table 4. 7: Median and (IQR) frequency of $CD4^+$Cytokine$^+$ T cells producing IFN-γ, TNF-α IL-2 and IL-17A cytokines and corresponding p-values.

Median (IQR)	HIV Infected Persons (n=12)				HIV Uninfected Persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	**1M**	**3M**	**6M**				
%$CD4^+$IFN-γ^+ IL-2^+ IL-$17A^-$ TNF-α^+	2.0 (0-10)	3.7 (0-15)	2.6 (0-11)	3.0 (0-18)	0.56 (0.024-1.)	0.3190	0.0121	0.0080
%$CD4^+$IFN-γ^+ IL-2^- IL-$17A^-$ TNF-α^+	0.93 (0-8.5.7)	2.2 (0-5.4)	2.0 (0-5.6)	2.6 (0-6.6)	1.4 (0.49-2.4)	>0.9999	0.2437	0.0520
%$CD4^+$IFN-γ^+ IL-2^- IL-$17A^-$ TNF-α^-	0.4 (0.0-1.4)	0.51 (0-0.96)	0.41 (0-1.4)	0.44 (0.075-1.7)	4.3 (2.7-18)	>0.9999	0.3662	<0.0001
%$CD4^+$IFN-γ^- IL-2^+ IL-$17A^+$ TNF-α^-	0.11 (0-1.7)	0.16 (0-0.67)	0.0 (0-0.56)	0.0 (0-0.28)	0.35 (0-0.51)	0.4070	0.0494	0.0219

Median (IQR)	HIV Infected Persons (n=12)				HIV Uninfected Persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
%$CD4^+$IFN-γ^- $IL\text{-}2^+$ $IL\text{-}17A^-$ $TNF\text{-}\alpha^+$	4.0 (2.3-7.1)	4.6 (2.2-8.5)	4.2 (1.6-9.2)	5.3 (1.7-8.2)	1.1 (0.53-2.0)	>0.9999	0.3905	<0.0001
%$CD4^+$IFN-γ^- $IL\text{-}2^+$ $IL\text{-}17A^-$ $TNF\text{-}\alpha^-$	11 (6.4-15)	4.7 (3.0-9.2)	4.4 (2.0-9.4)	2.8 (1.6-5.5)	3.4 (2.3-4.8)	<0.0001	<0.0001	0.4066
%$CD4^+$FN-γ^- $IL\text{-}2^-$ $IL\text{-}17A^+$ $TNF\text{-}\alpha^+$	0.0 (0-0.24)	0.0 (0-0.016)	0.0 (0-0.51)	0.15 (0-0.69)	0.41 (0.044-0.92)	0.5755	0.1421	0.2040
%$CD4^+$IFN-γ^- $IL\text{-}2^-$ $IL\text{-}17A^+$ $TNF\text{-}\alpha^-$	1.1 (0.24-3.4)	0.9 (0.46-3.7)	2.3 (0.62-3.7)	0.48 (0.23-2.6)	6.0 (3.5-7.1)	>0.9999	0.2643	<0.0001

4.4.2. Analysis of Mtb specific CD4⁺ T cells

Mtb specific cells were defined as at least 0.05% of $CD4^+$ T cells positive for either IFN-γ, TNF-α or IL-2 cytokine following subtraction of the unstimulated sample (i.e. *Mtb* specific cells= *Mtb* WCL - Nil ≥ 0.05%).

4.4.2.1 Memory phenotype of Mtb specific T cells in response to 6 months of ART

A summary of the *Mtb*-specific T cell memory phenotypes are shown in Figure 4.9. and Table 4.8. The proportion of naïve-like ($CD4^+Cytokine^+CD27^+CD45RA^+$), central memory ($CD4^+Cytokine^+CD27^+CD45RA^-$) and effector memory T cells ($CD4^+Cytokine^+CD27^-CD45RA^-$), remained unchanged in HIV infected persons during 6 months of ART (Figure 4.9. panels A – C respectively, p-values = 0.9893, 0.7796 and > 0.9999 respectively), the numbers of these cells tended to increase, although not significantly with the exception of the effector memory T cells (Figure 4.9. panels E - G, p-values = 0.2025, 0.0520 and 0.0297 respectively). Cross-sectional comparison with the HIV uninfected persons revealed the HIV infected persons at 6 months of ART to have a lower proportion and numbers of *Mtb*-specific naïve-like T cells (p-values = 0.4888 and 0.0077), while no difference in proportion of central memory and effector memory T cells was observed between the two groups (p-value = 0.9859 and > 0.9999). However, HIV uninfected persons had significantly higher numbers of *Mtb*-specific central memory and effector memory T cells (p-value = 0.0001 and 0.006) compared to HIV infected

persons at 6 months of ART. The proportion of terminal effector memory T cells ($CD4^+Cytokine^+CD27^-CD45RA^+$) tended to decrease in HIV infected persons during 6 months of ART (p-value = 0.1088), while their numbers tended to increase, although not significantly (p-value = 0.6385). Lastly, cross-sectional comparison with the HIV uninfected persons revealed no difference in proportion and numbers of *Mtb*-specific terminal effector memory T cells between the two groups (p-values = 0.2603 and 0.2374 respectively).

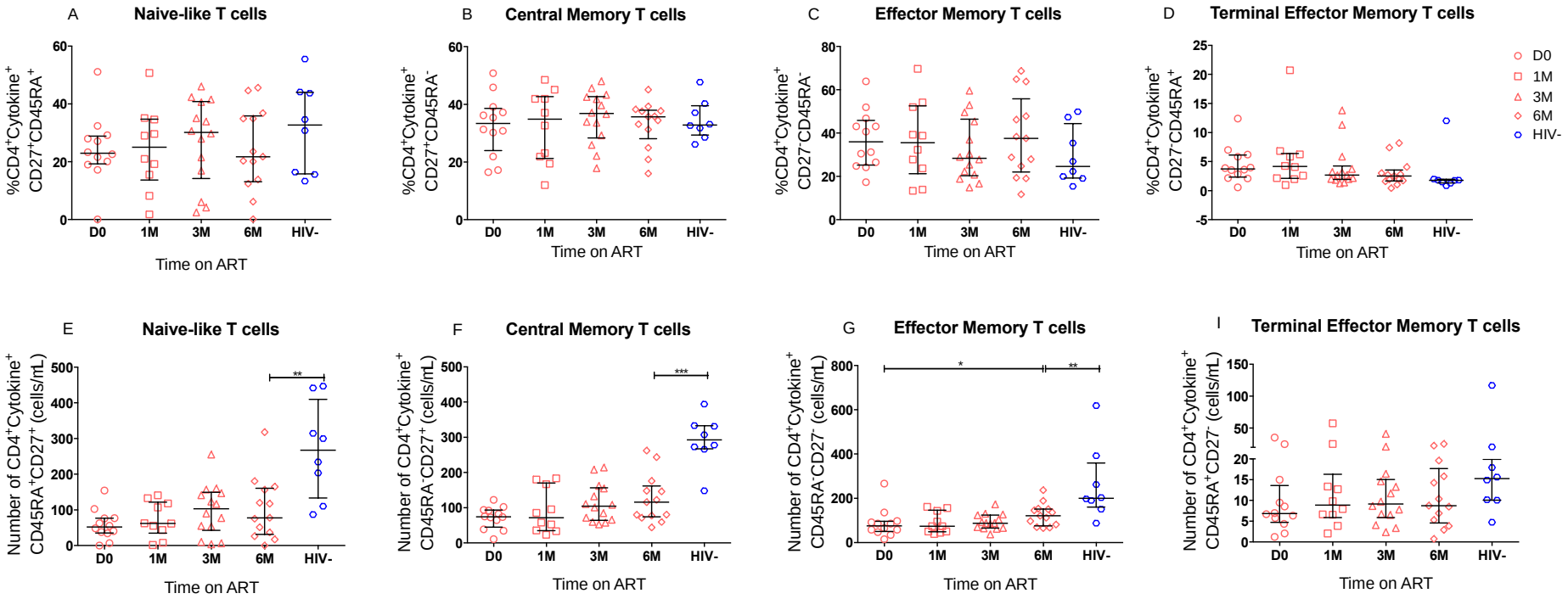

Figure 4. 9: Memory phenotype of *Mtb* specific CD4⁺ T cells as assessed by flow cytometry.

A – D: Proportion of CD4⁺ T cells expressing CD27 and CD45RA in HIV infected persons (red, n=12) after 6 months of ART, and HIV uninfected persons (blue, n=8). E – H: Number of CD4⁺ T cells expressing CD27 and CD45RA per 10^6 cells in HIV infected persons (red, n=12) after 6 months of ART, and HIV uninfected persons (blue, n=8). Graphs represent medians and IQR data.

Table 4. 8: Median and (IQR) frequency and number of *Mtb* specific T cells expressing CD27 and CD45RA and corresponding p-values.

Median (IQR)	HIV Infected Persons (n=12)				HIV Uninfected Persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
%$CD4^+$Cytokine$^+$ $CD27^+CD45RA^+$	23 (19-29)	25 (14-35)	30 (14-31)	22 (13-36)	33 (16-44)	>0.9999	0.9893	0.4888
%$CD4^+$Cytokine$^+$ $CD27^+CD45RA^-$	33 (24-39)	35 (21-43)	37 (28-43)	36 (26-38)	33 (29-40)	>0.9999	0.7796	0.9859
%$CD4^+$Cytokine$^+$ $CD27^-CD45RA^-$	36 (25-46)	36 (21-53)	28 (20-46)	38 (20-56)	25 (19-44)	>0.9999	>0.9999	0.3095
%$CD4^+$Cytokine$^+$ $CD27^-CD45RA^+$	3.8 (2.3-6.1)	4.2 (2.1-6.4)	2.7 (1.9-4.3)	2.5 (1.7-3.5)	1.8 (1.3-2.0)	0.5676	0.1088	0.2603

Median (IQR)	HIV Infected Persons (n=12)				HIV Uninfected Persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
Nrs/10^6 $CD4^+$Cytokine$^+$ $CD27^+$ $CD45RA^+$	52 (35-77)	62 (35-122)	103 (43-149)	78 (31-161)	267 (133-410)	0.8380	0.2025	0.0077
Nrs/10^6 $CD4^+$Cytokine$^+$ $CD27^+$ $CD45RA^-$	75 (45-93)	72 (35-170)	105 (64-157)	116 (74-162)	292 (267-333)	0.3260	0.0520	0.0001
Nrs/10^6 $CD4^+$Cytokine$^+$ $CD27^-$ $CD45RA^-$	76 (50-96)	74 (49-146)	88 (65-124)	121 (75-152)	200 (161-360)	0.1311	0.0297	0.0060
Nrs/10^6 $CD4^+$Cytokine$^+$ $CD27^-$ $CD45RA^+$	6.8 (4.7-14)	8.9 (5.9-16)	9.2 (5.9-15)	8.7 (4.6-18)	15 (10-20)	>0.9999	0.6385	0.2374

4.4.2.2. Activation and Senescence of Mtb specific CD4$^+$ T cells during the first 6 months of ART

A summary of the activation and senescence of CD4$^+$Cytokine$^+$ T cell phenotypes is shown in Figure 4.10. and Table 4.9. The proportion of activated T cells, characterized as CD4$^+$Cytokine$^+$HLA-DR$^+$, in HIV infected persons during 6 months of ART (Figure 4.10. panel A, p-value = 0.414) tended to decrease, (p-value = 0.4140) while their numbers (Figure 4.10. panel C) tended to increase, although not significantly (p-value = 0.2904). Cross-sectional comparison with the HIV uninfected persons revealed HIV infected persons at 6 months of ART still have significantly higher proportions (p-value = 0.0200) but not numbers (p-value = 0.3347) of HLA-DR expressing cells compared to HIV uninfected persons.

The proportion of senescent T cells, characterized as, CD4$^+$Cytokine$^+$KLRG-1$^+$, remained unchanged in HIV infected persons during 6 months of ART (Figure 4.10. panel B, p-value = 0.923 and > 0.9999 after correction for multiple comparisons), while their frequency tended to increase (Figure 4.10. panel D), although not significantly (p-value = 0.1502 and 0.7466 after correction for multiple comparisons). When compared with the HIV uninfected persons the proportion of senescent T cells did not differ between the groups (p-value = 0.8863) although HIV uninfected persons had significantly higher numbers of senescent T cells compared to HIV uninfected persons during 6 months of ART (p-value = 0.0302).

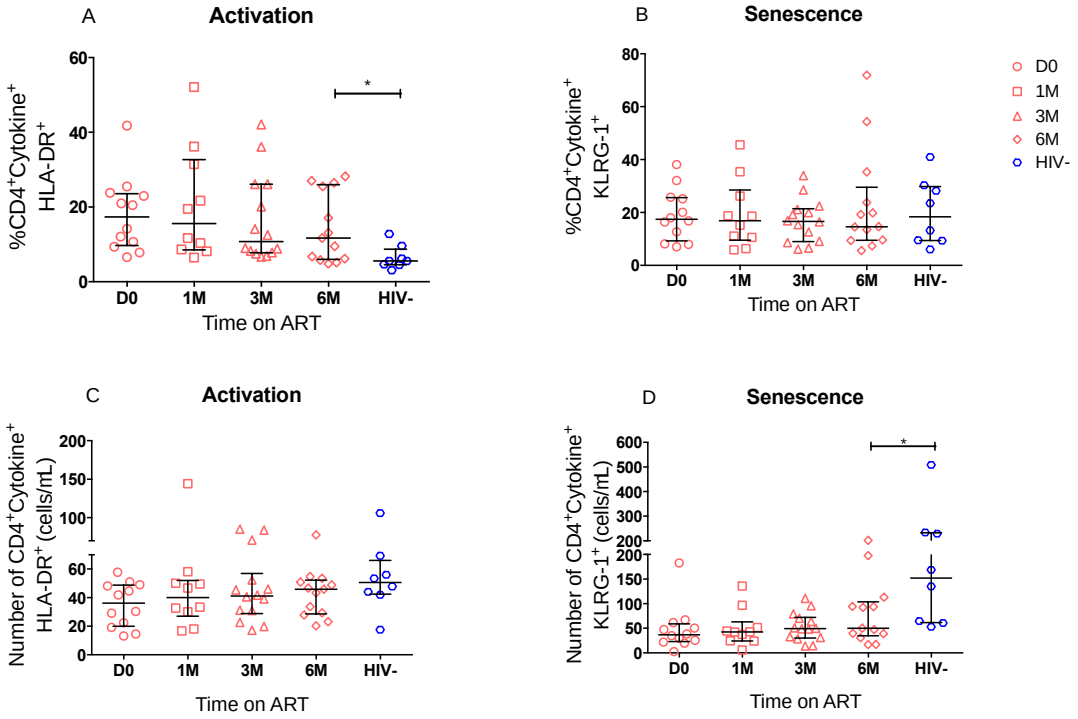

Figure 4. 10: Activation and Senescence of *Mtb* specific CD4⁺ T cells as measured by flow cytometry.

Proportion of CD4⁺Cytokine⁺ T cells expressing HLA-DR (A) and KLRG-1 (B) in HIV infected persons (red, n=12) after 6 months of ART, and HIV uninfected persons (blue, n=8). Number of CD4⁺ T cells expressing HLA-DR (C) and KLRG-1 (D) per 10^6 cells in HIV infected persons (red, n=12) after 6 months of ART, and HIV uninfected persons (blue, n=8). Graphs represent medians and IQR data for each cell subset.

Table 4. 9: Median and (IQR) frequency and number of *Mtb* specific $CD4^+$ T cells expressing HLA-DR and KLRG-1 with corresponding p-values.

Median (IQR)	HIV Infected persons (n=12)				HIV Uninfected persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
$\%CD4^+$ $HLA\text{-}DR^+$	17 (9.7-24)	16 (8.5-33)	11 (7.7-26)	12 (6-26)	5.6 (4.6-8.7)	>0.9999	0.4140	0.0209
$\%CD4^+$ $KLRG\text{-}1^+$	17 (9.3-26)	17 (9.5-29)	17 (9-21)	15 (9.5-30)	18 (9.4-30)	>0.9999	0.9230	0.8863
$Nrs/10^6$ $CD4^+HLA\text{-}DR^+$	36 (20-49)	40 (27-52)	41 (29-57)	46 (29-52)	51 (42-66)	>0.9999	0.2904	0.3347
$Nrs/10^6$ $CD4^+KLRG\text{-}1^+$	37 (23-59)	43 (24-63)	49 (30-72)	50 (35-104)	152 (62-232)	0.7466	0.1502	0.0302

4.4.2.3. Chemokine receptor expression on Mtb specific CD4$^+$ T cells during the first 6 months of ART

A summary of the expression of chemokine receptors, CXCR3, CCR4 and CCR6, on CD4$^+$ T cells is shown in Figure 4.11. and Table 4.10. The proportion of cells expressing CXCR3 (CD4$^+$Cytokine$^+$CXCR3$^+$), CCR4 (CD4$^+$Cytokine$^+$CCR4$^+$) and CCR6 (CD4$^+$Cytokine$^+$CCR6$^+$) remained unchanged during 6 months of ART (Figure 4.9. panels A – C with p-values = 0.5667, 0.2904 and 0.3168 respectively) however the numbers of these cells expanded, significantly for CCR4$^+$ and CCR6$^+$ T cells (Figure 4.11. panels D – F with p-values = 0.4951, 0.0006 and 0.0187 respectively). Cross-sectional comparison with the HIV uninfected persons revealed HIV uninfected persons to have a significantly higher proportions and numbers of CXCR3$^+$ T cells compared with HIV infected persons at 6 months of ART (p-values = 0.0013 and 0.0001 respectively). While the proportion of CCR4$^+$ and CCR6$^+$ T cells did not differ between the two groups (p-values = 0.5346 and 0.0558 respectively), the numbers of these cells were significantly higher in HIV uninfected persons (p-values = 0.0034 and < 0.0001 respectively) compared to HIV infected persons at 6 months of ART.

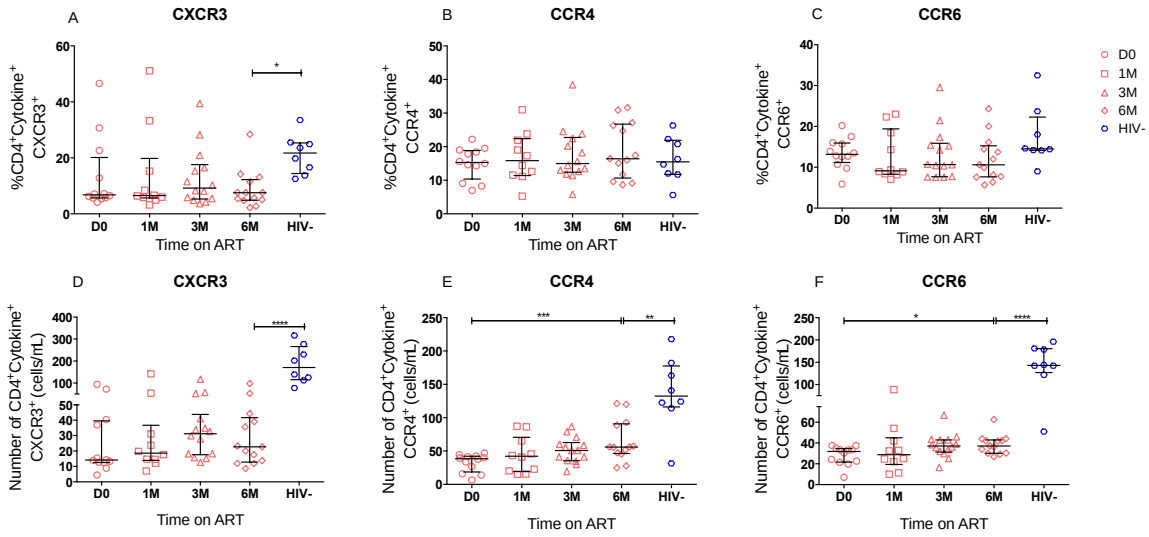

Figure 4. 11: Chemokine receptor expression on *Mtb* specific CD4⁺ T cells as measured by flow cytometry.

