The role of IL-4 and Th2-like cytokines in pulmonary tuberculosis

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DEDICATION

For my late father Vishnu, my mother Sintra, my sister Renata and my brother Andre who have always believed in me and supported me in all that I have done.

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2. **Title: The modulating effects of IL-4 on mycobacterial containment within monocyte derived macrophages in an *in vitro* model of human tuberculosis** (under preparation)

ABBREVIATIONS

Ab	Antibody
AFB	Acid fast bacilli
APC	Antigen presenting cell
-APC	Allophycocyanin
ATP	Adenosine triphosphate
BAL	Broncho-alveolar lavage
BCG	Bacillus Calmette-Guerin
Bcl-	Beclin
bp	Base pair
BSA	Bovine serum albumin
cat	Chloroamphenicol acetyl transferase
CCL	Chemokine C-C Motif Ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CFP-10	Culture filtrate protein 10
CFU	Colony forming units
COX-2	Cyclooxygenase 2
CPM	Counts per minute
Ct	Cycle threshold
C-terminus	Carboxyl terminus
CTL	Cytotoxic T-lymphocyte
CTLA	cytotoxic T lymphocyte antigen
CXCR	Chemokine CXC motif receptor
CXR	Chest X-ray
Cys	Cysteine
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonuleic acid
dNTP	Deoxynucleotide triphosphates
DR-TB	Drug resistant tuberculosis
DST	Drug susceptibility test
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ER	Endoplasmic reticulum
ESAT-6	Early secretory antigenic target 6

ETS	E26 transformation-specific
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FBS	Foetal bovine serum
FITC	Fluorescein
FoxP3	Forkhead box P3
FQ	Fluorescent quencher
FSC	Forward scatter
GATA3	Trans-acting T-cell-specific transcription factor-3
GFP	Green fluorescent protein
GluBP	Glutathione binding protein
GST	Glutathione-S-Transferase
HAART	Highly active antiretroviral therapy
His	Histidine
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
hsp65	Heat shock protein 65kDa
HuPO	Human ribisomal protein
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL-	Interleukin
IL-4δ2	Interleukin-4delta2
iNOS	Inducible nitric oxide synthase
IQR	Inter-quartile range
IRF4	Interferon regulatory factor 4
IRS	Insulin receptor substrate
JAK	Janus kinase
kb	Kilobase
kDa	Kilodalton
LAM	Lipoarabinomannan
LB	Luria broth
LC3	Microtubule-associated protein light chain 3
LPS	Lipopolysaccharide
LTBI	Latent tuberculosis infection
M.tb	*Mycobacterium tuberculosis*
MCP-1	Monocyte Chemoattractant protein-1
MDG	Millenium development goals
MDM	Monocyte derived macrophages
MDR-TB	Multi-drug resistant tuberculosis

MGIT	Mycobacterial growth indicator tube
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MR	Mannose receptor
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MVA85A	Modified-Vaccinia-Ankara 85A
MWCO	Molecular weight cut-off column
NAAT	Nucleic acid amplification technology
NK	Natural killer
NO	Nitric oxide
NTC	No template control
N-terminus	Amine terminus
NTM	Non tuberculous mycobacteria
NTP	National tuberculosis program
OADC	Oleate-Albumin-Dextrose-Catalase
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Programmed death 1
PE	Phycoerythrin
PE Cy	Phycoerythrin cytochrome
PerCP	Peridin chlorophyll protein
pfu	Plaque forming units
PGE2	Prostaglandin E2
PHA	Phytohaemmaglutinin
PI3K	Phosphatidylinositol-3-kinase
PMA	Phorbol myristate acetate
PPD	Purified protein derivative
PRR	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RBPJ	Recombination-signal-binding protein for immunoglobulin-κ J
RD1	Region of difference 1
RE	Restriction enzyme
RIN	RNA intergrity number
ROI/RNI	Reactive oxygen/nitrogen intermediates
RORγt	RAR-related orphan receptor gamma transcription
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sf	*Spodoptera frugiperda*
sIL-4R	Soluble interleukin-4 receptor
siRNA	Small interfering RNA
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TAE	Tris acetate
TB	Tuberculosis
T-bet	T-box expressed in T cells
TBST	Tris buffered saline - Tween
Tc cell	T-cytotoxic cell
TCR	T-cell receptor
TDR-TB	Totally drug resistant tuberculosis
TEV	Tobacco etch virus
TGFβ	T-cell growth factor beta
Th	Helper T-cell
TLR	Toll-like receptors
TNFR1	TNF receptor 1
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T-cell
TST	Tuberculin skin test
WHO	World Health Organization
XDR-TB	Extensively-drug resistant tuberculosis