A – C: Proportion of CD4⁺Cytokine⁺ T cells expressing CXCR3, CCR4 or CCR6 in HIV infected persons (red, n=12) after 6 months of ART, and HIV uninfected persons (blue, n=8). D – F: Number of CD4⁺ T cells expressing CXCR3, CCR4 or CCR6 per 10^6 cells in HIV infected persons (red, n=12) after 6 months of ART, and HIV uninfected persons (blue, n=8). Median and IQR data are represented on graphs, for each cell subset.

Table 4. 10: Median and (IQR) frequency and number of $CD4^+Cytokine^+$ T cells expressing CXCR3, CCR4 or CCR6 with corresponding p-values.

Median (IQR)	HIV Infected persons (n=12)				HIV Uninfected persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
$\%CD4^+Cytokine^+$ $CXCR3^+$	6.8 (5.7-20)	6.5 (5.7-20)	9.2 (5.3-18)	7.5 (4.9-12)	22 (14-25)	>0.9999	0.5667	0.0013
$\%CD4^+Cytokine^+$ $CCR4^+$	15 (10-19)	16 (11-22)	15 (12-23)	16 (11-27)	16 (12-22)	>0.9999	0.2904	0.5346
$\%CD4^+Cytokine^+$ $CCR6^+$	13 (11-16)	9.1 (8.3-19)	11 (7.7-16)	11 (7.6-15)	15 (14-22)	>0.9999	0.3168	0.0558

Median (IQR)	HIV Infected persons (n=12)				HIV Uninfected persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
Nrs/10^6 $CD4^+$ $Cytokine^+CXCR3^+$	14 (13-40)	19 (14-37)	31 (18-44)	23 (13-42)	171 (116-266)	>0.9999	0.4951	<0.0001
Nrs/10^6 $CD4^+$ $Cytokine^+CCR4^+$	38 (19-42)	42 (19-71)	51 (35-63)	56 (46-91)	133 (116-178)	0.0065	0.0006	0.0034
Nrs/10^6 $CD4^+$ $Cytokine^+CCR6^+$	32 (22-35)	29 (19-45)	37 (31-43)	37 (30-43)	143 (127-180)	0.1399	0.0187	<0.0001

4.4.2.4. Assessment of Mtb-specific CD4⁺ T cell functionality based on cytokine production

This section focuses on the qualitative cytokine data from *Mtb*-specific CD4⁺ T cells. Boolean gating was used in order to determine the functionality of *Mtb*-specific CD4⁺ T cells and this analysis was conducted in order to assess the functional capacity *of Mtb*-specific CD4⁺ T cells from HIV infected persons regained following 6 months of ART.

Our findings identified eight distinct cytokine-producing antigen-specific CD4⁺ T cell populations in HIV infected and uninfected persons, summarised in Figure 4.13. and Table 4.11. These populations were further grouped based on the number of cytokines each population was able to produce (i.e. 1, 2 or 3 cytokines), summarised in Figures 4.12. and 4.13. and Table 4.11.

Our *Mtb*-specific qualitative cytokine data showed that HIV infected persons at day 0 of ART had a single cytokine positive (IFN-γ⁺ or TNF-α⁺ or IL-2⁺) cell dominant response (44%), followed by the double positive cytokine producing cells (34%) and lastly the triple positive cytokine producing cells (22%). The proportion of triple positive cytokine producing cells significantly increased in HIV infected persons after 6 months of ART (p-value = 0.0273) and was significantly lower than proportions observed in the HIV infected persons (Figure 4.13. panel A, p-value = 0.0158).

Interestingly, the cytokine response at 6 months of ART in HIV infected persons was dominated, almost equally, by the *Mtb*-specific triple positive cytokine producing cells (45%) and the *Mtb*-specific double positive cytokine producing cells (42%). Both proportions of cells expanded throughout the 6 Months duration of ART in HIV infected persons. When compared with the HIV uninfected persons, higher proportions of the double positive cytokine producing cells were observed in the HIV infected persons at 6 months of ART. Further analysis revealed that the IL-2^+TNF-α^+ and the TNF-α^+IL-$17A^+$ *Mtb*-specific cytokine subsets (Figure 4.13. panels D and E respectively) may have contributed to the increased proportion of double positive cytokine producing cells as the proportion of these cells appear to increase, although not significantly (p-values = 0.4961 and 0.5781 respectively), in HIV infected persons throughout 6 months of ART. Proportions of *Mtb*-specific IL-2^+TNF-α^+ and the TNF-α^+IL-$17A^+$ double positive cytokine secreting cells were higher in HIV infected persons at 6 months of ART (p-values = 0.0018 and 0.9137 respectively) than proportions observed in HIV uninfected persons.

The single positive (IFN-γ^+ or TNF-α^+ or IL-2^+) cytokine producing cells dominated the response (81%) in HIV uninfected persons (Figure 4.13. panel C), followed by the double positive cytokine producing cells (17%) and lastly the single cytokine producing cells (2%). When we assessed the *Mtb*-specific $CD4^+$ single positive cytokine producing T cells individually, we noted that HIV uninfected persons had significantly higher proportions of *Mtb*-specific IFN-γ^+ single positive cells, and *Mtb*-specific IL-$17A^+$ single positive cells compared to HIV infected persons during 6 months of ART (Figure 4.13.

panel F and H, p-value = 0.0001 and p-value < 0.0001 respectively). The higher proportions of these single cytokine producing cells thus contribute to the dominance observed in HIV uninfected persons. The proportions of *Mtb*-specific IL-2^{+} single positive cells did not differ in HIV infected persons during 6 months of ART compared to HIV uninfected persons (Figure 4.13. panel G p-value = 0.8316).

Longitudinal analysis of the *Mtb*-specific single positive cytokine producing cells, revealed that the proportions of *Mtb*-specific IL-2 single positive, and IL-17A single positive cells showed no change in HIV infected persons during ART (Figure 4.13. panels G and H, p-values = 0.0078 and 0.9453 respectively). *Mtb*-specific single positive IFN-γ secreting cells also remained unchanged in HIV infected persons during ART (Figure 4.13. panel F, p-value = 0.8438).

Additionally, the longitudinal analysis of *Mtb*-specific IFN-γ^{+}TNF-α^{+} double positive cells remained unchanged in HIV infected persons during 6 months of ART (Figure 4.13. panel B, p-value = 0.5703), and proportions of *Mtb*-specific IFN-γ^{+}TNF-α^{+} double positive cells were similar in HIV infected persons (p-value = 0.2374). Lastly, the proportion of *Mtb*-specific IL-2^{+}IL-17A^{+} double positive cells significantly decreased in HIV infected persons during ART (Figure 4.13. panel C, p-value = 0.0078) however proportions of this cell subset did not differ significantly from that observed in HIV uninfected persons (p-value = 0.1522).

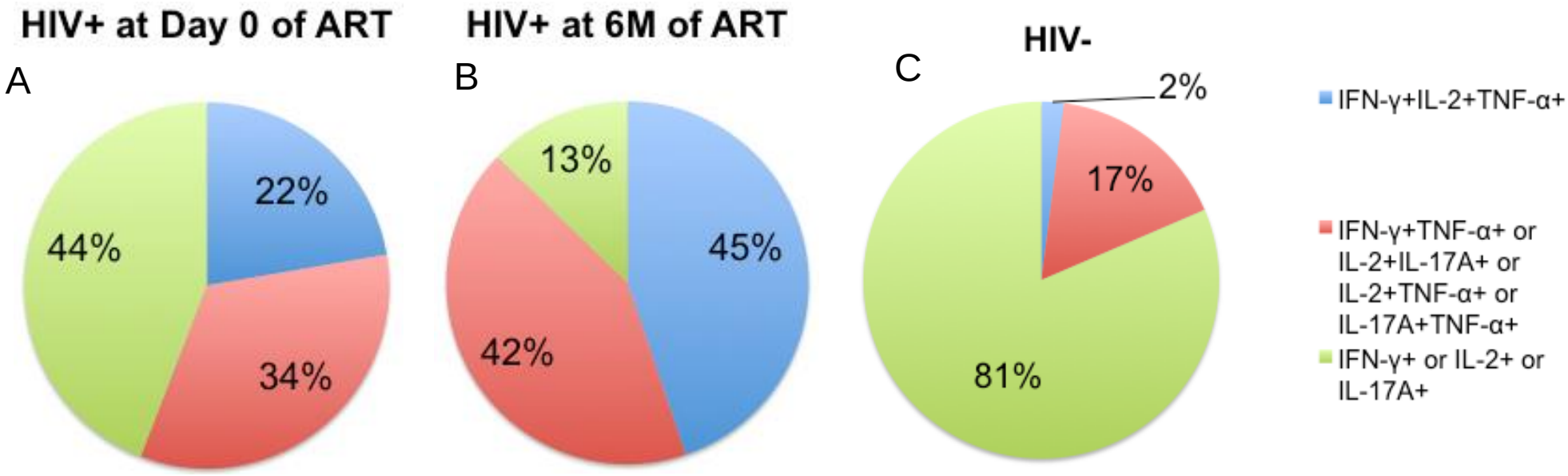

Figure 4. 12: Pie charts showing co-production of cytokines from *Mtb*-specific CD4⁺ T cells determined by flow cytometry analysis. Pie chart representing the polyfunctional capacity of *Mtb*-specific CD4⁺ T cells in (A) HIV infected persons at day 0 of ART, (B) HIV infected persons at 6 months of ART and (C) HIV uninfected persons. Blue slices represent the proportion of cells producing three of the cytokines measured, in this case IFN-γ^{+}TNF-α^{+}IL-2⁺ producing cells, while the red slices represent the proportion of cells producing a combination of any two cytokines and the green slices represent cells producing only one cytokine.

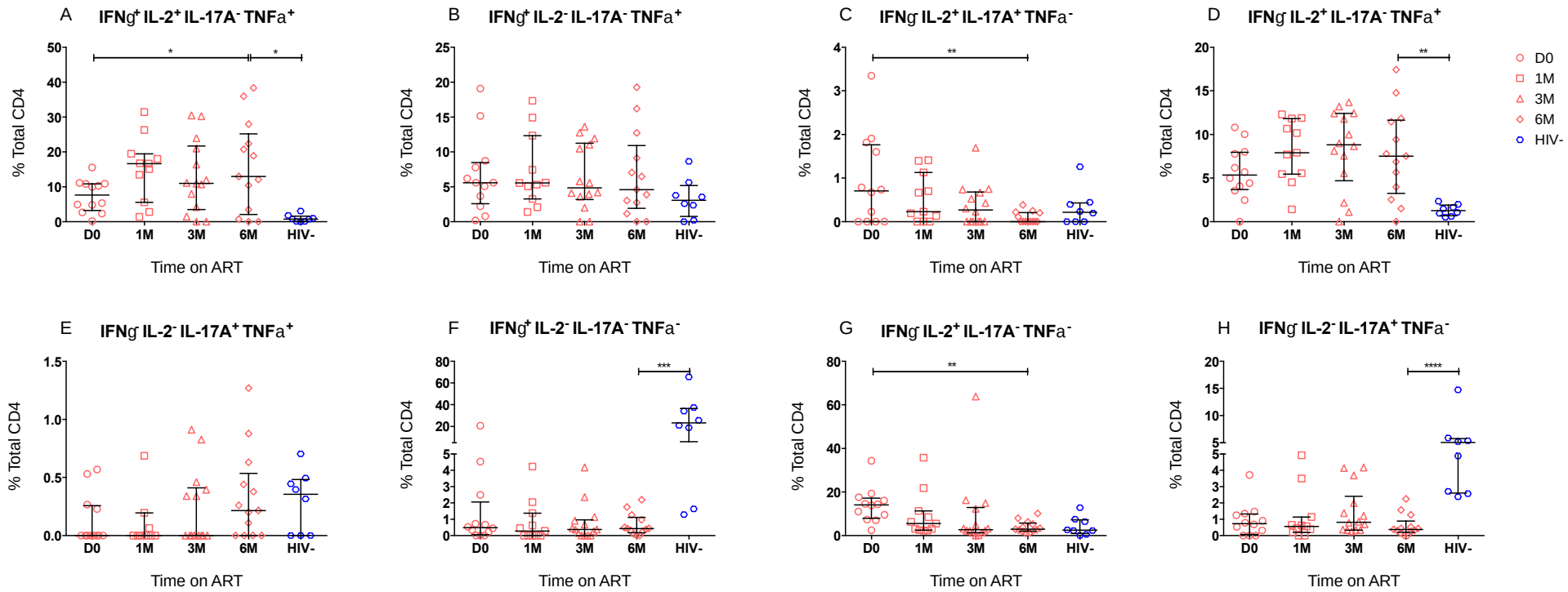

Figure 4. 13: Frequency of *Mtb* specific CD4⁺ T cells expressing IFN-γ, TNF-α, IL-2 and IL-17A cytokines as measured by flow cytometry.

Table 4. 11: Median and (IQR) frequency of $CD4^+Cytokine^+$ T cells producing IFN-γ, TNF-α IL-2 and IL-17A cytokines and corresponding p-values.

Median (IQR)	HIV Infected Persons (n=12)				HIV Uninfected Persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
$\%CD4^+Cytokine^+IFN\text{-}\gamma^+$ IL-2^+ IL-$17A^-$ TNF-α^+	7.7 (3.2-11)	17 (5.6-19)	11 (3.5-22)	13 (2.1-25)	0.77 (0.097-1.6)	0.4484	0.0273	0.0158
$\%CD4^+Cytokine^+IFN\text{-}\gamma^+$ IL-2^- IL-$17A^-$ TNF-α^+	5.6 (2.6-8.5)	5.6 (3.3-12)	4.9 (3.2-11)	4.6 (1.9-11)	3.1 (0.74-5.2)	>0.9999	0.5703	0.2374
$\%CD4^+Cytokine^+IFN\text{-}\gamma^+$ IL-2^- IL-$17A^-$ TNF-α^-	0.49 (0.057-2.1)	0.28 (0-1.4)	0.37 (0-0.97)	0.43 (0.18-1.1)	23 (5.9-37)	>0.9999	0.8438	0.0001
$\%CD4^+Cytokine^+IFN\text{-}\gamma^-$ IL-2^+ IL-$17A^+$ TNF-α^-	0.71 (0-1.8)	0.23 (0-1.1)	0.27 (0-0.68)	0.0 (0-0.21)	0.22 (0-0.43)	0.0514	0.0078	0.1522

Median (IQR)	HIV Infected Persons (n=12)				HIV Uninfected Persons (n=8)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	Day 0 of ART	1M	3M	6M				
%CD4$^+$Cytokine$^+$IFN-γ$^-$ IL-2$^+$ IL-17A$^-$ TNF-α$^+$	5.3 (3.7-7.9)	7.9 (5.5-12)	8.8 (4.7-12)	7.5 (3.3-12)	1.3 (0.68-1.9)	>0.9999	0.4961	0.0018
%CD4$^+$Cytokine$^+$IFN-γ$^-$ IL-2$^+$ IL-17A$^-$ TNF-α$^-$	14 (7.9-17)	5.6 (2.5-11)	2.7 (1.3-13)	2.9 (2-5.7)	2.5 (0.96-7.3)	0.0070	0.0078	0.8316
%CD4$^+$Cytokine$^+$FN-γ$^-$ IL-2$^-$ IL-17A$^+$ TNF-α$^+$	0.0 (0-0.26)	0.0 (0-0.2)	0.0 (0-0.41)	0.22 (0-0.54)	0.36 (0-0.48)	0.6296	0.5781	0.9137
%CD4$^+$Cytokine$^+$IFN-γ$^-$ IL-2$^-$ IL-17A$^+$ TNF-α$^-$	0.74 (0.079-1.3)	0.56 (0.2-1.1)	0.82 (0.34-2.4)	0.38 (0.21-0.89)	5.0 (2.6-5.7)	>0.9999	0.9453	<0.0001

4.5. Discussion

Using flow cytometry, we determined the memory profile of reconstituting $CD4^+$ T cells in HIV infected persons sensitised by *Mtb*, and compared this to the memory profile of $CD4^+$ T cells in the HIV uninfected control group. We also determined the expression of certain chemokine receptors, the activation and senescence profile and the overall cytokine secreting capacity of $CD4^+$ T cells in both cohorts.

The proportions and numbers of naïve and central memory $CD4^+$ T cells significantly expanded in HIV infected persons on ART, while the proportions and numbers of effector memory and terminally differentiated effector $CD4^+$ T cells decreased significantly. Additionally, these trends were reflected in the case of the *Mtb* specific $CD4^+$ T cells, with the exception of effector memory $CD4^+$ T cells; the frequencies of this cell population significantly expanded in HIV infected persons during ART. Wilkinson *et al.* first demonstrated the significant expansion in the proportion of CD4 central memory T cells in HIV infected persons with LTBI, receiving ART [12]. Additionally they reported a significant decrease in the proportion of effector memory CD4 T cells. These phenomena were noted at 12 weeks of ART. Furthermore Riou *et al.* demonstrated that pathogen-specific early differentiated, such as *Mtb*-specific, CD4 T cells have a higher capacity to replenish in HIV infected persons receiving ART, compared to pathogen-specific late differentiated CD4 T cells [16]. Two studies in addition to those described in section 4.1. above, Kipnis *et al.* and Anderson *et al.*, have also

demonstrated, by use of the murine model, the importance of memory T cells in TB protection [4, 17]. Both made use of adoptive transfer techniques to transfer $CD4^{+}CD44^{lo}CD62L^{hi}$ central memory T cells from BCG-vaccinated mice [4] or TB infected mice [17] to naïve mice and illustrated a significant decline in the bacterial burden in the lungs of mice post *Mtb* challenge, suggesting that these cells mediate protection.

The significant decrease in the proportion, but not the numbers, of activated and senescent $CD4^{+}$ T cells was observed in HIV infected persons on ART but the numbers of activated T cells observed in the HIV infected persons remained unchanged during ART. This was also observed in the *Mtb* specific $CD4^{+}$ T cells. The significant decrease in the proportion of senescent $CD4^{+}$ T cells indicates that cells display a less differentiated phenotype thereby indicating that the cells may possibly be exhausted. Similarly Riou *et al.* demonstrated a significant decrease in CD4 T cells co-expressing CD38 and HLA-DR markers, and cells expressing HLA-DR only, LTBI HIV infected persons [16]. A study conducted in HIV uninfected, BCG vaccinated infants, without any chronic disease and household exposure to an adult with active TB, revealed that HLA-DR^{+} $CD4^{+}$ T cells associate with increased TB risk [18]. This further substantiates our findings that the decrease in $CD4^{+}$ HLA-DR^{+} T-cells in HIV infected persons, undergoing immune reconstitution through ART, may be identified as a correlate of protective immunity to TB. In keeping with our results, a recent study by Wilkinson *et al.* further demonstrated that LTBI HIV infected persons exhibited a higher expression of HLA-DR on *Mtb*-specific cells, compared to HIV uninfected persons,

similar to the expression of HLA-DR in the CD4 T cell compartment [19]. We observed an expansion in the CD4 T cell compartment, of the proportion of activated CD4 T cells significantly declined while the actual numbers of activated CD4 T cells remained unchanged. This may be attributed to a decreased need for activated CD4 T cells and thus a decline in the proportion of activated cells, and corresponding proportional increase in the proportion of cells that may be needed.

The numbers of $CD4^+$ T cells expressing CXCR3, CCR4 and CCR6 chemokine receptors remained significantly lower than that observed in HIV uninfected persons, indicating that the homing potential of these $CD4^+$ T cells may not be completely restored by 6 months of ART thus inhibiting their trafficking from the bloodstream into peripheral organs. However, the proportions of CCR4 positive cells significantly expanded during 6 months of ART to the levels seen in the HIV uninfected persons. The observations made regarding the frequencies of these cells were mirrored in the *Mtb* specific $CD4^+$ T cells. Cecchinato *et al*. illustrated that the long term use of ART (greater than 3 years) in HIV infected persons, and the corresponding significant decrease in viral load to levels below the detection limit, is not sufficient to restore the ability of cells to respond to stimuli and in turn not being able to traffic from the bloodstream to peripheral organs [20]. Riou *et al.* also noted unchanged expression of CXCR3, CCR4 and CCR5 chemokines in *Mtb*-specific CD4 T cells in LTBI and HIV co-infected persons [21] thus further substantiating our findings.

The overall cytokine secreting capacity of $CD4^+$ T cells was determined for all HIV infected persons and indicated that following 6 months of ART, HIV infected persons showed a significant decrease in proportion of $CD4^+$ T cells producing TNF-α, $CD4^+$ T cells producing IL-2 and $CD4^+$ T cells producing IL-17A, however the numbers of $CD4^+$ T cells producing IL-2 and $CD4^+$ T cells producing IL-17A increased in this group of persons. The proportion of $CD4^+$ IFN-γ producing T cells remained unchanged, however the numbers of $CD4^+$ IFN-γ producing T cells significantly expanded.

Lastly we assessed the qualitative polyfunctional cytokine secreting capacity of $CD4^+$ T cells and *Mtb*-specific $CD4^+$ T cells and observed that the proportion of $CD4^+$ T cells with the ability to secrete three cytokines (IFN-γ^+TNF-α^+IL-2^+) simultaneously, significantly expanded in HIV infected persons following 6 months of ART. Additionally, HIV uninfected persons had a significantly lower proportion of IFN-γ^+TNF-α^+IL-2^+ polyfunctional CD4 T cells compared to HIV infected persons after 6 months of ART. While the role of polyfunctional cells has yet to be clearly defined in TB [22] Day *et al*. illustrated that PPD-specific polyfunctional T cells are associated with active TB disease and bacterial load [23]. However, our results are similar to those demonstrated by Bunjun *et al*. in which they found LTBI and HIV co-infected persons to have a higher proportion of IFN-γ^+TNF-α^+IL-2^+ polyfunctional T cells compared to HIV uninfected persons [24]. Conversely, Kalsdorf *et al*. and Jambo *et al.* found lower proportions of IFN-γ^+TNF-α^+IL-2^+ polyfunctional T cells in HIV infected persons compared to HIV uninfected persons [25, 26].

Sutherland and colleagues also demonstrated an expansion in *Mtb*-specific polyfunctional CD4 T cells, in HIV infected persons, after ART [27].

Additionally the single cytokine secreting cells dominated the cytokine response in HIV infected persons at day 0 of ART, in *Mtb*-specific $CD4^+$ T cells. Interestingly at 6 months of ART, the cytokine response was dominated by single positive and cells secreting two cytokines in *Mtb*-specific $CD4^+$ T cells. These results were similarly reported by Bunjun *et al*., Jambo *et al*. and Kalsdorf *et al*. in which HIV infected persons displayed an *Mtb*-specific profile consisting of monofunctional and bifunctional cytokine expression [24-26]. Both IFN-γ and TNF-α play pivotal roles in macrophage activation, and in turn preventing the growth of *Mtb* [28]. Murine and human studies have additionally demonstrated the requirement of IFN-γ for resistance to *Mtb* infection [29-33]. IL-2 is known for its proliferative role [34, 35] and is required by antigen-specific cells to undergo clonal expansion [36]. However a definitive role of IL-2 in LTBI and TB disease has yet to be established.

Overall, a lower *Mtb*-specific response was detected in our cohort of HIV infected persons receiving ART. A possible reason for the reduced detection is the absence of *Mtb*-specific cells to begin with, due to early depletion of this cell subset as a result of HIV infection [24, 37-40]. A limitation in this study is the reliance on antigenic stimulation in order to measure a *Mtb*-specific response, thus limiting the analysis solely to persons that are able to mount a detectable *Mtb*-specific response. We opted for the use of *Mtb* WCL, a pool of lipids, proteins and carbohydrates present within the *Mtb*

H37Rv strain, in order to maximise our ability to detect a response and at the same time without compromising the specificity of the response. Overcoming the reliance of antigenic stimulation may be possible through the use of a novel technology, or analysis program, that will facilitate the detection of antigen specific responses that are below current levels of detection based on available technologies. Additionally, it is important to note that these individuals are immune compromised and therefore pre-determined sensitisation cut-off values may not be applicable to this cohort. This was further observed by Esmail *et al* [41]. We defined the memory response based on the use of CD45RA and CD27 markers, which unfortunately cannot identify stem cell memory T cells ($CD45RA^{+}CD45RO^{-}CCR7^{+}CD27^{+}$) [42] that are the intermediate subpopulation between naïve-like and central memory T cells. Inclusion of the CCR7 and CD62L markers would have been beneficial and would further allow the identification of this subpopulation. Also, although we were powered to conduct the above assays the loss of persons due to incomplete timepoints resulted in a lower overall number of samples available for analysis. Furthermore the cryopreservation of samples may have affected cell viabilities and cell numbers also affecting the ability to detect a *Mtb*-specific response in immune-compromised persons.

This study focused on the characterisation of *Mtb*-specific CD4 central memory T cells as potential correlate of protection to TB. Effector molecules are also sourced from CD8 T cells and may therefore play a vital role in TB immunity and thus it may be worthwhile looking at the CD8 compartment in LTBI HIV infected persons receiving ART. Further experiments are needed

to confirm the findings in a larger cohort, including a control cohort of individuals with active TB disease. A longitudinal study looking at *Mtb*-specific responses in individuals that progress to active disease may provide better insights into protection in TB.

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Chapter 5: Analysis of soluble markers from QFT supernatants of HIV Infected persons sensitised by *Mycobacterium tuberculosis*, receiving antiretroviral therapy

5.1. Introduction

Tuberculosis remains one of the highest causes of mortality with an estimated 10.4 million people developing active TB in 2016 [1]. The BCG vaccine is currently the only available vaccine against TB, but it lacks effectiveness for lifelong protection against TB infection. The Quantiferon TB Gold assay is used in research and as a diagnostic test for *Mtb* that measures IFN-γ responses against *Mtb* specific antigens [2]. This test however, is unable to differentiate between the active form of TB and latent TB infection.

Upon entry into the pulmonary alveolus, *Mtb* interacts with the airway epithelial cells, macrophages, dendritic cells and neutrophils [3]. This interaction triggers a host immune defence comprised of cytokines, chemokines and other inflammatory molecules to kill and contain the bacteria [4]. Infected dendritic cells (DC) migrate to the lymph nodes where they initiate an adaptive immune response to control the growth of *Mtb* [5]. T and

B cells also play a role in the control and elimination of *Mtb* by antigen presentation and antibody production [6]. Many of these events are initiated by cytokines that have an influence on surrounding cells. The type of cytokines that are produced is dependent on the type of immune cell and their interaction with the bacteria [7]. T cells, DCs, macrophages and other immune cell signals are mediated by cytokines, such as IFNγ, TNFα, IL-2 and IL-10, and these interactions have an impact on disease severity and outcome [8]. Cytokines play a key role at sites in which *Mtb* is present and cytokine signalling is representative of a checkpoint of the immune response against *Mtb* [7]. Several studies using gene knockout murine models have illustrated that cytokines such as IFN-γ, TNF-α and IL-12 play and important role in *Mtb* control [9-14]. IL-1 has also been shown to be required for host resistance to *Mtb* infection and studies in mice have found that a deficiency of IL-1 leads to susceptibility to infection [15].

IFN-γ production by CD4 T_H1 cells is important for a protective immune response against *Mtb* [13]. DCs, activated by *Mtb*, produce IL-12 which plays an important role in the induction of protective IFN-γ T cell responses against primary infection [13, 16]. Apart from the induction of IFN-γ, IL-12p40 plays an important role in the continuous production of IL-12p70 to sustain T_H1 responses in the lung to control chronic infection [17]. A study in IFN-γ deficient mice showed IFN-γ and IL-17 producing T cells were induced following infection with *Mtb* and furthermore, IFN-γ limited the IL-17 producing T cell population thereby suggesting that the IFN-γ pathway is potentially an important factor in limiting mycobacterial associated immune-

mediated pathology [18]. Although IL-23 is essential for a IL-17 response during *Mtb* infection, it is not required for IFN-γ responses in the presence of IL-12p70 [19]. It has been shown that the central role of these cytokines is to initiate a T_H1 phenotype whereby downstream effector functions ensure the death of *Mtb* bacilli [20].

IL-10 plays a role in immune suppression and is produced by many cell types such as macrophages, DCs, neutrophils, T and B cells. IL-10 has an essential role in regulating the immune response and limiting immunopathology [21]. It inhibits the production of IL-12, thereby inhibiting T_H1 responses [22]. IL-10 also plays a role in inhibiting macrophage killing of intracellular pathogens and production of TNF-α [23], therefore IL-10 plays a role in regulating the immune response to *Mtb* and ultimately disease outcome [24] .

TNF-α is produced by many immune cells and plays a role in the activation of macrophages, induction of chemokines as well as the upkeep of the granuloma [13, 25]. The role of TNF-α in the control of *Mtb* infection in humans was highlighted in a study whereby patients, who were latently infected with *Mtb*, receiving anti-TNF therapy had an increased rate of reactivation of active TB [26]. TNF-α is important in providing resistance to *Mtb* infection and may also have a role in the promotion of phagocytosis and intracellular killing of *Mtb* [27]. Although IFN-γ, TNF-α and IL-1 have been shown to have protective roles for the host, if left unregulated, they can be detrimental to the host.

Type 1 IFN is well known for its role in antiviral immunity but the overexpression of type 1 IFN following *Mtb* infection has a detrimental effect on the host [28, 29]. Type 1 IFN has also been shown to express the production of IL-1 and IL-12 after *Mtb* infection and therefore, type 1 IFN may increase susceptibility of an individual to *Mtb* infection [30]. More importantly Berry *et al*. revealed a 393 transcript whole-blood signature for active TB that was dominated by a IFN-inducible gene profile, consisting of type 1 IFN-αβ and IFN-γ genes [29] and furthermore revealed that this signature was resolved in persons during TB therapy. This suggests that this signature may be used to measure efficacy of treatment, and may also provide insight into the host response to *Mtb* infection [29]. The uncontrolled production of TNF-α may cause an increased pathology at higher levels [27].

Following determination of *Mtb* sensitisation, QFT supernatants were used to measure *Mtb*-specific soluble markers using multiplex Luminex analysis.

5.2. Patient characteristics

Thirty-two HIV infected persons and 12 HIV uninfected persons were included in this arm of the study based on availability of stored QFT supernatants for all longitudinal follow up timepoints. All HIV infected persons and HIV uninfected persons included in this aim were determined to be *Mtb* sensitised based on a positive IFN-γ response determined by either the QFT, ELISpot or the Luminex assay at at least one timepoint. Therefore all

persons were considered to be sensitised to *Mtb*. The characteristics of all patients included are summarized in Table 5.1. Although the median age of HIV infected persons differed significantly from the median age of HIV uninfected persons, we do not believe that this difference impacted downstream statistics and subsequent significant differences in immunological markers measured.

Table 5. 1: Characteristics of patients included in the analysis of soluble markers using Luminex

	HIV Infected Persons (n=32)	HIV Uninfected Persons (n=12)	p-value
Median Age (years)	35 (29-40)	25 (20-38)	0.019
Gender Male/Female	10/22	5/7	0.77

Table 5. 2: Median and (IQR) CD4 counts and Viral loads of patients included

Median (IQR)	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)
	Day 0 of ART	**1M**	**3M**	**6M**	
CD4 count	201 (140-240)	270 (175-343)	299 (181-353)	294 (218-426)	1 041 (784-1 134)
Viral Load	62792 (35 662-181 693)	262 (105-574)	40 (39-68)	40 (39-40)	N/A

5.3. Methods and statistical analysis

Frozen QFT plasma from 32 HIV infected and 12 HIV uninfected persons was thawed and used for multiplex analysis. 25µL of sample was used at a 1:3 dilution ratio to determine the concentrations of the analytes IL-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6 IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, EOTAXIN, basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, PDGF, TNF-α and VEGF using the Bio-Plex Pro Human Cytokine 27-plex kit (Bio-Rad Laboratories, Hercules, CA). The analytes IL-12p40, IL-22, IL-23, IL-1α, FGF-2 and IFN-α were measured using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (Merck Millipore). Multiplex assays were carried out according to the manufacturer's instructions detailed in chapter 2, section 2.6. Acquisition was performed on the Bio-Plex 200 system with Luminex xMap Technology (Bio-Rad Laboratories, Hercules, CA) using the Bio-Plex Manager Software (version 6.1). The response of the analyte was defined as the concentration of the analyte in the nil tube plasma subtracted from the concentration of the analyte in the *Mtb* antigen (Ag) tube plasma (Ag-Nil), with resulting negative values being assigned a value of 0. Ag-Nil values were assessed using statistics, described below, and plotted on subsequent graphs.

Statistical analysis was performed using GraphPad Prism (version 6) using methods for non-parametric data. The Friedman test, with correction for multiple comparisons, was used for paired data comparison across all

sampling time points in HIV infected individuals. There were four sampling timepoints (k=4) thus the Friedman test was performed with d.f. = 3 ('degrees of freedom' = k-1 and alpha = 0.05) in order to determine p-values. The Kruskal Wallis test with correction for multiple comparisons was used for unpaired data comparisons across all time points in HIV infected persons. The Wilcoxon test (equivalent of the non-parametric student t-test) was used to compare paired data in HIV infected persons at day 0 and 6 months of ART. Additionally we also compared cross-sectional data from the HIV infected persons at 6 months of ART with HIV uninfected persons using the Mann-Whitney test, used for unpaired data comparisons. A p-value of less than or equal to 0.05 was considered statistically significant. P-values are illustrated in subsequent figures by asterixes and mean the following: ns refers to a p-value > 0.05, a single asterisk (*) refers to a p-value ≤ 0.05, two asterix (**) refer to a p-value ≤ 0.01, three asterix (***) refer to a p-value ≤ 0.001 and four asterix (****) refer to a p-value ≤ 0.0001.

5.4. Results

The analytes measured were broadly grouped based on their functions into 5 groups namely; pro-inflammatory, T cell growth factors, chemoattractants, growth factors and anti-inflammatory. First we explored each analyte individually in order to determine how the concentrations changed over time on 6 months of ART.

5.4.1. Pro-inflammatory analytes

A summary of the pro-inflammatory cytokines measured is shown in Figure 5.1. and Table 5.3. The Friedman test of differences among repeated measures was conducted in order to determine the difference in concentration of analytes across all timepoints of sampling, for HIV infected persons. This test also corrects for multiple comparisons. A Friedman test statistic of 14 (d.f. = 3) which was statistically significant (p-value = 0.002) for the decreasing concentration of IFN-γ produced by HIV infected individuals after 6 months of ART, was calculated. We also assessed the concentration of IFN-γ at day 0 and after 6 months of ART and found that the concentration of IFN-γ decreased significantly in HIV infected persons, during 6 months of ART (Figure 5.1. panel A, p-value = 0.0498). Lastly, a cross sectional comparison between HIV infected persons at 6 months of ART with HIV uninfected persons, revealed no significant difference in the concentration of IFN-γ produced by either group of individuals (p-value = 0.409).

Friedman tests, with d.f. = 3, were conducted for the longitudinal analysis for the remaining analytes, and generated statistically significant test statistics for IL-1α (27), IL-1β (25), IL-6 (16), IL-17A (17) and TNF-α (17) with p-values < 0.0001, < 0.0001, 0.0014, 0.0009 and 0.0008 respectively. The Friedman test statistic generated for IL-12p70 was 2.3, and was not statistically significant (p-value = 0.522).

When we compared the concentrations of analytes IL-1α (Figure 5.1. panel B), IL-1β (Figure 5.1. panel C), IL-6 (Figure 5.1. panel D), IL-17A (Figure 5.1. panel F) and TNF- α (Figure 5.1. panel G) at day 0 and after 6 months of ART, we found a significant decrease in the abovementioned analytes in HIV

infected persons during ART (p-values < 0.0001, 0.0009, 0.006, 0.006 and 0.0007 respectively). However the concentration of IL-12p70 remained unchanged in HIV infected persons for the duration of therapy (Figure 5.1. panel E, p-value = 0.784).

Lastly, comparison with the HIV uninfected persons revealed a number of differences: HIV infected persons produced significantly higher concentrations of IL-1α (p-value = 0.017) and IL-6 (p-value = 0.0005) compared to HIV uninfected persons, even after 6 months of ART. Although not significant, HIV infected persons also produced higher concentrations of IL-1β (p-value = 0.104) compared to HIV uninfected persons. IL-17A, TNF-α and IL-12p70 concentrations measured in both cohorts were indistinguishable between them (p-values = 0.173, 0.439 and 0.484 respectively).

Table 5. 3: Median and IQR of pro-inflammatory cytokines measured in HIV infected and HIV uninfected cohorts and corresponding p-values.

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
IFN-γ	326 (172-534)	178 (64-535)	115 (27-349)	185 (1.9-404)	278 (109-688)	0.002	0.0498	0.409
IL-1α	1691 (62-3371)	96 (0-1369)	51 (0-798)	152 (0-478)	0.0 (0-0)	< 0.0001	< 0.0001	0.017
IL-1β	2921 (1175-7157)	739 (176-2746)	319 (0-1529)	965 (0-2106)	129 (0-1257)	< 0.0001	0.0009	0.104

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
IL-6	26007 (7066-74573)	7097 (2991-26852)	3428 (124-23448)	4690 (502-27330)	0.0 (0-0)	0.0014	0.006	0.0005
IL-12p70	0.0 (0-17)	0.0 (0-4.8)	0.0 (0-12)	0.0 (0-14)	0.0 (0-61)	0.522	0.784	0.484
IL-17A	120 (63-151)	72 (28-130)	43 (0-62)	45 (7-96)	25 (0-50)	0.0009	0.006	0.173
TNF-α	406 (2.9-1429)	217 (0-547)	27 (0-191)	34 (0-204)	146 (0-387)	0.0008	0.0007	0.439

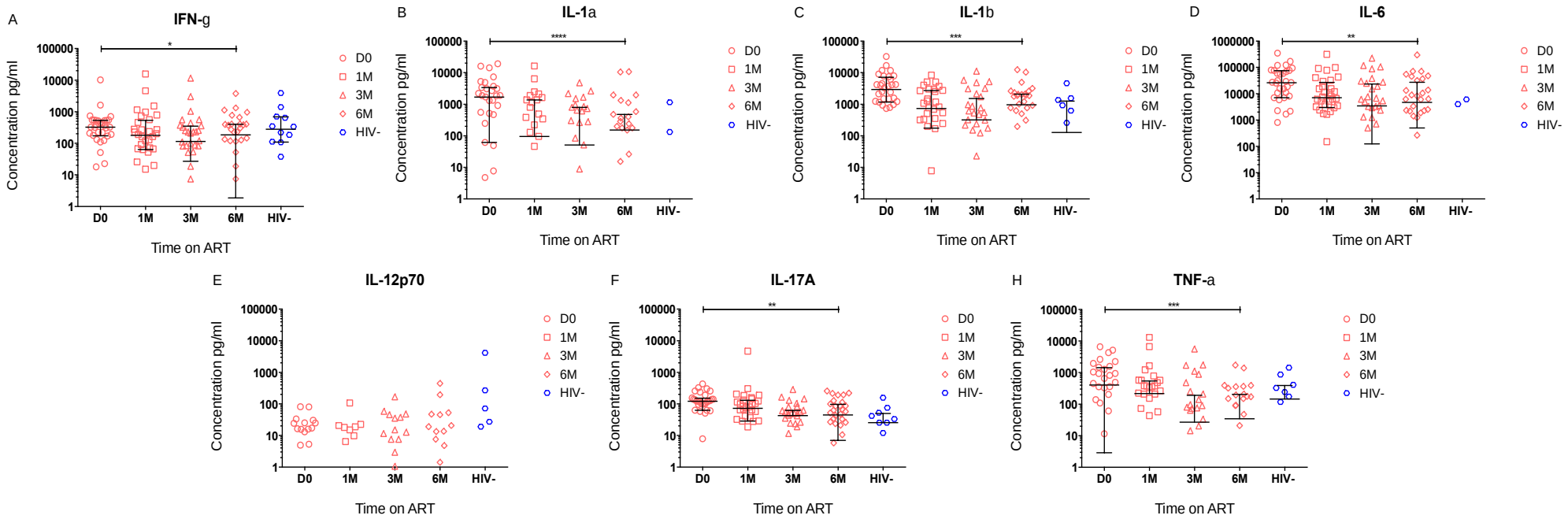

Figure 5. 1: Pro-inflammatory cytokines, (A) IFN-γ, (B) IL-1α, (C) IL-1β, (D) IL-4, (E) IL-12p70, (F) IL-17A and (G) TNF-α, measured in QFT supernatants of HIV infected persons during 6 months of ART, and HIV uninfected persons using Luminex. Data points on graphs represent log transformed detectable concentrations of analytes for patients.