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ABSTRACT

Background: Tuberculosis (TB) vaccine candidates have been mostly ineffective and it remains unclear as to what constitutes protective host immunity. Despite high levels of IFN-γ at the site of disease, the Th1 cytokine associated with protection, many individuals still have progressive active TB. Whether a Th2-like immune response (IL-4; IL-4δ2; IL-9) can subvert protective host immunity requires clarification.

Methods: Blood and/or broncho-alveolar lavage fluid (BAL) were obtained from individuals with confirmed pulmonary TB [TB; n=51(BAL=11; Blood=40)] and presumed latent TB infection [LTBI; n=38 (BAL=14; Blood=24)]. Expression of mRNA encoding IFN-γ (Th1), IL-4 and IL-4δ2 (Th2) from whole blood and alveolar lavage cells was determined by quantitative real-time PCR. Human recombinant IL-4 (rIL-4) and IL-4δ2 (rIL-4δ2) proteins were expressed in a baculovirus system and functionally validated using ^{3}H-thymidine proliferation and B-cell flow cytometric assays. The effect of IL-4 on mycobacterial containment (colony-forming units (CFU)/ml) was evaluated by co-culturing effector T-cells (pre-primed with rIL-4 and/or PPD) with H37Rv-infected monocyte-derived-macrophages. Flow cytometry was used to evaluate cell surface and intracellular biomarker expression in co-cultured cells. The impact of neutralizing IL-4 (anti-IL4 antibody) was also evaluated in this model. T-helper cytokine levels were determined in RD1-stimulated cell culture supernatants using a Luminex multiplex assay. TB-antigen driven IL-9 producing T-cell populations were investigated by flow cytometry.

Results: IFN-γ and IL-4 responses in TB were compartment-specific (high IFN-γ levels in BAL and high IL-4 levels in blood). In whole blood, TB patients expressed higher median IL-4 mRNA levels (p=0.02) and a lower IFN-γ/IL-4 ratio (p=0.01) compared to LTBI controls. Functionally active rIL-4 increased T-cell proliferation and B-cell CD23 expression in a dose-dependent manner. rIL-4δ2 inhibited IL-4-induced T-cell proliferation but protein yield was insufficient for further downstream experiments. In the mycobacterial containment model, addition of rIL-4 was associated with a reduction

in mycobacterial containment (CFU/ml), increased levels of regulatory T-cells (CD4+CD25+FoxP3+; p=0.0006), decreased CD4+ Th1 cytokines (CD4+IFN-γ, p=0.0005; CD4+TNFα, p=0.01), and increased macrophage DC-SIGN expression (p=0.02) in a dose-dependent manner. Anti-IL-4 antibodies abrogated the effect of IL-4 on mycobacterial containment and the expression of CD4+IFN-γ+ (p=0.03) and regulatory T-cells (p=0.03). Soluble IL-9 was increased in RD-1-stimulted BAL supernatants from patients with TB versus LTBI (p=0.02). CD4+ and CD8+ IL-9-producing lymphocytes exhibited a Th9 and Tc9 phenotype (IL9+$IL13_{neg}IL17_{neg}$), respectively and Th9 and Tc9 levels higher in TB versus LTBI in both BAL (p=0.02) and blood (p=0.03).

Conclusions: Patients with TB from Cape Town, South Africa, have a compartmentalized and Th2-skewed (IL-4 and IL-9) host immune response. It is shown for the first time that IL-4 is associated with subversion of mycobacterial containment in human monocyte-derived macrophages, and this seems to occur, at least in part, through a regulatory T-cell-related mechanism. These data have implications for selection of effective vaccine candidates and the design of appropriate TB-specific immunotherapeutic interventions.