5.4.2. TH_2 cytokines

A summary of the TH_2-associated soluble markers measured are shown in Figure 5.2. and Table 5.4. A Friedman test statistic of 2.5 (d.f. = 3) was not statistically significant (p-value = 0.48) for the change in concentration of IFN-α2 produced by HIV infected individuals after 6 months of ART. An increase was seen in the concentration of IFN-α2, although not significant, in HIV infected persons during 6 months of ART (Figure 5.2. panel A, p-value = 0.86). A cross sectional comparison between HIV infected persons at 6 months of ART with HIV uninfected persons, revealed that HIV infected persons produce significantly higher concentrations of IFN- α2 compared to that of HIV uninfected persons (p-value = 0.039). A Friedman test (d.f. = 3) rendered a test statistic of 8.7, which was statistically significant (p-value = 0.034) only for IL-4 concentrations in HIV infected persons receiving ART. Friedman test statistics for IL-5 (2.9), IL-13 (4.4), IL-10 (6.2), IL-1RA (6.5) and IL-9 (6.8) were not statistically significant (p-values = 0.4, 0.218, 0.103, 0.089 and 0.077 respectively) in HIV infected persons during ART. Although not significantly, concentrations of IL-4 (Figure 5.4. panel B, p-value = 0.072), IL-5 (Figure 5.2. panel C, p-value = 0.226), IL-10 (Figure 5.2. panel E, p-value = 0.366), IL-13 (Figure 5.2. panel F, p-value = 0.529) and IL-1RA (Figure 5.2. panel G, p-value = 0.814) tended to decrease in HIV infected persons during 6 months of ART, while concentrations of IL-9 (Figure 5.2. panel D, p-value = 0.02) declined significantly in the same cohort of individuals.

Comparison with the HIV uninfected persons indicated that HIV infected persons produced significantly higher IL-4 (p-value = 0.041) concentrations, and higher, but not significant, concentrations of IL-10 (p-value = 0.603) and IL-1RA (p-value = 0.738) than HIV uninfected persons. Interestingly, HIV uninfected persons produced significantly higher concentrations of IL-5 (p-value = 0.021) and IL-13 (p-value = 0.032) compared to HIV infected persons. Lastly, HIV infected and uninfected persons produced similar amounts of IL-9 (p-value = 0.434).

Table 5. 4: Median and IQR of TH_2 cytokines measured in HIV infected and HIV uninfected cohorts and corresponding p-values.

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
IFN-α2	13 (0-306)	0.0 (0-300)	40 (0-380)	21 (0-343)	0.0 (0-18)	0.48	0.86	0.039
IL-4	16 (7.8-21)	7.8 (2.1-14)	4.8 (1.6-13)	9.6 (1.8-19)	0.0 (0-13)	0.034	0.072	0.041
IL-5	4.0 (0-11)	2.0 (0-10)	1.6 (0-12)	2.4 (0-9)	16 (2.6-32)	0.4	0.226	0.021
IL-9	64 (17-110)	43 (0-82)	42 (0.24-78)	17 (0-64)	14 (0-43)	0.077	0.02	0.434

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
IL-10	9.1 (0-28)	2.4 (0-15)	0.68 (0-11)	2.3 (0-20)	0.0 (0-25)	0.103	0.366	0.603
IL-13	62 (13-148)	46 (5.6-202)	33 (1-96)	43 (2.6-200)	266 (43-586)	0.218	0.529	0.032
IL-1RA	423 (195-769)	171 (4-514)	233 (9.7-599)	356 (29-732)	286 (125-1550)	0.089	0.814	0.738

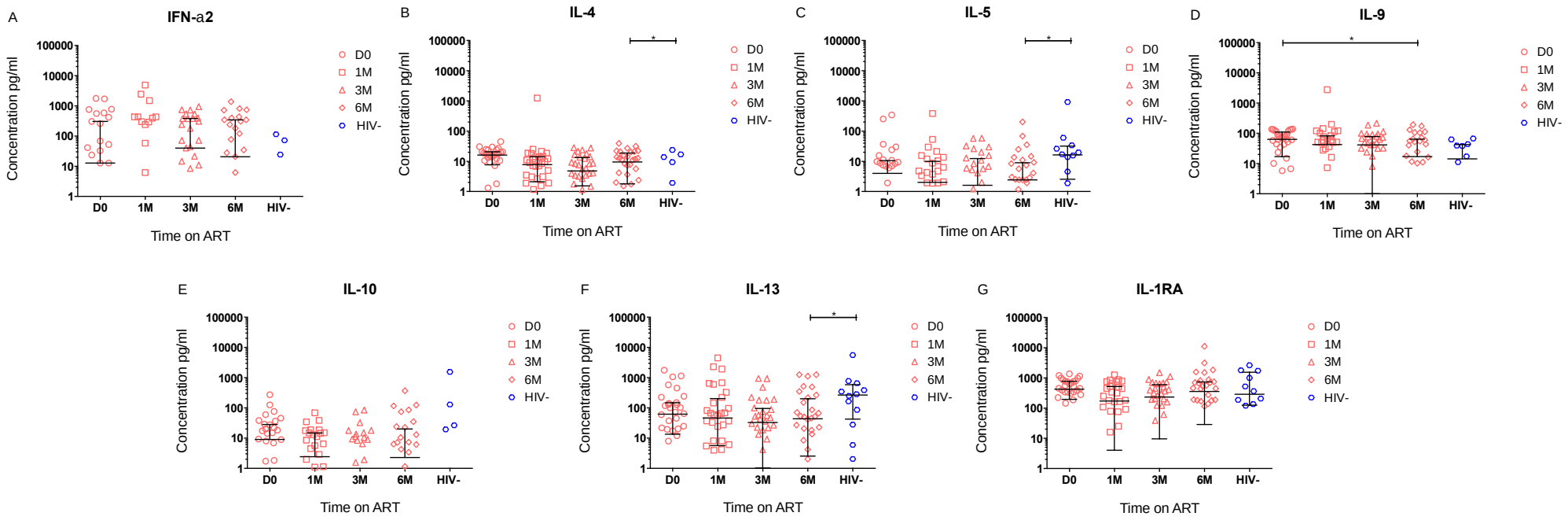

Figure 5. 2: TH_2 cytokines, (A) IFN-α2, (B) IL-4, (C) IL-5, (D) IL-9, (E) IL-10, (F) IL-13 and (G) IL-1RA, measured in QFT supernatants of HIV infected persons during 6 months of ART, and HIV uninfected persons using Luminex. Data points on graphs represent log transformed detectable concentrations of analytes for patients.

5.4.3. T cell growth factors

A summary of the soluble T cell growth factors measured are shown in Figure 5.3. and Table 5.5. Friedman test statistics (d.f. = 3) of 9.5 for IL-2 and 5.4 for IL-15 were calculated indicating a statistically significant increase for IL-2 concentrations (p-value = 0.023) but not for IL-15 (p-value = 0.145) concentrations in HIV infected persons during ART (p-value = 0.145). The concentrations of IL-2 (Figure 5.3. panel A) and IL-15 (Figure 5.3. panel C) tended to increase in HIV infected persons sensitised by *Mtb*, during 6 months of ART (p-values = 0.253 and 0.197) although not significantly as determined by paired t-test.

A cross sectional comparison between HIV infected persons at 6 months of ART with HIV uninfected persons, revealed no difference in IL-2 concentrations between HIV uninfected persons and HIV infected persons (p-value = 0.079), while the opposite was observed for IL-15. HIV infected persons produced significantly higher concentrations of IL-15 compared to HIV uninfected persons (p-value = 0.021). IL-7 concentrations measured in HIV infected persons during 6 months of ART did not differ significantly (Figure 5.3. panel B, p-value = 0.978). Comparison with the HIV uninfected persons revealed no difference in IL-7 concentrations compared to HIV infected persons (p-value = 0.275) and the Friedman test statistic of 3.6 (d.f. = 3) also indicated the difference was not statistically significant (p-value = 0.312).

Table 5. 5: Median and IQR of T cell growth factors measured in HIV infected and HIV uninfected cohorts and corresponding p-values.

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
IL-2	52 (21-98)	44 (14-140)	38 (5-73)	70 (10-229)	109 (57-449)	0.023	0.253	0.079
IL-7	8.4 (0-119)	1.3 (0-94)	12 (0-195)	12 (0-217)	78 (0.84-361)	0.312	0.275	0.978
IL-15	17 (0.15-57)	31 (10-60)	14 (0-48)	34 (2.8-59)	9.5 (0-20)	0.145	0.197	0.021

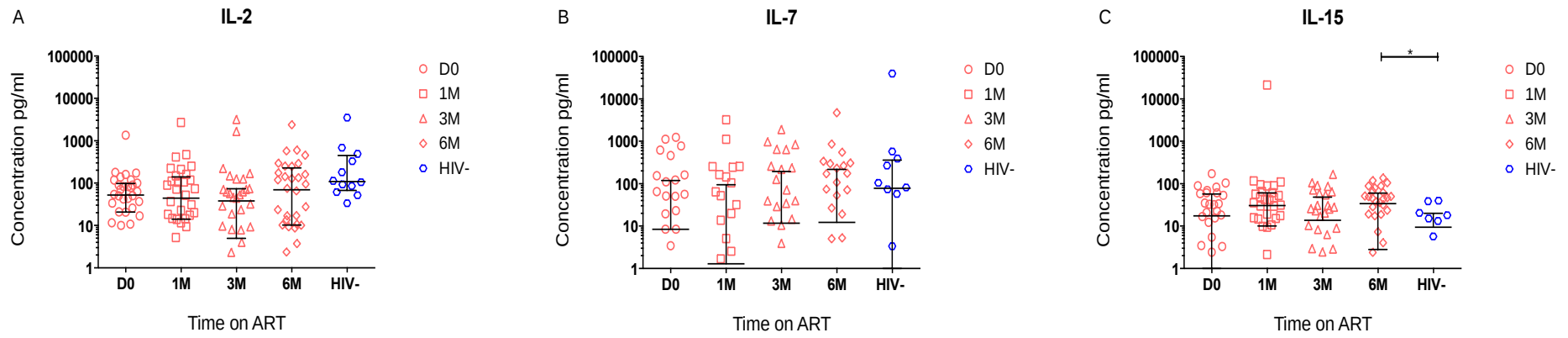

Figure 5. 3: T cell growth factors, (A) IL-2, (B) IL-7 and (C) IL-15, measured in QFT supernatants of HIV infected persons during 6 months of ART, and HIV uninfected persons using Luminex. Data points on graphs represent detectable log transformed concentrations of analytes for patients.

5.4.4. Chemoattractant analytes

A summary of soluble chemoattractant markers measured are shown in Figure 5.4. and Table 5.6. A Friedman test statistic of 6.3 (d.f. = 3), which was not statistically significant (p-value = 0.098), was calculated for Eotaxin concentrations produced by HIV infected individuals after 6 months of ART. Concentrations of Eotaxin in HIV infected persons declined during ART (Figure 5.4. panel A, p-value = 0.781) although not significantly while the comparison between HIV infected and HIV uninfected persons revealed that HIV infected persons produced higher concentrations of Eotaxin, although not significant, (p-value = 0.164).

Friedman test statistics, with d.f. = 3, for IL-8 (5.6), MIP-1α (5.9), MIP-1β (6.6) and Rantes (6.3) concentrations were not statistically significant (p-values = 0.135, 0.117, 0.085 and 0.099 respectively) in HIV infected persons after 6 months of ART. Meanwhile, IL-8 (Figure 5.4. panel B), MIP-1α (Figure 5.4. panel E), MIP-1β (Figure 5.4. panel F) and RANTES (Figure 5.4. panel G) concentrations decreased in HIV infected persons during ART (p-values = 0.386, 0.069, 0.36 and 0.725 respectively). Comparison with the HIV uninfected persons revealed that HIV infected persons produced significantly higher MIP-1α and MIP-1β concentrations than HIV uninfected persons (p-values = 0.006 and 0.0002), while IL-8 concentrations were also higher in HIV infected persons (p-value = 0.293) although not significant. RANTES concentrations in HIV infected persons did not differ significantly from that produced by HIV uninfected persons (p-value = 0.376).

Friedman test statistics (d.f. = 3) of 9.9 and 4.5 were generated for IP-10 and MCP-1, with the test statistic being statistically significant for IP-10 (p-value = 0.019) but not for MCP-1 (p-value = 0.208). The concentrations of IP-10 increased significantly in HIV infected persons (Figure 5.4. panel C, p-value = 0.005) and MCP-1 concentrations in HIV infected persons followed the same increasing trend, although not significant (Figure 5.4. panel D, p-value = 0.467). Lastly IP-10 concentrations in HIV uninfected persons were significantly higher than concentrations observed for HIV infected persons (p-value = 0.003), while the opposite was observed for MCP-1 concentrations (p-value = 0.078).

Table 5. 6: Median and IQR of chemoattractant analytes measured in HIV infected and HIV uninfected cohorts and corresponding p-values.

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
Eotaxin	38 (0-77)	26 (1.1-44)	7.3 (0-64)	27 (0-92)	0.70 (0-43)	0.098	0.781	0.164
IL-8	17528 (0-37283)	12045 (0-26839)	1732 (0-20409)	5360 (0-21996)	0.0 (0-24364)	0.135	0.386	0.293
IP-10	11589 (4619-34514)	12122 (1676-84556)	5322 (2245-22599)	24011 (4739-133594)	147526 (87963-177604)	0.019	0.005	0.003
MCP-1	1342 (298-2913)	1238 (594-1981)	608 (35-1719)	1548 (303-3109)	458 (111-1516)	0.208	0.467	0.078

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
MIP-1α	11608 (0-50695)	7873 (0-18472)	1749 (0-4871)	3995 (21-13929)	0.0 (0-0)	0.117	0.069	0.006
MIP-1β	19349 (0-44525)	8033 (0-34251)	6433 (0-22752)	13283 (1045-30129)	0.0 (0-460)	0.085	0.36	0.0002
RANTES	5091 (0-21730)	0.0 (0-3389)	817 (0-13512)	5563 (0-27645)	312 (0-17464)	0.099	0.725	0.376

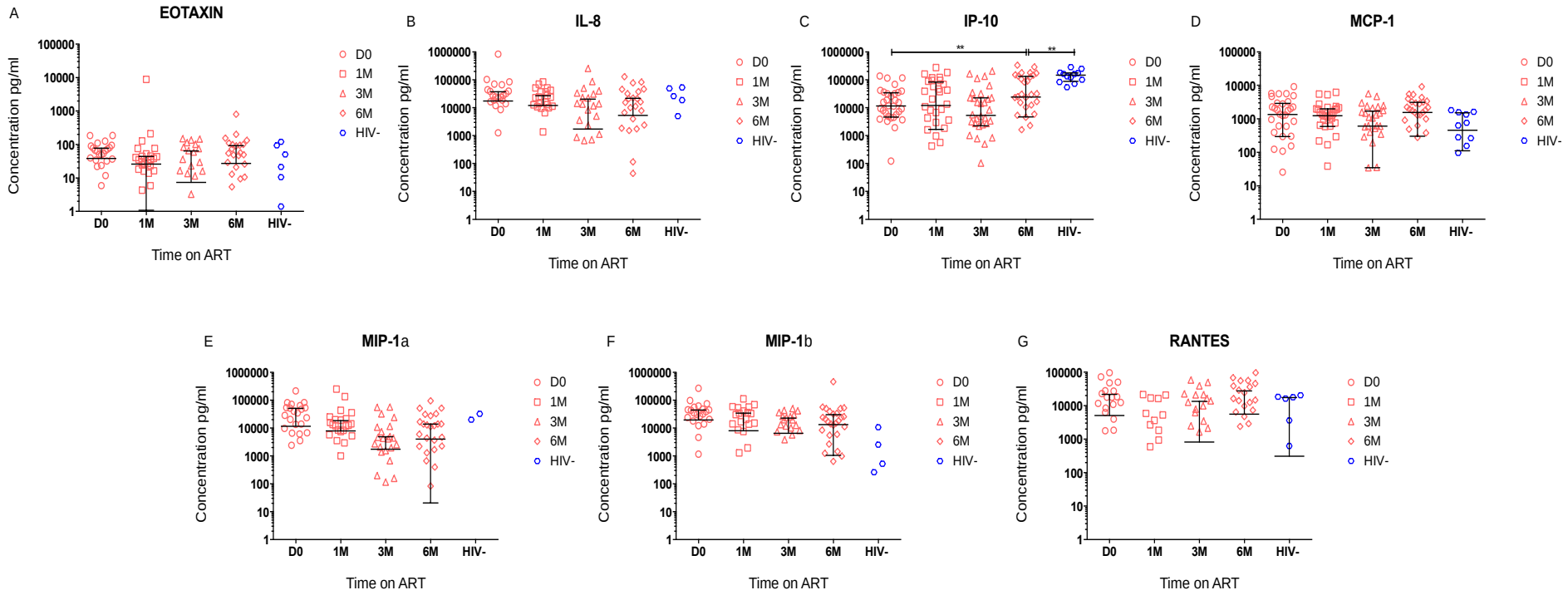

Figure 5. 4: Chemoattractant analytes, (A) Eotaxin, (B) IL-8, (C) IP-10, (D) MCP-1, (E) MIP-1α, (F) MIP-1β and (G) Rantes, measured in QFT supernatants of HIV infected persons during 6 months of ART, and HIV uninfected persons using Luminex. Data points on graphs represent log transformed detectable concentrations of analytes for patients.

5.4.5. Growth factors

A summary of soluble growth factors measured are shown in Figure 5.5. and Table 5.7. A Friedman test statistic of 9.8 (d.f. = 3) which was statistically significant (p-value = 0.02) was calculated for the concentration of basic FGF produced by HIV infected individuals after 6 months of ART. Concentrations of basic FGF in HIV infected persons following 6 months of ART declined although not significantly (Figure 5.5. panel A, p-value = 0.154). Comparison with the HIV uninfected persons revealed that HIV infected persons produced similar concentrations, after 6 months of ART, of basic FGF (p-value = 0.247).

Friedman test statistics (d.f. = 3) determined for FGF-2 (7.8) and G-CSF (19) were statistically significant (p-values = 0.05 and 0.0002), while Friedman statistics for VEGF (4.8) and PDGF (3.8) were not statistically significant (p-values = 0.183 and 0.279). Similar to basic FGF, FGF-2 (Figure 5.5. panel B) and PDGF (Figure 5.5. panel E) concentrations declined in HIV infected persons (p-values = 0.132 and 0.765), while G-CSF (Figure 5.5. panel C), and VEGF (Figure 5.5. panel F) concentrations significantly decreased in HIV infected persons (p-values = 0.0006 and 0.039). Comparisons with the HIV uninfected persons revealed that FGF-2 concentrations produced by HIV infected and uninfected persons were indistinguishable (p-value = 0.9749), while HIV infected persons produced significantly higher concentrations of G-CSF (p-value = 0.002) and VEGF (p-value = 0.279) although not significant. Conversely, HIV uninfected persons produced higher concentrations of PDGF (p-value = 0.339) than HIV infected persons.

The median concentration of GM-CSF (Figure 5.5. panel D) was undetectable in both HIV infected persons following 6 months of ART, and HIV uninfected persons included in this analysis.

Table 5. 7: Median and IQR of growth factors measured in HIV infected and HIV uninfected cohorts and corresponding p-values.

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
Basic FGF	137 (53-212)	82 (0.46-205)	25 (0-119)	84 (4-153)	22 (0-100)	0.02	0.154	0.247
FGF-2	357 (0-817)	0.0 (0-736)	0.0 (0-558)	0.0 (0-262)	0.0 (0-332)	0.05	0.132	0.9749
G-CSF	269 (99-480)	94 (40-205)	76 (0-126)	81 (0-157)	0.0 (0-3.1)	0.0002	0.0006	0.002

	HIV Infected Persons (n=32)				HIV Uninfected Persons (n=12)	HIV Infected D0 vs. 6M (with multiple comparisons)	HIV Infected D0 vs. 6M (paired t test)	HIV Infected at 6M vs. HIV Uninfected (unpaired t test)
	D0 of ART	**1M**	**3M**	**6M**				
GM-CSF	0.0 (0-66)	0.0 (0-30)	0.0 (0-13)	0.0 (0-0)	0.0 (0-10)	0	0	0
PDGF	619 (0-2385)	415 (0-775)	248 (0-849)	542 (0-1622)	1352 (91-2320)	0.297	0.765	0.339
VEGF	64 (0-184)	9.0 (0-86)	0.0 (0-50)	0.0 (0-40)	0.0 (0-0)	0.183	0.039	0.279

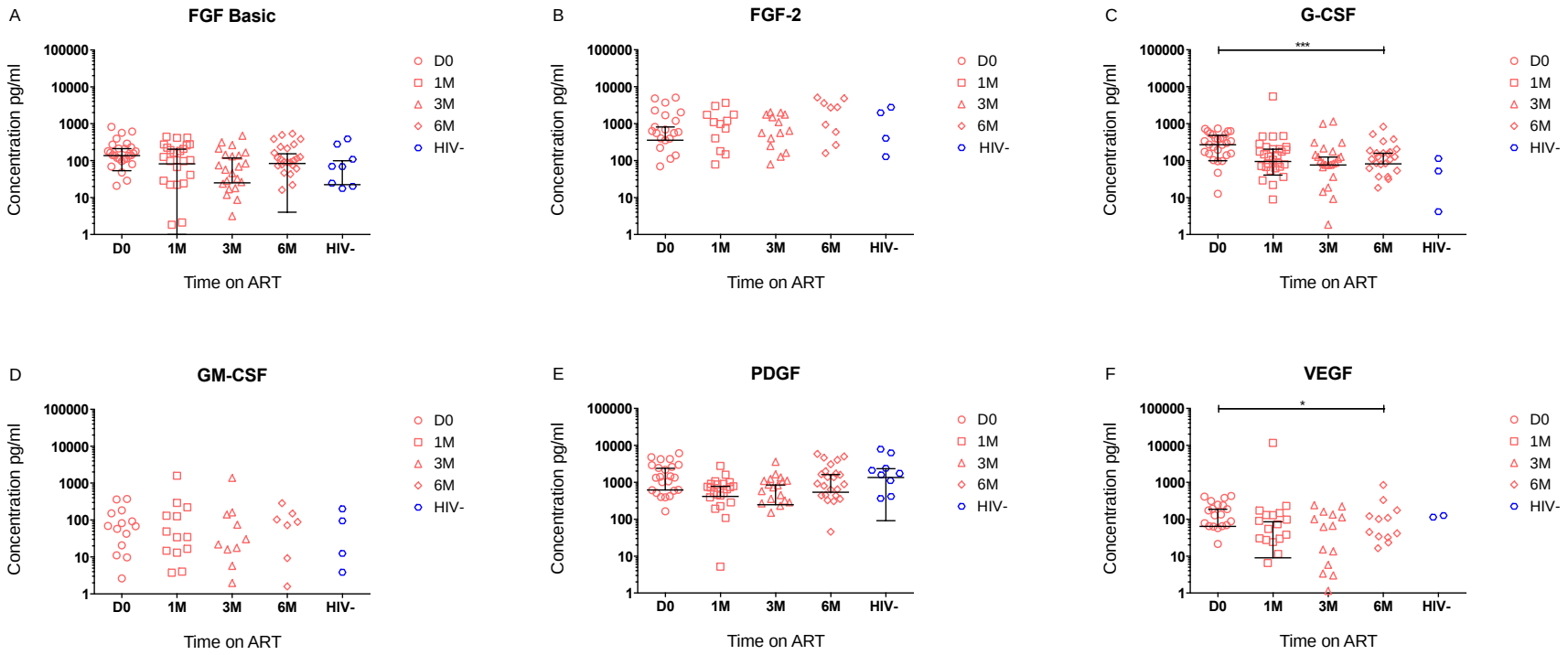

Figure 5. 5: Growth factors, (A) FGF basic, (B) FGF-2, (C) G-CSF, (D) GM-CSF, (E) PDGF and (F) VEGF measured in QFT supernatants of HIV infected persons during 6 months of ART, and HIV uninfected persons using Luminex. Data points on graphs represent detectable log transformed concentrations of analytes for patients.

Following this analysis method, we decided to determine whether the pro-inflammatory analyte concentrations correlated with each other and whether this changed during 6 months of ART.

5.4.6. Correlations of analyte concentrations measured in HIV infected persons during 6 months of ART

A correlation matrix was generated for all pro-inflammatory analytes measured at day 0 and after 6 months of ART. The Spearman correlation values and corresponding p-values are summarised in Figure 5.7. Spearman correlations revealed that IL-1β correlated positively with IL-6, IL-17A and TNF-α (p-values < 0.0001, 0.011 and 0.001 respectively). IL-1α also positively correlated with TNF-α (p-value = 0.027) and IL-12p70 positively correlated with IL-17A and TNF-α (p-values = 0.001 and 0.034 respectively) in HIV infected persons at day 0.

During 6 months of ART, Spearman correlations revealed that IL-1β remained positively correlated with IL-17A, TNF-α and IL-6 (p-values = 0.0022, 0.005 and < 0.0001 respectively) and IL-17A remained positively correlated with TNF-α (p-value = 0.024). Additionally it was determined that IL-6 now positively correlated with IL-17A (p-value = 0.004), whereas IL-12p70 no longer correlated with IL-17A and TNF-α; and IL-1α no longer correlated with TNF-α as previously noted.

For an unbiased analysis, we conducted paired t-tests and ANOVA statistical analysis, for each timepoint comparison, followed by correction for multiple comparison using the Benjamini-Hochberg procedure. The p-values generated from these tests were used to calculate and plot the corresponding 1 minus p-value numerical figures, as coloured dots on a set of axes, that are displayed in Figure 5.7. A vertical line was placed at 0.95 on the x-axis representing the cut-off value for statistical significance, and thus all coloured dots to the right of the line represent comparisons for which there was a significant difference between any two timepoints. This analysis was performed with the help of the statistician, Dr. Maia Lesosky.

Following correction for multiple comparisons it was evident that the pro-inflammatory group of analytes, specifically IFN-γ, TNF-α, IL-6, IL-17A and IL-1β (Figure 5.6.), had changed significantly at various timepoints during ART, in line with the correlation analysis above. Additionally this unbiased analysis had also indicated that there were significant differences in the concentrations of IL-4, G-CSF, basic FGF and IP-10 at, at least one timepoint comparison.

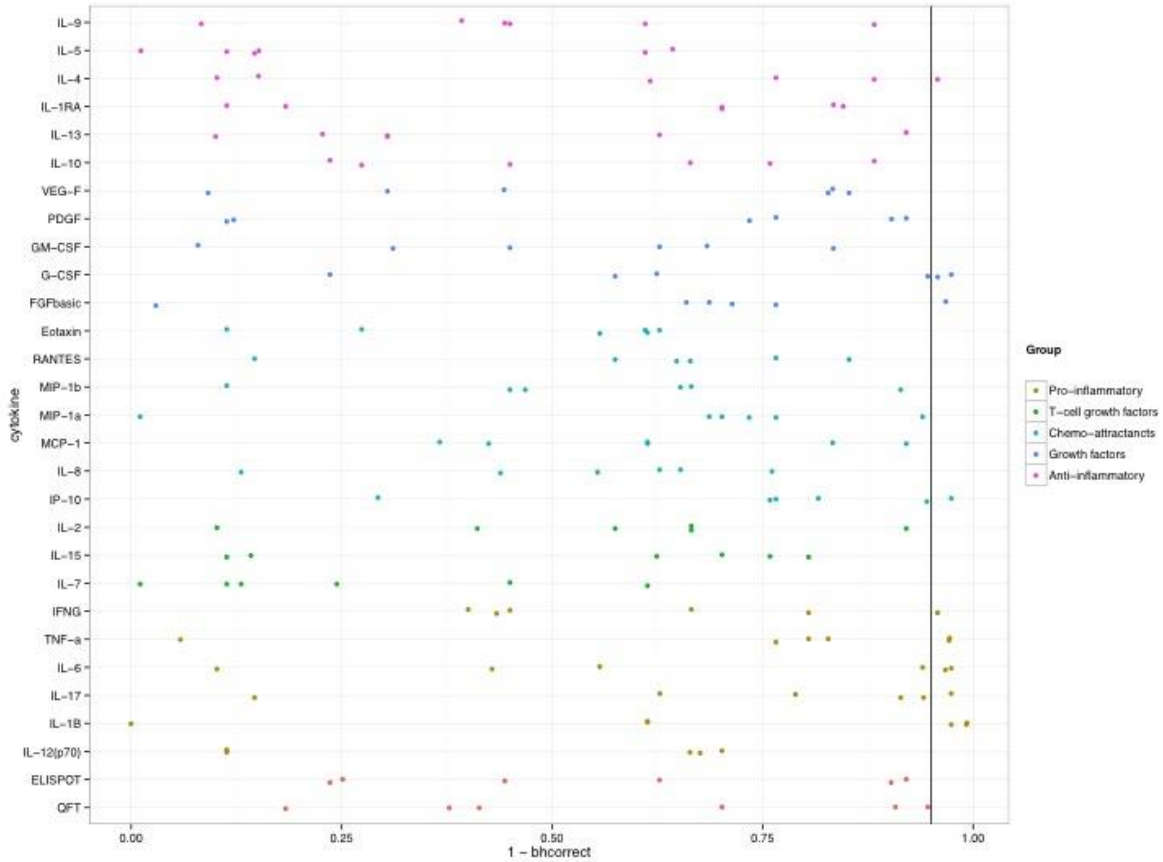

Figure 5. 6: Figure representing 1-p-values conducted for comparisons of each timepoint, after correction for multiple comparisons, in HIV infected persons during 6 months of ART.

A

D0	IL-1a	IL-1b	IL-6	IL-12p70	IL-17A	TNF-a
IL-1a		0.308	0.162	-0.108	0.126	0.394
IL-1b	0.308		0.707	0.068	0.442	0.535
IL-6	0.162	0.707		-0.156	0.211	0.330
IL-12p70	-0.108	0.068	-0.156		0.534	0.375
IL-17A	0.126	0.442	0.211	0.534		0.462
TNF-a	0.394	0.535	0.330	0.375	0.462	

B

6M	IL-1a	IL-1b	IL-6	IL-12p70	IL-17A	TNF-a
IL-1a		-0.246	-0.027	-0.279	0.047	-0.195
IL-1b	-0.246		0.818	-0.068	0.402	0.479
IL-6	-0.027	0.818		0.027	0.486	0.269
IL-12p70	-0.279	-0.068	0.027		0.132	-0.086
IL-17A	0.047	0.402	0.486	0.132		0.396
TNF-a	-0.195	0.479	0.269	-0.086	0.396	

C

D0	IL-1a	IL-1b	IL-6	IL-12p70	IL-17A	TNF-a
IL-1a		0.091	0.382	0.561	0.498	0.027
IL-1b	0.091		< 0.0001	0.708	0.011	0.001
IL-6	0.382	< 0.0001		0.391	0.244	0.064
IL-12p70	0.561	0.708	0.391		0.001	0.034
IL-17A	0.498	0.011	0.244	0.001		0.007
TNF-a	0.027	0.001	0.064	0.034	0.007	

D

6M	IL-1a	IL-1b	IL-6	IL-12p70	IL-17A	TNF-a
IL-1a		0.180	0.882	0.128	0.801	0.291
IL-1b	0.180		< 0.0001	0.710	0.022	0.005
IL-6	0.882	< 0.0001		0.881	0.004	0.135
IL-12p70	0.127	0.710	0.881		0.470	0.636
IL-17A	0.801	0.022	0.004	0.470		0.024
TNF-a	0.291	0.005	0.135	0.636	0.024	

Figure 5. 7: Correlation matrix of pro-inflammatory analytes measured in HIV infected persons. Spearman r values at (A) day 0 and (B) after 6 months of ART and corresponding p-values at (C) day 0 and (D) after 6 months of ART, with significant values in red.

5.5. Discussion

33 markers were analysed using the Luminex assay in HIV infected persons undergoing immune reconstitution through antiretroviral treatment, to identify potential soluble markers of protective immunity to TB, in HIV infected persons sensitised by *Mtb*.

Our results revealed a significant decrease in the pro-inflammatory cytokines IFN-γ, IL-1α, IL-1β, IL-6, IL-17A and TNF-α in HIV infected persons after 6 months of ART. Although not significant, IL-12p70 mirrored this trend. Additionally our results indicated that after 6 months of ART, concentrations of pro-inflammatory cytokines fell to concentrations similar to, or lower than that observed in HIV uninfected persons. Xiong and colleagues found significantly higher concentrations of pro-inflammatory cytokines, TNF-α, IL-6 and IP-10, in patients with pulmonary TB compared to patients with no TB [31].

The release of IFN-γ is mediated by innately-derived TNF-α, IL-2 and the autocrine binding of itself [32]. IFN-γ in turn plays a pivotal role in immune activation as it mediates the transcription of at least 200 genes via the Janus kinase/signal transducers and activators of JAK/STAT transcription pathway [33]. Moreover IFN-γ is required for the attraction of immune cells to the sites of inflammation and is also vital in order for lymphocyte-endothelial interactions to occur. This is achieved by the upregulation of adhesion molecules and the secretion of the chemoattractants RANTES, IP-10, MIP-

1α, MIP-1β and MCP-1 [33]. IFN-γ produced by T_H1 cells leads to macrophage activation thus resulting in the death of *Mtb* bacilli and increased MHC class II molecule expression, in turn promoting antigen recognition. IL-6 serves a double role in mediating inflammation and the host response. It therefore plays an essential role in the immune response after *Mtb* infection [34, 35].

IL-17 is characterised as a pro-inflammatory cytokine and has a role in inflammation initiation and in the induction of chemokine gradients [7, 36, 37]. IL-17 has also been shown to play a role in macrophage accumulation [38] thus Khader and Cooper have suggested that IL-17 may play a role in the accumulation of polymorphonuclear cells [39]. Based on this evidence, it has been suggested that IL-17 has an impact on the inflammatory response to mycobacteria [40, 41].

Once activated, by the ingestion of neutrophils containing dead bacilli, macrophages release TNF-α [42] while IL-1β is released upon ingestion of live bacilli [7]. TNF-α is required for granuloma formation and in the lymph node, TNF-α acts with an array of cytokines (including IL-1β and IL-6) to promote T_H17 cell differentiation [7]. TNF-α is known to promote phagocytosis in macrophages resulting in the death of *Mtb* bacilli, however increased levels of TNF-α have been associated with increased pathology [43]. IL-1β contributes towards the resistance to *Mtb* infection, during the early stages of infection [44], although increased concentrations may be

detrimental to the host [45] and IL-1β has been associated with acute chronic inflammation [46].

Interestingly we noted IP-10 concentrations significantly increased in HIV infected persons during 6 months of ART. Xiong and colleagues found significantly higher levels of IP-10 in patients with TB compared to healthy patients [31]. IP-10 is currently the leading alternative biomarker for the detection of *Mtb* antigen specific responses for diagnostic purposes [33] having been shown to have a greater sensitivity than IGRAs in HIV infected adults and children [33, 47, 48]. IP-10 is a chemoattractant that is secreted by antigen-presenting cells (APCs) and is also induced via cell surface receptor interactions between APCs and T cells [49-51]. It binds to the CXCR3 receptor that is involved in regulating innate and adaptive immune responses by chemotaxis [52, 53]. Chemokines, such as IP-10, induce inflammation thereby mediating a protective anti-TB immune response, however excessive chemokine-induced lung inflammation can drive lung pathology [54]. Additionally increased chemokine levels may promote *Mtb* proliferation and therefore increased bacterial burdens [55].

The concentrations of the TH_2-associated cytokines; IL-4, IL-5, IL-10, IL-13, IL-1RA and IL-9, chemoattractant soluble markers; EOTAXIN, IL-8, MIP-1α, MIP-1β and RANTES, growth factor analytes; basic FGF, FGF-2, PDGF, G-CSF and VEGF tended to decline in HIV infected persons during 6 months of ART. G-CSF and VEGF significantly declined after ART, with VEGF concentrations falling to undetectable levels. GM-CSF was undetectable in

both cohorts. Conversely the concentrations of IFN-α2, T cell growth factors IL-2, lL-7 and IL-15, chemoattractant MCP-1 tended to increase in the same cohort of individuals.

While overall, we show a decline in concentrations in the majority of analytes measured in our HIV infected persons receiving ART cohort, our study has several limitations, including a relatively small sample size and a heterogeneous cohort. While we recruited a sufficient number of HIV infected (n=50) and HIV uninfected persons (n=55) in order to be powered for this study, we were unable to include all recruited persons for these analyses due to missing time points during follow up of ART, and the costs associated with these assays. Currently there is no gold standard for the detection of latent TB infection thus some discordant results, between the QFT and ELISpot, from the same person is expected due to assay limitations [56, 57]. Moreover while our results reflect an *Mtb*-specific response, we are unable to determine the cellular source of each analyte due to limitations of this assay. Therefore our results are representative of the innate and adaptive immune response to LTBI. We were also unable to assess every potential soluble marker, in order to perform an unbiased assessment, due to a limited number of markers being incorporated into a single kit panel.

In conclusion our results point to a decrease in immune activation as demonstrated by the decline in the majority of soluble analytes detected. Importantly we observed a significant decrease in the pro-inflammatory analytes that are predominantly responsible for chemokine induction,

downstream activation of immune cells and the orchestration of immune cells to the site of infection.

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Chapter 6: Transcriptomic Analysis of Central Memory Cell-specific and Effector Memory Cell-specific genes in Whole Blood from HIV Infected persons, sensitised by *Mycobacterium tuberculosis*

6.1. Introduction

Following antigen exposure, memory T cells are classified into either central memory T cells or effector memory T cells based on the expression of certain markers that are indicative of their functions. These functions include proliferative capacity, differentiation capacity, cytokine secretion, senescence profile and the ability to home or migrate. Central memory T cells are early differentiated $CD27^+$ memory T cells, that have been shown to home to the secondary lymphoid organs [1]. Conversely effector memory T cells are late differentiated $CD27^-$ memory T cells that exhibit effector functions, such as the ability to produce high levels of IFN-γ, and have been shown to migrate into peripheral sites of inflammation [1].