1. CHAPTER 1: Literature Review

1.1 Study rationale and objectives

Tuberculosis (TB) is one of most widespread infectious diseases globally and, despite slowly declining incidence, still causes 9 million new cases and 1.5 million deaths every year [1]. This is none more apparent than on the African continent, contributing to one quarter of global cases, where inefficient control programmes, high levels of poverty, emerging drug resistance and HIV are fuelling the epidemic. Furthermore, current vaccine candidates, such as BCG and MVA85A, do not offer sufficient protection to prevent disease progression [2-4]. Thus novel approaches to therapy and the design of effective vaccine candidates are urgently needed but will require a comprehensive understanding of the host‟s immune response to TB. However, despite substantial research on the subject, a definitive answer as to how the *Mycobacterium tuberculosis* (*M.tb*) pathogen is able to evade the immune system and cause active disease remains elusive.

The spectrum of disease caused by *M.tb* is more extensive than previously thought. Indeed, the outcome of infection is heavily dependent on the host-pathogen interaction or, more precisely, the nature of the immune response evoked by *M.tb*, but this interaction is not well understood [5]. Most exposed individuals do not develop TB and the bacilli may either be completely eradicated from the host or remain in a latent non-replicative state under the control of the immune system [5, 6]. On the opposite end of the spectrum, development of active TB may manifest as a subclinical infection or overt cavitatory disease with extensive tissue damage [7, 8]. However, many of these individuals have no obvious defects in immunity and actually have very strong measurable Th1 responses, characterized by high levels of the type I cytokine IFN-γ, at the site of disease [9-15]. Indeed, the current TB vaccines have been designed to evoke such a response but still fail to provide adequate protection. Therefore a strong Th1 response, although important, is clearly not enough for a successful host defense and the question remains: Why do some individuals exposed to *M.tb* develop active disease whereas others, despite repeated exposure, remain healthy? The work presented in this thesis attempts to provide some insight into this crucial question.

The host immune response to *M.tb* involves a complex cascade of innate and adaptive mechanisms that work in conjunction to combat the infection. Various innate responses, elicited mainly by macrophages, neutrophils and specialized T-cells, are the „first responders" that attempt to eliminate the pathogen, but are often circumvented by *M.tb* specific mechanisms [8]. As such, adaptive immunity is initiated to reassert immune control and involves an interaction of various helper T-cell subsets and cytokines responses. However, dysregulation of T-cell mediated immunity may also lead to immunopathological consequences. Indeed, there are a number of hypotheses that may explain how *M.tb* is able to evade and sabotage a protective host response. One such theory is that in TB susceptible patients, mycobacterial antigens induce a small but significant Th2 response within a dominant Th1 environment, which disrupts protective immunity allowing *M.tb* to evade anti-mycobacterial cellular mechanisms [16]. Indeed, there is considerable evidence to show that IL-4 (the signature Th2 cytokine) is increased in TB patients and these levels correlate with immunopathology [9, 17-21] and disease susceptibility [22, 23]. However, IL-4 is a very low expressing cytokine and its measurement can be technically challenging [24-27]. Also, the existence of IL-4δ2, an alternatively spliced variant and natural antagonist of IL-4, must be considered when evaluating IL-4 responses but this cytokine has not been well described in TB. Evidence suggests increased IL-4δ2 mRNA expression is associated with a protective disease phenotype [28, 29] and response to anti-TB treatment [9, 30]. Furthermore, IL-4δ2 may exist as a functional protein and acts like a Th1 cytokine in *in vitro* culture [31, 32]. Consequently, other studies failed to detect differences in the IL-4 levels of TB patients and controls, which may be due to the use of insensitive assays or failure to distinguish between IL-4 and IL-4δ2 [12, 33-37]. Even now, commercial immunoassays are not available to differentiate between the two isoforms at a protein level due to a lack of discriminatory antibodies and the only currently available method involves measuring mRNA by quantitative PCR assay.

Another caveat of previous studies and more recent ones as well, is that most researchers tend to focus on the peripheral compartment to evaluate cytokine responses rather than the site of disease as blood samples are more easily acquired. This is surprising because the

immune profile and cellular environment of the lungs in pulmonary TB can differ significantly from the circulating blood due to cell migration and enhanced cellular activity at the site of host-pathogen interactions [38]. Indeed, only one previous study, performed in a low TB endemic setting, measured IL-4 and IL-4δ2 in both compartments of TB patients and controls [9]. There are none that have measured the compartment-specific levels of these cytokines in a high burden setting.