A study conducted by our collaborators, Dintwe PhD, 2014, [2] aimed to characterize naïve-like *Mtb*-specific T cells at the transcriptional level by measuring the expression of genes that are differentially expressed between naïve, effector, central memory and stem cell like memory T cells. They

therefore selected genes, based on literature available, that were differentially expressed between the aforementioned memory T cell subsets. Genes translated into products such as cytokines and cytokine receptors, chemokine receptors, inhibitory molecules, co-stimulatory molecules, kinases, transcription factors, molecules that play a role in homing and migration and effector molecules were selected. They subsequently determined genes that were differentially expressed in the memory T cell sub-populations. Thus, ICOS, SELL, PRKCA, TCF7L, LEF1, NFκβ, CD38, ITK, IGF1R, ARHGEF18, AXIN2, CCR7 and CD27 had a higher expression in central memory T cells compared to effector memory T cells while the genes; GNLY, RORC, TGF-β, PRF1, CCL5, IFN-γ, CCR2, GATA, GZMA, GZMB, GZMK, PRR5L, TXB21, IL-2Rβ, CCR4, CCR5 and FAM129A had a higher expression in effector memory T cells compared to central memory T cells. We determined the expression of these T cell memory genes during ART in collaboration, with the hypothesis that the transcriptomic signature associated with central memory T cells will increase over time in HIV infected persons on ART, while the signature associated with effector memory T cells will decrease. We therefore compared gene transcriptional signatures characteristic of central memory and effector memory T cells in HIV infected persons starting ART and sampled at day 0, 1 month, 3 months and 6 months of ART.

6.2. Patient characteristics of individuals included in analysis

Forty-five HIV infected persons were included in this arm of the study based on availability of blood stored in Tempus® tubes for RNA extraction at all follow up timepoints. An additional 23 patients recruited from the same site, and using the same inclusion criteria, were also included in this arm of the study thus bringing the total number of HIV infected persons to 68, with median age 33 (29-37) years, 49 males/ 19 females. All HIV infected persons and HIV uninfected persons included in this aim were determined to be *Mtb* sensitised based on a positive IFN-γ response determined by either the QFT, ELISpot or the Luminex assay at at least one timepoint. Therefore all persons were considered to be sensitised to *Mtb*. Blood for RNA isolation was not collected from the HIV uninfected persons. The characteristics of all patients included in this arm of the study are summarized in Tables 6.1. and 6.2.

Table 6. 1: Characteristics of patients included in the transcriptomic analysis of CM- and EM- T cell specific genes in whole blood

	HIV Infected Persons (n=68)
Median Age (years)	33 (29-37)
Gender Male/Female	49/19

Table 6. 2: Median and (IQR) longitudinal CD4 counts and Viral loads of patients included

Median (IQR)	**HIV Infected Persons (n=68)**			
	Day 0 of ART	**1M**	**3M**	**6M**
CD4 count	199 (115-252)	272 (207-347)	297 (189-363)	294 (240-406)
Viral Load	76869 (36 559-224 000)	300 (123-725)	40 (39-109)	40 (39-40)

6.3. Methods and statistical analysis

RNA from blood stored in Tempus™ Blood RNA tubes was isolated using the Tempus™ Spin RNA Isolation Kit, according to the manufacturer's instruction, after tubes were allowed to thaw overnight at room temperature. Following isolation the RNA quantity and quality was determined using Nanodrop spectrophotometry and the Agilent Bioanalyzer. Briefly the 260/280 ratio, determined by the Nanodrop, was used to assess the purity of the RNA with a ratio of between 1.8 and 2.0 being accepted as pure RNA. The 260/230 ratio was used to assess the possibility of contamination and RNA with a 260/230 ratio lower than 2.2 was considered to be contaminated. Using the electrophoretic trace of the RNA, the Bioanalyzer assigned RIN values to all RNA patient samples based on the integrity. RIN values >8 indicate high quality RNA.

RNA was reverse transcribed to generate cDNA using the SuperScript® III First-Strand Synthesis System for RT-PCR kit, according to the manufacturer's instructions. Taqman probes were used to detect the Ct values of exponentially amplified cDNA using RT-PCR. The data generated for each gene of interest was normalised to 3 housekeeping genes, 18S, β-actin and GAPDH, and corrected for CD4 counts by further normalising to Log_2[CD4] for each patient, in Microsoft Excel 2011 and subsequent data analysis was performed using GraphPad Prism (version 6.0).

Statistical analysis was performed using GraphPad Prism (version 6) using methods for non-parametric data. The Friedman test, with correction for

multiple comparisons, was used for paired data comparison across all sampling time points in HIV infected individuals. There were four sampling time points (k=4) thus the Friedman test was performed with d.f. = 3 ('degrees of freedom' = k-1 and alpha = 0.05) in order to determine p-values. The Kruskal Wallis test with correction for multiple comparisons was used for unpaired data comparisons across all time points in HIV infected persons. The Wilcoxon test (equivalent of the non-parametric test) was used to compare paired data in HIV infected persons at day 0 and 6 months of ART. A p-value of less than or equal to 0.05 was considered statistically significant. P-values are illustrated in subsequent figures by asterixes and mean the following: ns refers to a p-value > 0.05, a single asterisk (*) refers to a p-value ≤ 0.05, two asterix (**) refer to a p-value ≤ 0.01, three asterix (***) refer to a p-value ≤ 0.001 and four asterix (****) refer to a p-value ≤ 0.0001.

6.4. Results

The results of the RT-PCR experiments were analysed longitudinally, after being normalized to the three housekeeping genes 18S, β-actin and GAPDH in order to generate ΔCt values. The genes we selected were T cell-specific genes and since RT-PCR was conducted on RNA isolated from whole blood, as opposed to RNA of specific memory T cells, we corrected for CD4 counts in order to represent the data with respect to the number of CD4 T cells. This was achieved by dividing the ΔCT value at each timepoint by the Log_2[CD4] count of the patient at the corresponding timepoint. We then represented the

data using bar graphs and compared the resulting medians at day 0 to medians after 6 months of ART, for each gene normalised to each housekeeping gene. A summary of all comparisons is shown in Table 6.5. We then grouped the genes based on whether there was a significant difference in gene expression levels during ART when normalised to each of the three housekeeping genes. It is important to note that a decrease in the ΔCT/CD4 indicates an increase in expression of the particular gene, and vice versa.

Following analysis the genes GNLY, PRF1, RORC and TCF7L2 showed no significant change in expression, in HIV infected persons during 6 months of ART, when normalised to 18S, β-actin and GAPDH (Figure 6.1. Panels A-D). The genes ICOS, ITK, NFκβ1, PRKCA, ARHGEF18, AXIN2, CCR7, CD27, IGF1R, LEF1, CD38, SELL and TCF7L2 were shown to be more highly expressed in central memory CD4 T cell subsets as opposed to effector memory CD4 T cell subsets (Mpanda *et al.* unpublished). Similarly, we observed a significant increase in the expression of ICOS, ITK, NFκβ1 and PRKCA (p-values = 0.029, 0.0001, 0.0011 and 0.0017 respectively) when normalised to the 18S housekeeping gene (Figure 6.2. Panels A-D). The expression of ARHGEF18, AXIN2, CCR7, CD27, IGF1R and LEF1 significantly increased after 6 months of ART (Figure 6.3. Panels A-H) when normalised to 18S and β-actin housekeeping genes (p-values summarised in Table 6.3.), while CD38 and SELL expression significantly increased when normalised to 18S and GAPDH (p-values summarised in Table 6.3.).

Conversely the genes CCL5, CCR2, CCR4, CCR5, FAM129A, GATA3, GZMA, GZMB, GZMK, IFN-γ, IL-2RB, PRF1, PRR5L, RORC, TBX21 and TGFβ1 were shown to be more highly expressed in effector memory CD4 T cell subsets (Mpanda *et al.* unpublished). With the exception of TBX21, the expression of aforementioned genes significantly increased, in HIV infected persons during 6 months of ART, when normalised to the 18S housekeeping gene (p-values = 0.003, 0.0012, 0.0001, 0.009, 0.0189, 0.0073 and 0.0022 respectively, Figure 6.4. Panels A-H). TBX21 exhibited a decrease in gene expression when normalised to the housekeeping gene GAPDH (p-value = 0.0176, Figure 6.3. Panel I) while expression remained relatively consistent when normalised to the other housekeeping genes.

Table 6. 3: P-values for comparing changes of central memory CD4 T cell specific gene expression between day 0 and 6 months of ART in HIV infected persons.

Gene	Normalised to 18S	Normalised to β-actin	Normalised to GAPDH
ARHGEF18	<0.0001	0.0399	0.7218
AXIN2	<0.0001	0.0165	0.0754
CCR7	<0.0001	0.0051	0.0608
CD27	<0.0001	0.0012	0.073
IGF1R	<0.0001	0.0005	0.0084
LEF1	0.0005	0.0058	0.1568
CD38	0.0094	0.8935	0.0395
SELL	0.0264	0.3725	0.0001

Lastly the expression of the effector memory CD4 T cell specific genes CCR2, FAM129A, GZMB, GZMK and IL-2RB, in HIV infected persons after 6 months of ART, significantly increased when normalised to the 18S and β-actin housekeeping genes but not to GAPDH (Figure 6.5. Panels A-E, p-values summarised in Table 6.5.).

Table 6. 4: P-values for comparing changes of effector memory CD4 T cell specific gene expression between day 0 and 6 months of ART in HIV infected persons.

Gene	Normalised to 18S	Normalised to β-actin	Normalised to GAPDH
CCR2	0.0001	0.0028	0.6296
FAM129A	<0.0001	0.0036	0.068
GZMB	<0.0001	0.0424	0.6715
GZMK	0.0003	0.0033	0.0586
IL2RB	<0.0001	0.0092	0.1571

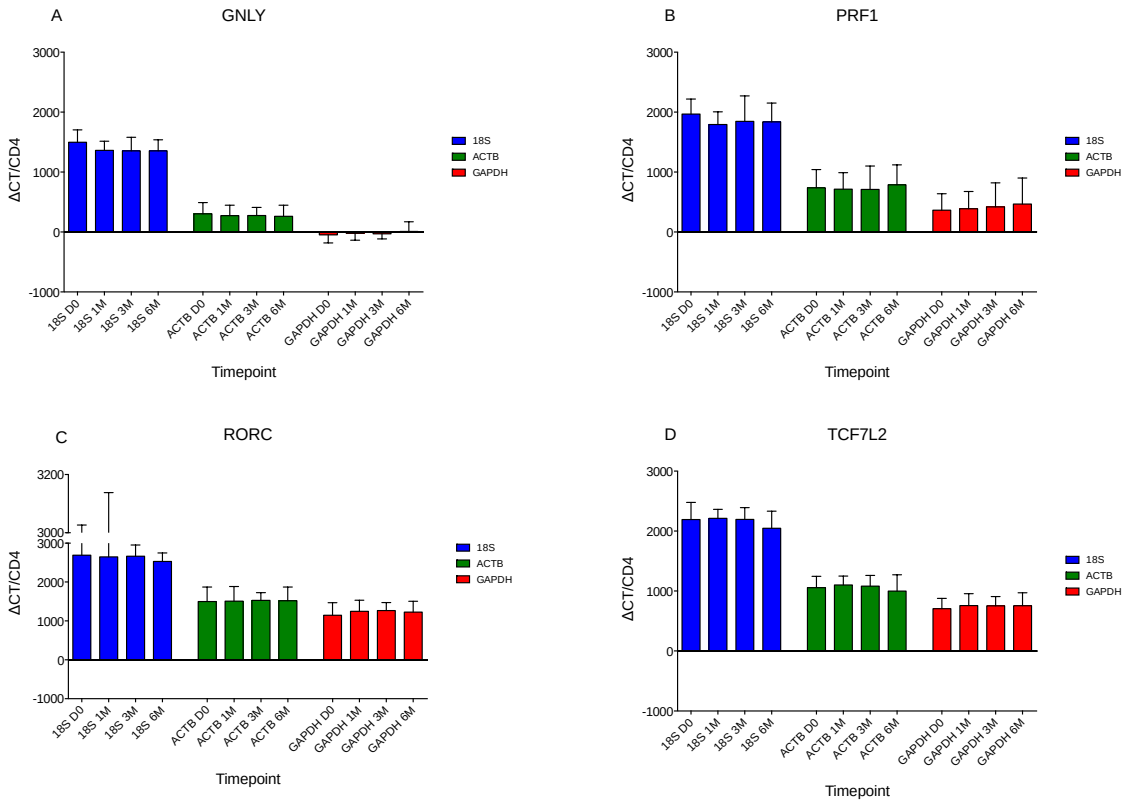

Figure 6. 1: Gene expression of (A) GNLY, (B) PRF1, (C) RORC and (D) TCF7L2 showing no significant change after 6 months of ART compared to day 0 of ART, when normalised to 3 housekeeping genes using RT-PCR. Graphs are a measure of ΔCt normalised to CD4 counts, a decrease in the ΔCT/CD4 indicates an increase in expression of the particular gene.

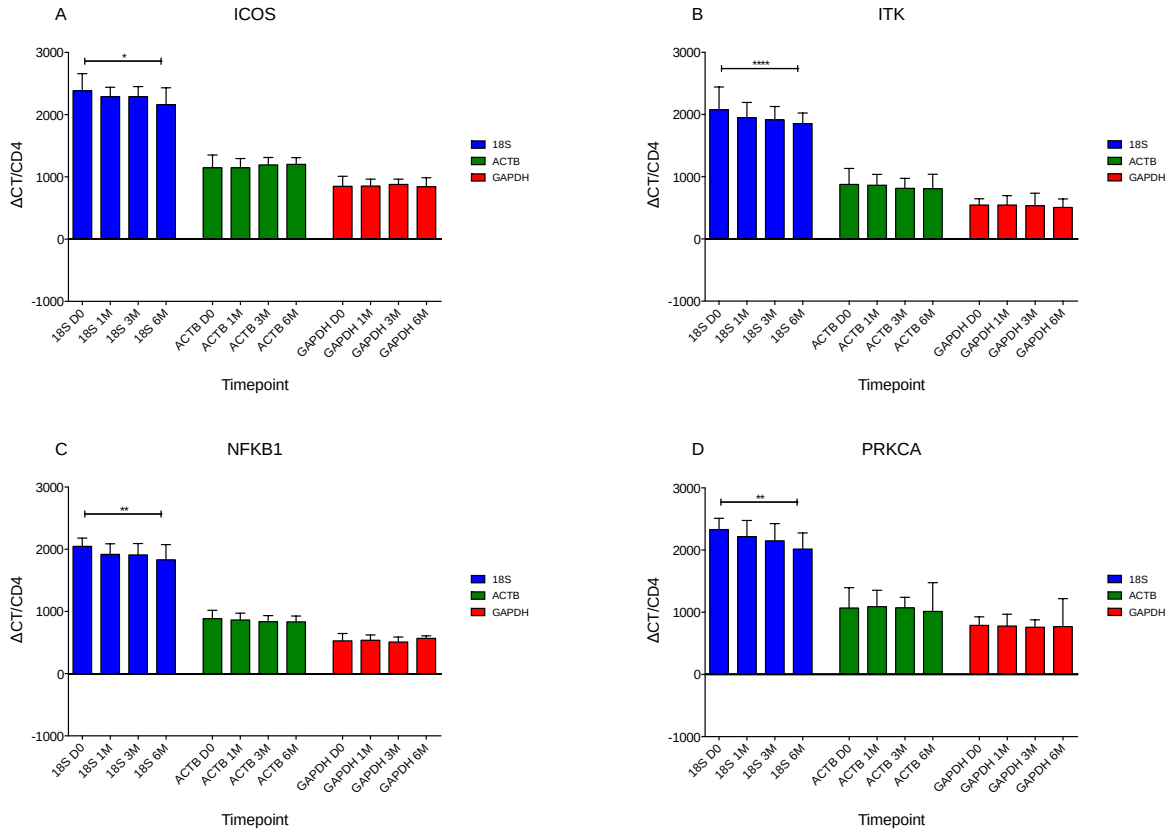

Figure 6. 2: Gene expression of central memory CD4 T cell-specific genes (A) ICOS, (B) ITK, (C) NFKβ1 and (D) PRKCA showing a significant change after 6 months of ART compared to day 0 of ART, when normalised to 18S housekeeping gene using RT-PCR. Graphs are a measure of ΔCt normalised to CD4 counts, a decrease in the ΔCT/CD4 indicates an increase in expression of the particular gene.

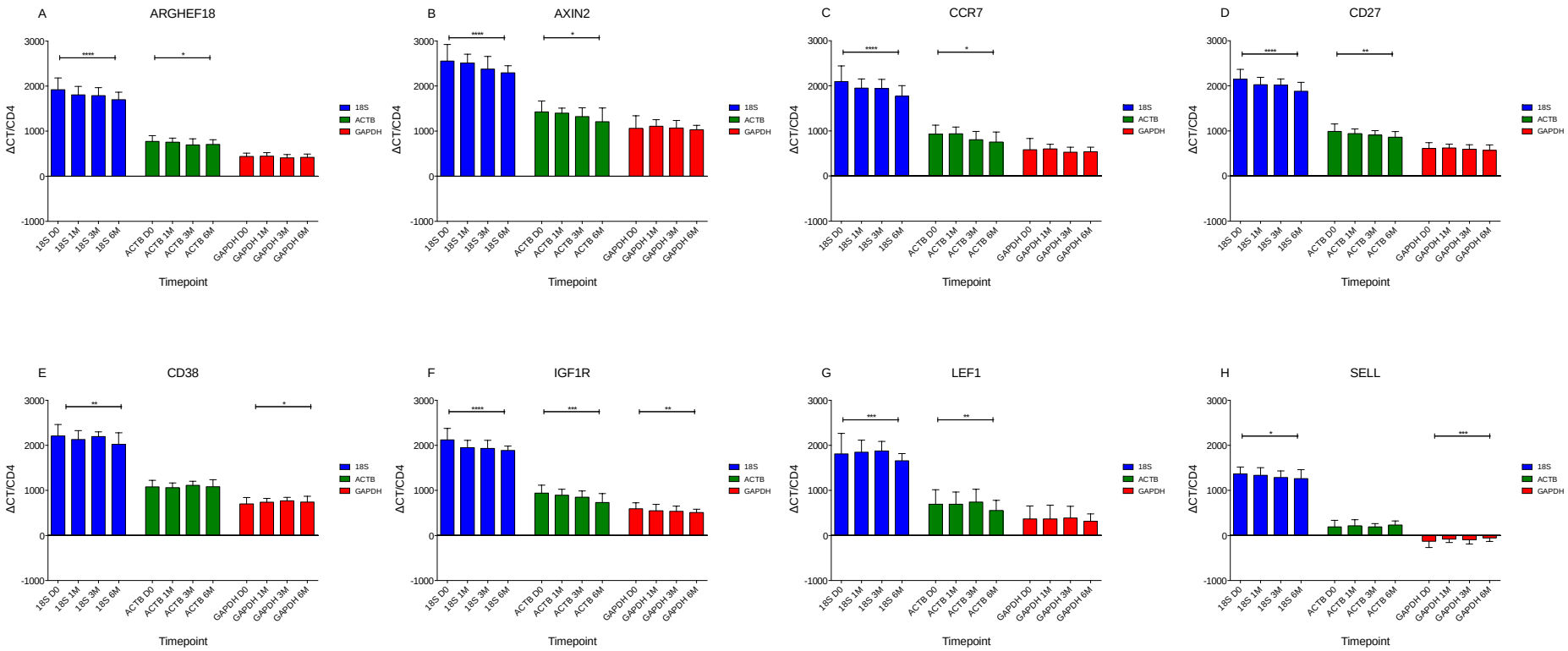

Figure 6. 3: Gene expression of central memory CD4 T cell-specific genes (A) ARHGEF18, (B) AXIN2, (C) CCR7, (D) CD27, (E) CD38, (F) IGF1R, (G) LEF1 and (H) SELL showing a significant change after 6 months of ART compared to day 0, when normalised to two housekeeping genes using RT-PCR. Graphs are a measure of ΔCt normalised to CD4 counts, a decrease in the ΔCT/CD4 indicates an increase in expression of the particular gene.

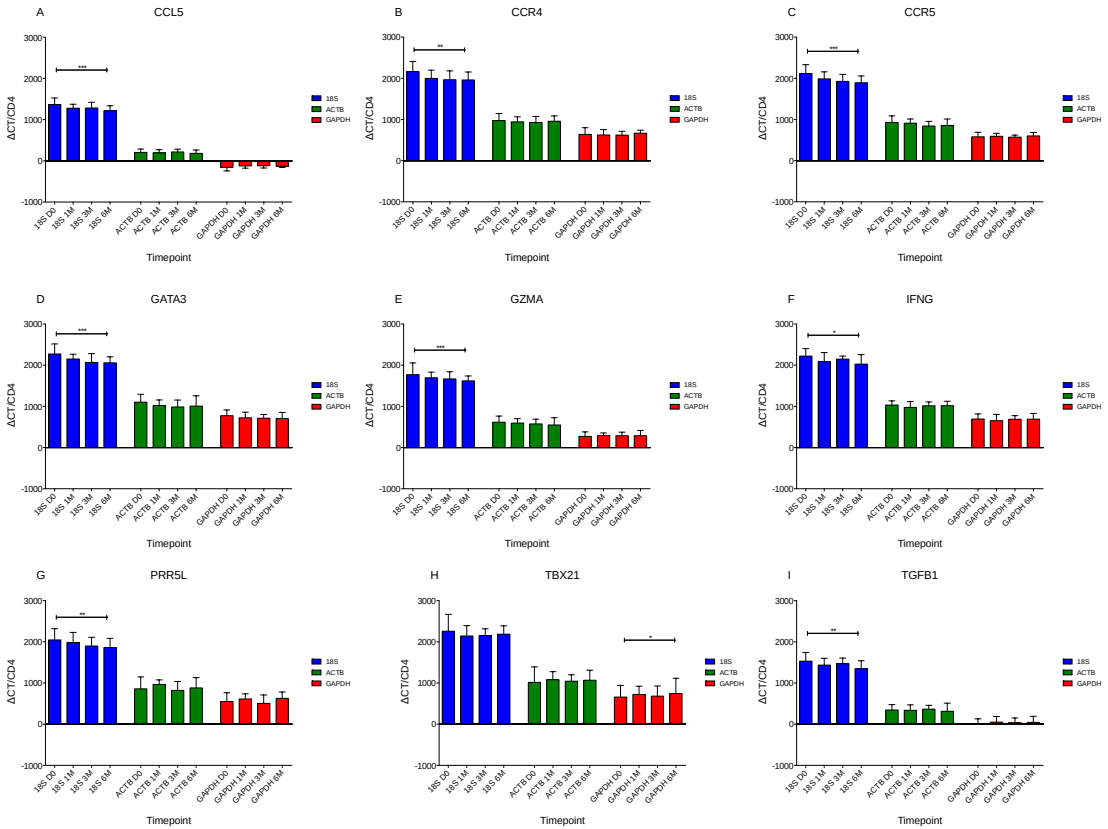

Figure 6. 4: Gene expression of effector memory CD4 T cell-specific genes (A) CCL5, (B) CCR4, (C) CCR5, (D) GATA3, (E) GZMA, (F) IFN-γ, (G) PRR5L, (H) TBX21 and (I) TGFβ1, showing a significant change after 6 months of ART compared to day 0, when normalised to one housekeeping gene using RT-PCR. Graphs are a measure of ΔCt normalised to CD4 counts, a decrease in the ΔCT/CD4 indicates an increase in expression of the particular gene.

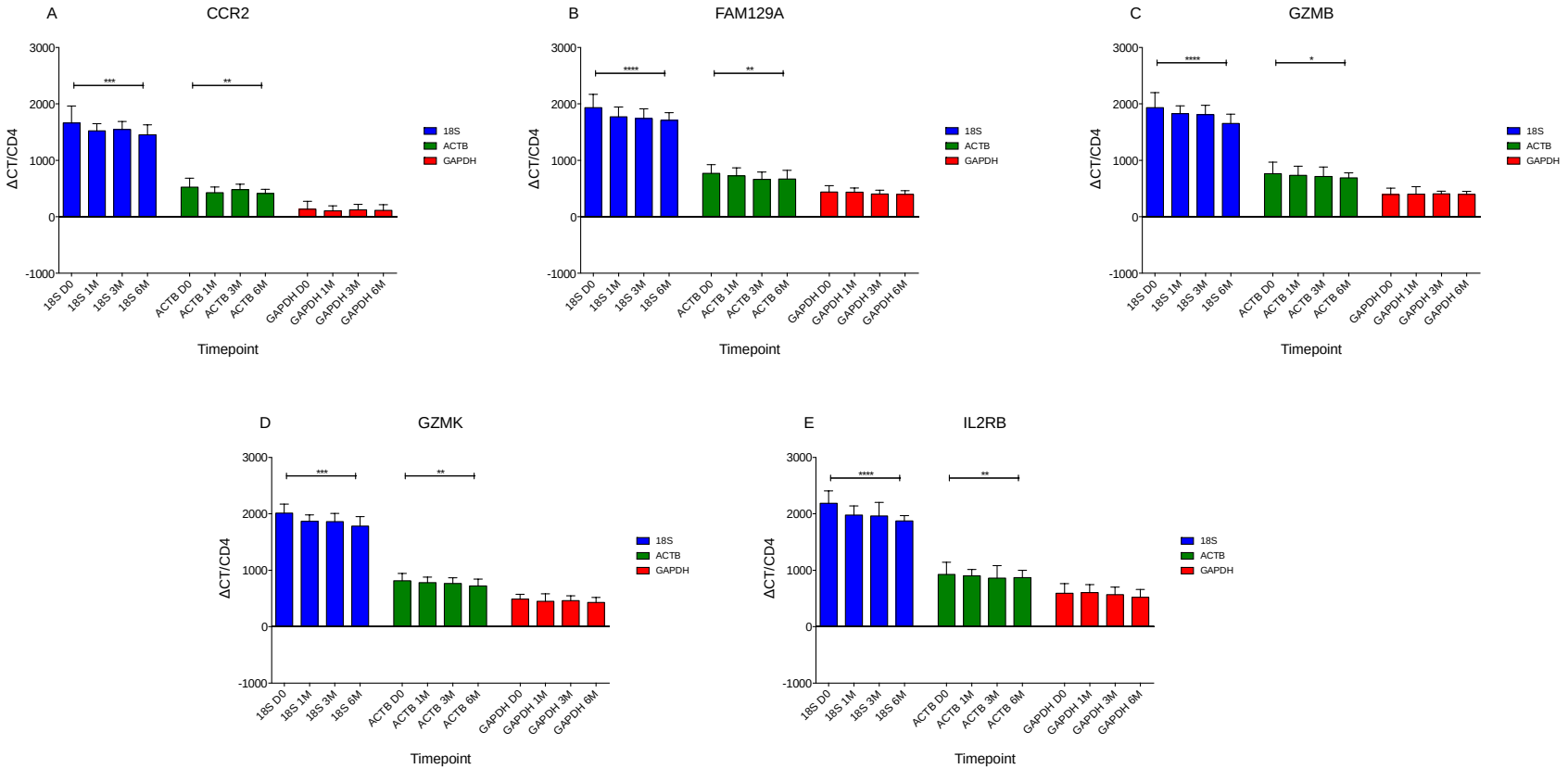

Figure 6. 5: Gene expression of effector memory CD4 T cell-specific genes (A) CCR2, (B) FAM129A, (C) GZMB, (D) GZMK and (E) IL-2RB showing a significant change after 6 months of ART compared to day 0, when normalised to two housekeeping genes using RT-PCR. Graphs are a measure of ΔCt normalised to CD4 counts, a decrease in the ΔCT/CD4 indicates an increase in expression of the particular gene.

Table 6. 5: Median and p-values for comparing the expression level of genes at day 0 and 6 months of ART, normalised to 18S

Gene/Median	Day 0 of ART	1M	3M	6M	D0 of ART vs. 6M p-value
ARHGEF18	1923	1807	1790	1699	<0.0001
AXIN2	2554	2510	2377	2293	<0.0001
CCL5	1367	1277	1282	1215	0.0003
CCR2	1664	1522	1545	1452	0.0001
CCR4	2162	1997	1964	1960	0.0012
CCR5	2115	1984	1926	1894	0.0001
CCR7	2096	1951	1941	1772	<0.0001
CD27	2148	2026	2017	1880	<0.0001
CD38	2210	2130	2197	2021	0.0094
FAM129A	1935	1773	1742	1709	<0.0001
GATA3	2275	2151	2066	2059	0.0001
GNLY	1499	1364	1354	1354	0.0524
GZMA	1771	1697	1669	1623	0.0009
GZMB	1935	1827	1809	1654	<0.0001
GZMK	2013	1867	1862	1782	0.0003
ICOS	2384	2289	2288	2160	0.0292
IFN-γ	2221	2089	2148	2028	0.0189
IGF1R	2124	1949	1933	1887	<0.0001
IL-2RB	2187	1977	1963	1873	<0.0001
ITK	2077	1950	1915	1856	0.0001
LEF1	1811	1849	1876	1657	0.0005
NFKβ-1	2048	1919	1910	1833	0.0011
PRF1	1968	1795	1848	1838	0.2939
PRKCA	2330	2216	2147	2015	0.0017
PRR5L	2045	1983	1898	1860	0.0073
RORC	2690	2646	2666	2532	0.0897
SELL	1365	1334	1286	1264	0.0264
TBX21	2257	2143	2153	2185	0.1696
TCF7L2	2193	2211	2195	2048	0.1664
TGFβ-1	1529	1435	1466	1344	0.0022

Table 6. 6: Median and p-values for comparing the expression of genes at day 0 and 6 months of ART, normalised to β-actin.

Gene/Median	Day 0 of ART	1M	3M	6M	Day 0 of ART vs. 6M p-value
ARHGEF18	775	757	698	708	0.0399
AXIN2	1425	1399	1321	1208	0.0165
CCL5	206	192	218	183	0.127
CCR2	524	427	481	413	0.0028
CCR4	976	943	925	959	0.1905
CCR5	929	909	842	855	0.083
CCR7	929	936	804	754	0.0051
CD27	990	940	911	862	0.0012
CD38	1076	1060	1112	1081	0.8935
FAM129A	771	726	664	670	0.0036
GATA3	1103	1022	985	1007	0.1229
GNLY	304	272	276	263	0.1989
GZMA	617	596	574	549	0.1647
GZMB	763	736	714	691	0.0424
GZMK	813	780	766	724	0.0033
ICOS	1147	1147	1193	1202	0.6801
IFN-γ	1032	976	1014	1019	0.5229
IGF1R	938	897	850	732	0.0005
IL-2RB	926	901	861	869	0.0092
ITK	877	865	815	808	0.0514
LEF1	692	695	743	555	0.0058
NFKβ-1	884	864	834	834	0.0848
PRF1	738	717	712	787	0.7193
PRKCA	1068	1089	1071	1013	0.7469
PRR5L	858	964	820	880	0.7887
RORC	1496	1511	1529	1519	0.7859
SELL	191	213	191	234	0.3725
TBX21	1015	1078	1043	1067	0.6002
TCF7L2	1054	1103	1081	999	0.6906
TGFβ-1	345	338	365	313	0.5013

Table 6. 7: Median and p-values for comparing the expression of genes at day 0 and 6 months of ART, normalised to GAPDH

Gene/Median	Day 0 of ART	1M	3M	6M	Day 0 of ART vs. 6M p-value
ARHGEF18	440	448	410	420	0.7218
AXIN2	1063	1109	1071	1032	0.0754
CCL5	-158	-123	-121	-130	0.1091
CCR2	134	105	123	114	0.6296
CCR4	639	625	622	668	0.8857
CCR5	581	592	572	599	0.8752
CCR7	584	601	528	538	0.0608
CD27	613	623	596	569	0.073
CD38	702	738	771	743	0.0395
FAM129A	440	436	403	399	0.068
GATA3	775	721	713	704	0.3107
GNLY	-183	-136	-114	-119	0.1437
GZMA	271	289	288	288	0.4721
GZMB	397	400	409	398	0.6715
GZMK	487	451	460	430	0.0586
ICOS	850	854	879	844	0.68
IFN-γ	694	653	690	691	0.6806
IGF1R	592	547	538	510	0.0084
IL-2RB	593	604	568	520	0.1571
ITK	545	547	535	508	0.8693
LEF1	369	369	387	316	0.1568
NFKβ-1	528	537	511	567	0.4757
PRF1	364	389	421	468	0.4757
PRKCA	789	777	758	769	0.8135
PRR5L	552	611	505	625	0.0793
RORC	1145	1246	1269	1227	0.523
SELL	-127	-80	-94	-56	0.0001
TBX21	658	722	681	742	0.0176
TCF7L2	706	757	754	756	0.155
TGFβ-1	5.0	51	36	45	0.117

6.4. Discussion

In order to identify a transcriptomic signature that may provide insights to protective immunity to TB, we used RT-PCR to determine the expression of selected genes in HIV infected *Mtb* sensitised persons during ART. The selection of genes was based on results from a previous study, Dintwe PhD 2014, in which the genes were shown to be differentially expressed between central memory and effector memory T cells. Genes selected included cytokines and cytokine receptors, chemokine receptors, inhibitory molecules, co-stimulatory molecules, kinases, transcription factors, molecules that play a role in homing and migration and effector molecules, in which the genes; ICOS, SELL, PRKCA, TCF7L, LEF1, NFκβ, CD38, ITK, IGF1R, ARHGEF18, AXIN2, CCR7 and CD27 were shown to have a higher expression in central memory T cells while the genes; GNLY, RORC, TGF-β, PRF1, CCL5, IFN-γ, CCR2, GATA, GZMA, GZMB, GZMK, PRR5L, TXB21, IL-2Rβ, CCR4, CCR5 and FAM129A had a higher expression in effector memory T cells.

Our results indicated a significant increase in expression of the central memory-specific genes: ARHGEF18, AXIN2, CCR7, CD27, CD38, IGF1R, LEF1 and SELL when normalised to two housekeeping genes. In the case of the gene IGF1R, a significant increase in gene expression was noted irrespective of the housekeeping gene used for normalisation.

While the expression and role of these genes in LTBI infection has yet to be determined, a number of the aforementioned genes play a role in cell trafficking and the ability of cells to home, or the modulation of intercellular

proteins leading to the transcription of genes. The product of ARHGEF18, Rho guanine nucleotide exchange factor 18, is responsible for proteins involved in the regulation of cellular processes such as cytoskeletal rearrangements, gene transcription, cell growth and motility [3]. AXIN2 (axis inhibition protein 2) has been shown to play an important role in the regulation of the stability of β-catenin in the Wnt signalling pathway [4]. The activation of the Wnt signalling pathway has also been shown to elevate AXIN2 expression. CCR7 (chemokine receptor type 7) is the receptor for CCL19 and CCL21 chemokines, and plays a role in the migration of memory T cells to secondary lymphoid organs [5]. Additionally CCR7 knockout mice were found to have activated, naïve Ag85B-specific CD4 T cells that were able to proliferate in the lung. These mice also displayed compromised resistance to *Mtb* infection compared to wildtype mice [6]. CD27 is present on the surface of CD4 T cells and is a receptor involved in co-stimulation. CD27 downregulates as naïve T cells differentiate into central memory T cells and subsequently effector memory T cells, thus central memory T cells express CD27 while effector memory T cells lack the CD27 receptor [7]. Additionally, Petruccioli *et al*. observed a significantly higher proportion of $CD45RA^{-}$ $CD27^{+}$ T cells in LTBI and persons cured of TB compared to persons with active disease [8]. They therefore concluded that increased $CD27^{+}$ is a marker of TB containment as they found a higher frequency of $CD4^{+}$ IFN-γ^{+} $CD27^{+}$ T-cells in LTBI while a higher frequency of $CD4^{+}$ IFN-γ^{+} $CD27^{-}$ T-cells was associated with active TB [8].

CD38 is found on the surface of many immune cells and plays a role in the regulation of intracellular calcium, that in turn controls numerous processes such as cell activation and proliferation, hormone secretion and immune responses to name a few [9]. IGF1R, insulin-like growth factor, has tyrosine kinase activity and plays a critical role in transformation events. IGF1R knockout mice displayed signs of growth deficiency with some mice dying shortly after birth [10]. LEF1 (Lymphoid enhancer-binding factor 1) is a transcription factor that mediates the Wnt signalling pathway, leading to the expression of Wnt target genes [11, 12]. SELL is the gene responsible for the transcription of L-selectin, also known as, CD62L. The protein product of SELL is a homing receptor required by lymphocytes to enter secondary lymphoid tissues. CD62L is expressed on the surfaces of T cells with central memory T cells expressing L-selectin to localize in secondary lymphoid organs, and effector memory T cells not expressing CD62L [1].

The functions of 2 out of the 8 central memory-specific genes, AXIN2 and LEF1, that were found to change significantly during 6 months of ART in our cohort, indicate that they play an important role in the canonical Wnt signalling pathway. The Wnt signalling pathway has been associated with motility, cellular proliferation, apoptosis, differentiation and polarization of cells, in invertebrates and mammals [13], and involves the LEF1/TCF family of transcription factors and β-catenin. In the absence of Wnt ligands the phosphorylation of β-catenin leads to ubiquitylation and proteosomal breakdown thus preventing nuclear translocation of β-catenin and in turn preventing enhanced transcription of Wnt-dependent genes [14]. The

presence of Wnt ligands leads to the inactivation of the destruction of β-catenin through the action of AXIN, among other proteins, thus β-catenin levels accumulate leading to nuclear translocation. Once in the nucleus β-catenin drives the expression of TCF/LEF-dependent genes, that play a role in survival, cell differentiation and proliferation [15] (Figure 6.6).

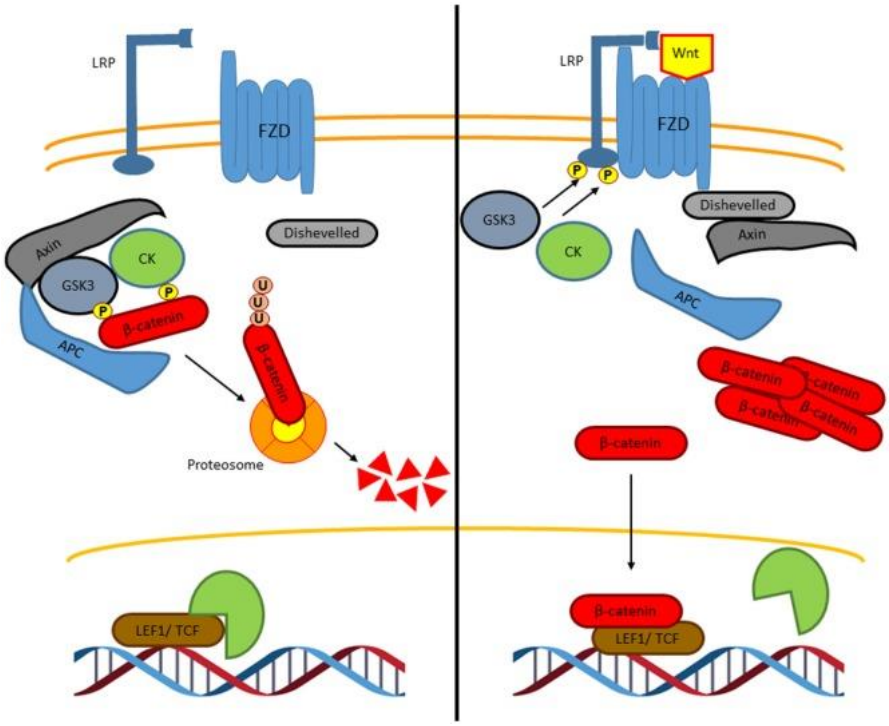

Figure 6. 6: The canonical Wnt signalling pathway. (Image sourced from Villaseñor *et al*. [15]). Left: the absence of Wnt ligands the phosphorylation of β-catenin results in reduced Wnt-dependent gene expression. Right: Wnt ligands inactivate the β-catenin destruction complex leading to nuclear translocation of β-catenin and the expression of LEF-dependent genes.

The roles of Wnt family proteins in *Mtb* infection remains controversial. It has been demonstrated *in vitro* that Wnt3 reduced the secretion of TNF-α

cytokine, but did not influence expression of the TNF-α gene in *Mtb* infected macrophages. This was mediated via the canonical pathway as Wnt3 was shown to induce β-catenin stabilisation and AXIN2 expression in this system [16]. Conversely Neumann *et al*. demonstrated using a murine model that Wnt3 promoted the expression of ARGINASE 1 in *Mtb* infected macrophages [17]. ARGINASE 1 has been shown to promote anti-inflammatory effects. Furthermore, Wnt3 has also been shown to inhibit IL-6 production in murine macrophages, during *Mtb* infection, therefore further supporting the anti-inflammatory phenotype [17]. Baba *et al.* illustrated that the Wnt signalling pathway has a role in maintaining an undifferentiated cell state by showing that expression of β-catenin in lymphoid or myeloid cells generated uncommitted cells with the potential to differentiate into multilineages [18]. This further supports the expression of these genes that showed a significantly increased expression in central memory T cells, which are early differentiated cells. Overall, we observed an increase in T cell memory specific gene expression in HIV infected persons receiving ART and our results illustrate that 8 out of the 13 central memory CD4 T cell-specific genes showed a significantly increased expression when normalised to at least 2 reference genes. After applying the most stringent analysis (i.e. normalisation to at least two reference genes), the genes that exhibited significant increase were specific to central memory T cells, thereby supporting our hypothesis that the expression of central memory T cell specific genes increase over 6 months of ART. Our findings support the expansion of early differentiated T cells during ART induced protective

immune reconstitution and may offer insight into the mechanisms of ART-mediated prevention of TB in HIV-1 infected persons.

There were several limitations to our study: firstly the genes included for analysis were based on studies in HIV uninfected persons while our results depict expression in HIV infected persons during ART, therefore it is important to acknowledge the effect of HIV infection during analysis. Secondly while blood samples were collected from HIV uninfected persons for soluble and cell marker analysis, we did not collect blood for transcriptomic analysis thus we lacked a control group for analysis of this arm of the study. We therefore were unable to determine the expression of our set of genes in HIV uninfected persons and use this cohort for comparison purposes. Additionally we initially planned to conduct either microarray or RNA-seq experiments, as opposed to RT-PCR, in order to identify a transcriptomic signature indicative of protective immunity in our cohort. However due to insufficient RNA quality, we opted to measure the expression of selected genes instead. Our RNA was extracted from whole blood as opposed to selected cell populations. The RT-PCR results may thus have been more unambiguous and specific to each cell subset if we were able to conduct experiments using RNA from separated T cell subsets.

Despite this, we observed an overall increase in gene expression in HIV infected persons receiving ART and our results illustrate that out of the 13 central memory CD4 T cell-specific genes, 8 showed a significantly increased expression when normalised to at least 2 housekeeping genes.

After applying the most stringent analysis (i.e. normalisation to at least two housekeeping genes), we observed that the genes that exhibited significant increase were central memory specific genes (Figure 6.7), thereby supporting our hypothesis that the expression of central memory T cell specific genes increases over 6 months of ART.

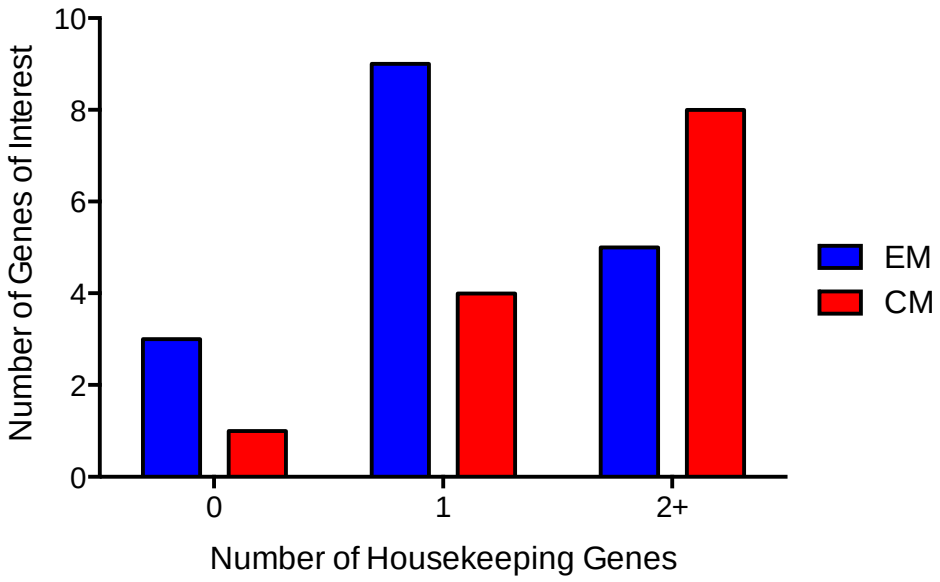

Figure 6. 7: The number of central memory- (CM) or effector memory (EM) CD4 T cell -specific genes that significantly increased in HIV infected persons during 6 months of ART after normalisation to either 1, 2 or more housekeeping genes.

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Chapter 7: Overall Discussion and Conclusion

The objectives of this thesis are summarised below:

i. We firstly characterised *Mtb*-specific CD4 T cells in HIV infected persons, sensitised by *Mtb*, during the first 6 months of antiretroviral treatment induced immune reconstitution. This was achieved using a 15 colour flow cytometry panel assessing T cell memory phenotype, senescence profile, homing marker expression and cytokine secretion to assess their functional capacity. We hypothesised that central memory CD4 T cells with high proliferative capacity and the ability to home to the site of infection upon re-exposure to antigen will expand significantly, based on previous findings, and therefore may play a protective role against TB in HIV infected persons.

ii. Secondly, using ELISA and multiplex assays, we aimed to evaluate soluble markers in plasma from HIV infected, *Mtb* sensitised individuals starting ART and followed up for 6 months. Analytes assessed included pro-inflammatory, anti-inflammatory, chemo-attractant, growth factors and T cell growth factors. We hypothesised that soluble factors associated with central memory T cell function will be detectable in plasma and that their concentrations will change over time on ART in HIV infected *Mtb* sensitised individuals.

iii. Lastly, we aimed to compare gene transcriptional signatures characteristic of central memory and effector memory T cells using quantitative RT-PCR in HIV infected persons starting ART and sampled at day 0, 1 month, 3 months and 6 months of ART.

Using flow cytometry we characterised *Mtb*-specific CD4 T cells in HIV infected persons and observed an expansion in the number and proportion of central memory CD4 T cells, and a corresponding decrease in the proportion of effector memory CD4 T cells. We were thus able to confirm the findings of a previous study in our group, in a larger cohort of individuals [1]. Riou *et al*. also identified pathogen-specific early differentiated CD4 T cells to have a higher capacity to replenish in HIV infected persons receiving ART, compared to pathogen-specific late differentiated CD4 T cells [2]. This further supports the concept of different T cell subpopulations possessing diverse potentials to be replenished during ART.

Additionally we observed a significant decrease in the proportion of activated CD4 T cells, and senescent CD4 T cells in our cohort. Similarly Riou *et al*. demonstrated a significant decrease in CD4 T cells co-expressing CD38 and HLA-DR, and cells expressing HLA-DR only, in HIV infected LTBI individuals [2].

We also assessed the numbers and proportions of CD4 T cells expressing single cytokines and observed that the proportions of cells producing either IL-2, IL-17A or TNF-α decreased significantly during ART. The comparator group of HIV uninfected persons had significantly lower proportions of IL-2 or TNF-α producing cells, and significantly higher proportions of IL-17A producing cells compared to HIV infected persons. Strickland *et al*. described that HIV uninfected persons had higher proportions of TNF-α producing cells

compared to HIV infected persons while the proportions of IL-2 producing cells could not be distinguished between the two cohorts [3].

Lastly, we demonstrated that the proportion of $CD4^+$ T cells with the ability to secrete three cytokines (IFN-γ^+TNF-α^+IL-2^+) simultaneously significantly expanded in HIV infected persons during 6 months of ART. HIV uninfected persons had a significantly lower proportion of IFN-γ^+TNF-α^+IL-2^+ polyfunctional CD4 T cells compared to HIV infected persons after 6 months of ART. The significant increase in the proportion of these cells in our cohort may be attributed to the overall increased proportion of CD4 T cells as a result of ART induced immune reconstitution, and thus these cells may have expanded as a result of clonal expansion. Our finding is in line with that of Bunjun *et al.* in which they found LTBI HIV infected persons to have a higher proportion of IFN-γ^+TNF-α^+IL-2^+ polyfunctional T cells compared to HIV uninfected persons [4]. Bell and Westermann have proposed that in the absence of antigen contact antigen-primed T cells, such as polyfunctional T cells, return to a resting state [5]. Thus LTBI persons would be expected to have lower proportions of CD4 T cells secreting IFN-γ, TNF-α and IL-2 compared to TB infected persons. Caccamo *et al*. and Sutherland *et al*. illustrated that persons with active TB disease had significantly higher levels of *Mtb*-specific $CD4^+$ T cells secreting IFN-γ, TNF-α and IL-2 cytokines simultaneously compared to persons with LTBI [6, 7]. Thus the inclusion of a control group of TB infected individuals, in our study, would have been beneficial in order to compare the polyfunctional capacity of CD4 T cells to patients with active disease in our setting.

More importantly, we observed a significant increase in the proportion of CD4 T cells with the ability to produce 2 cytokines in HIV infected persons after therapy. After 6 months of ART, the quality of the immune response was dominated by bifunctional CD4 T cells ($CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$, $CD4^+IL\text{-}2^+TNF\text{-}\alpha^+$ and $CD4^+IL\text{-}17A^+TNF\text{-}\alpha^+$ cells). This finding is central to the fact that CD4 T cells are imperative for a protective response to TB. Furthermore, these findings were similar to that of Sutherland *et al*. [8] as they demonstrated a dominant polyfunctional CD4 T cell profile after ART whereby the cytokines TNF-α and IL-2 were produced by polyfunctional CD4 T cells. Together these findings indicate that the increased polyfunctional profile associates with a reduced TB prevalence, and that CD4 polyfunctional T cells are therefore a key player in mediating protection to TB infection.

Using multiplex assays we demonstrated a significant decline in concentrations of the pro-inflammatory cytokines, IFN-γ, IL-1α, IL-1β, IL-6, IL-17A and TNF-α, in HIV infected persons after 6 months of ART. Comparison with the HIV uninfected persons revealed no significant difference between the concentrations of cytokines between the two groups, indicating that the concentrations of analytes observed at 6 months of ART in the HIV infected persons, were similar to those observed in the control cohort, thus indicating effective ART. Pro-inflammatory cytokines play important roles in TB-HIV pathogenesis. IFN-γ contributes to the recruitment of cells to the sites of inflammation, and to phagocyte and DC activation leading to bacterial control. IL-1α and IL-1β have been shown to play roles in

autophagy and bacterial control [9] while TNF-α is responsible for granuloma formation, maintenance and bacterial control. Thus the decrease in concentrations of these cytokines during ART, indicate a decrease in immune activation, in parallel with ART induced reduction in HIV viral load.

Lastly, using RT-PCR, we demonstrated a significant increase in the expression of central memory-specific genes, compared to effector memory-specific genes, in HIV infected persons during 6 months of ART. More importantly, this increased expression was observed regardless of the housekeeping gene used for normalisation during data analysis thus illustrating a robust increase in expression of 8 central memory-specific genes.

The products of these genes have been shown to play a role in processes such as cytoskeletal rearrangements, gene transcription, homing and migration, activation and proliferation. The AXIN2 and LEF1 gene products play an important role in the canonical Wnt signalling pathway, in which Wnt signalling has a role in conferring a more undifferentiated state [10]. This is in keeping with our data as central memory T cells are early differentiated cells, that have the ability to further differentiate into effector memory T cells upon receipt of appropriate signals and specific antigen presentation for which the cell has been primed. Okamura *et al*. demonstrated that *LEF1*$^{-/-}$ and *TCF1*$^{-/-}$ double knockout mice have a block in T cell differentiation indicating the importance of this gene in the interaction with the Wnt mediator, β-catenin [11]. This further demonstrates the crucial role that the Wnt signalling

pathway plays in delivering proliferative signals to immature T cells [12]. However the involvement of the Wnt signalling pathway in the T cell differentiation pathway can also be regulated at many different levels; and the assessment of protein markers, specifically the proteins translated from the aforementioned genes, is needed in order to fully determine the role of these genes in the context of ART induced immune reconstitution in HIV infected persons sensitised by *Mtb*.

Therefore we observed indications of a decrease in immune activation in our cohort of HIV infected persons, using two technologies. Firstly the significant decrease in pro-inflammatory cytokines was observed during the assessment of soluble markers via Luminex, and secondly we also observed a significant decrease in the proportions of cells producing the same single cytokines in the same cohort of persons using flow cytometry. Furthermore activation, measured by the expression of the marker HLA-DR, was determined using flow cytometry and our results indicate a significant decrease in the proportion of activated CD4 T cells in HIV infected persons during ART. Adekambi *et al*. demonstrated that the activation markers CD38 and HLA-DR expressed on *Mtb*-specific CD4 T cells, were able to distinguish between LTBI and active TB disease [13] with LTBI persons having a significantly lower proportion of HLA-DR$^+$IFN-γ$^+$ and CD38$^+$ IFN-γ$^+$ cells than persons with active TB disease. Additionally Adekambi and colleagues illustrated that the proportions of HLA-DR$^+$IFN-γ$^+$ and CD38$^+$ IFN-γ$^+$ *Mtb*-specific T cells in LTBI persons were comparable to persons who had resolved TB successfully after TB treatment. This further supports our finding

of a decrease in immune activation during ART-mediated immune reconstitution in HIV infected persons. Lastly our RT-PCR results describe a significantly increased expression of central memory-specific genes, when normalised to at least 2 housekeeping genes, in our patient cohort.

A number of limitations of the study include: i) the reliance on antigenic stimulation in order to measure a *Mtb*-specific response. Persons that were unable to mount a detectable IFN-γ response were not included in the study; ii) additionally samples with missing timepoints resulted in a further decreased number of samples available for analysis; iii) this study would have also benefitted from the inclusion of an additional control group consisting of TB-HIV co-infected persons, sampled once, as a comparison to determine the loss of immune control. This would be of particular interest as a previous study in our group, Wilkinson *et al*. observed a significantly higher proportion of $CD4^+IFN\text{-}\gamma^+HLA\text{-}DR^+$ CD4 T cells in TB-HIV co-infected persons compared to HIV infected persons [14] with the proportion of $CD4^+IFN\text{-}\gamma^+HLA\text{-}DR^+$ CD4 T cells in HIV infected persons also being significantly higher than that of HIV uninfected persons.

Two ways in which the findings of this PhD ought to be validated in furture studies include:

1) Confirming that the finding of $CD4^+$ T cell activation ($HLA\text{-}DR^+$ and/or secreted as well as intracellular IL-2, IFN-γ and TNF-α) as being a marker of bacterial load and that may potentially identify persons with latent TB infection who would benefit from isoniazid preventive

treatment. In doing so, IPT would provide protection to TB by preventing the progression of latent TB to active TB disease in this group of persons at risk.

2) More importantly the findings ought to be validated through the use of mouse models. Knocking out the gene(s), responsible for the production of potential markers of protection to *Mtb* (as identified in this PhD), in mice will allow us to determine whether it is actually these markers that are responsible for the protection provided. Furthermore, over-expressing these genes will also substantiate the abov. In order to achieve this, a conditional knockout mouse model ought to be used, as tissue-specific and inducible knockouts will be possible and will allow us to have control over the location and timing of the expression of these genes. Since these are vital genes, we need to control the timing of the deletion of the gene(s) in order to prevent a lethal phenotype.

In conclusion we have shown that early differentiated CD4 T cells exhibiting a less activated phenotype, with the ability to secrete at least two cytokines, have the potential to serve as a correlate of decreased susceptibility to TB disease in HIV infected persons. This might aid in vaccine efficacy testing, vaccine design and host directed therapies. Moreover the correlate of protection could be used to identify potential vaccine candidates, optimise vaccine dose, the number of doses required and to provide preliminary evidence of immunogenicity amongst other functions. The use of blood-based markers also has the ability to rapidly identify persons that have a

delayed or no response to vaccines and/or TB therapy, in the case where the markers will be used to measure treatment response. Finally this subset of CD4 T cells as a correlate of protection has the potential to be a marker indicative of bacterial load, thereby providing the ability to identify persons that will benefit from Isoniazid Preventative Therapy (IPT) in order to prevent disease progression from latent to active TB disease (Figure 7.1). Thus the validation of early differentiated CD4 T cells exhibiting a less activated phenotype, with the ability to secrete at least two cytokines as a potential correlate of protection is required in future clinical studies.

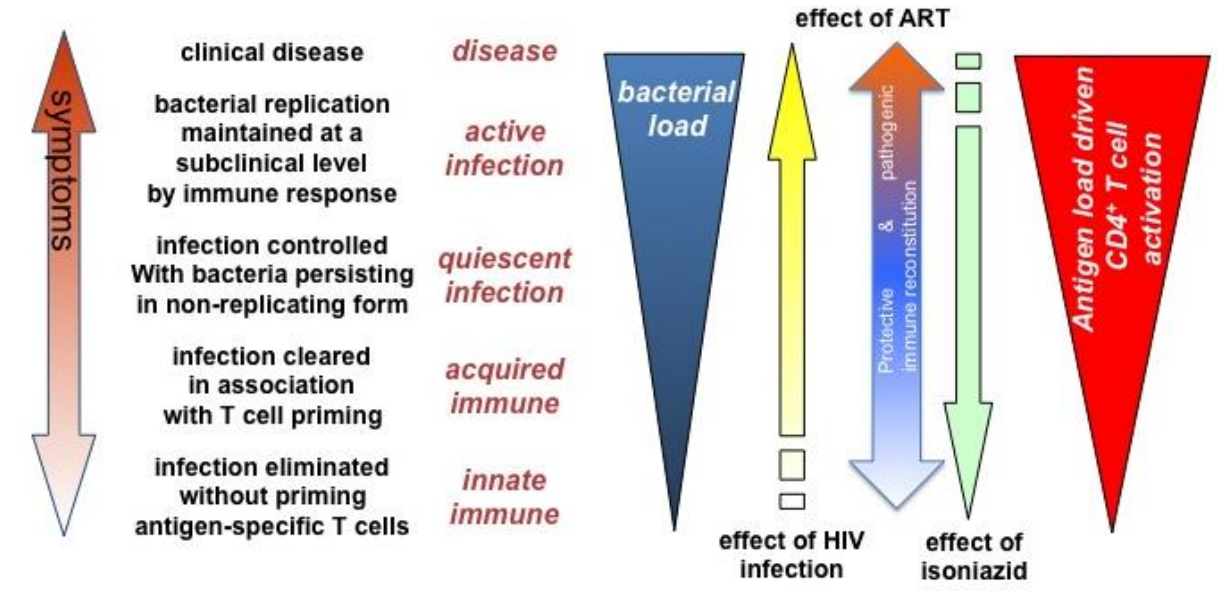

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Figure 7. 1: *Mtb* Infection: A Spectrum of Immune Responses. The spectrum describes persons with an effective immune response, thus enabling the clearing of infection, persons with subclinical active disease in which persons harbour replicating bacteria but do not exhibit clinical symptoms of disease and, persons with active TB disease presenting with clinical symptoms. We demonstrated that *Mtb* senstised, HIV infected persons display a decreased activation due to ART Induced immune reconstitution. A higher bacterial load increases CD4 T cell activation. Therefore the use of HLA-DR as a marker of activation may be useful in identifying a key population of persons that will benefit from IPT and in doing so, prevent disease progression to active TB disease (image from A/Prof. Katalin A. Wilkinson, based on Young *et al*., Barry *et al*., Lawn *et al*. and Esmail *et al*. [14-18].)

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