Despite evidence of increased IL-4 in tuberculosis, it is still unclear whether it is causing the immunopathology or is merely a consequence of excessive inflammation. Some studies have reported an inhibitory effect of IL-4 on known anti-mycobacterial mechanisms in macrophages and T-cells [39-44]. However, many of these studies have been reported in murine models which have limited bearing in human TB. For example, most murine models fail to develop cavitatory disease (a hallmark of transmissible disease) and lack the structural complexity of granuloma formation and the wide spectrum of disease phenotypes which occurs in humans [6, 45, 46]. Furthermore, there are no studies that have directly assessed the impact of IL-4 on mycobacterial survival in a human TB model.

A better understanding of the host response is important in developing new approaches to treating and preventing TB. The failure of current vaccines suggests that simply inducing protective Th1 responses may not be the only answer. If IL-4 does contribute to the development of immunopathology then it would be useful to identify the specific IL-4-inducing components of *M.tb* so that they may be incorporated into the design of prophylactic and therapeutic vaccines. Additionally, immune-modulators targeting IL-4 itself may be useful as an immunotherapeutic intervention to decrease treatment duration or as an adjunct in cases where treatment options are limited, such as drug-resistant TB.

Thus the overall objective of the thesis was:

To determine the role of Th2 and Th-2 like cytokines in the host immune response to tuberculosis and to determine if IL-4, the signature Th2 cytokine, is causal in subverting TB immunity.

The specific objectives of this thesis were as follows:

(i) To evaluate the mRNA expression levels of IFN-γ, IL-4 and IL-4δ2 in *ex vivo* bronchoalevolar (BAL) cells and peripheral whole blood of pulmonary TB patients and presumed latently infected controls. A multi-compartment approach to assessing the expression of IL-4 and IL-4δ2 in a high burden TB setting has not been previously performed. A compartment-specific profile of IFN-γ and IL-4 mRNA expression was observed and increased IL-4 levels were found in TB patients compared to LTBI controls (Chapter 3).

(ii) In order to determine if the role of IL-4 and IL-4δ2 in TB is causal or a by-product of disease, it was first necessary to clone and express human recombinant IL-4 and IL-4δ2 (rIL-4 and rIL-4δ2) in a baculovirus-insect cell expression system. IL-4δ2 has been previously expressed in yeast [47] and mammalian cells [32] but never in an insect cell system. Both rIL-4 and rIL-4δ2 was successfully expressed in insect cells but low quantities of rIL-4δ2 were obtained which limited its use in downstream assays (Chapter 4).

(iii) Following successful protein expression, it was essential to validate rIL-4 and rIL-4δ2 function using a ^{3}H thymidine proliferation assay and CD23 flow cytometric assay, as performed in previous studies [47, 48]. Functionality was confirmed as rIL-4 induced proliferation of T-cells and CD23 expression on B-cells whereas rIL-4δ2 inhibited IL-4-mediated T-cell proliferation. However, the low quantity of rIL-4δ2 obtained and the high concentrations required to produce a visible effect prevent further assessment of rIL-4δ2 in immunological assays (Chapter 5).

(iv) The next step was to determine the effect of rIL-4 on the ability of PPD pre-primed effector T-cells to control *M.tb* survival in monocyte derived macrophages in an *in vitro* TB infection model. rIL-4 subverted the ability of effector T-cells to contain *M.tb* within macrophages in an IL-4-concentration dependent manner and neutralization of IL-4 abrogated these effects (Chapter 6).

(v) The next logical step was to assess the effect of rIL-4 on various cellular mechanisms potentially involved in the IL-4-associated subversion of *M.tb* containment. This was determined by performing a mycobacterial containment assay (as performed in (iv)) where cells were stained for surface marker and intracellular cytokine expression and measured by flow cytometry. Addition of rIL-4 resulted in decreased T-cell expression of Th1 cytokines, increased regulatory T-cell expression and increased expression of DC-SIGN on macrophages. These effects were blocked upon the addition of anti-IL4 antibodies (Chapter 7).

(vi) In parallel, the production of other Th1, Th2 and Th2-like cytokines were assessed in RD-1 antigen stimulated supernatants of bronchoalveolar lavage and peripheral blood mononuclear cells from pulmonary TB patients and LTBI controls by a Luminex multiplex assay. Compartment specific differences in cytokine expression were observed in TB patients compared to LTBI controls, most notably characterized by increased levels of IL-9 in BAL and decreased IFN-γ in blood (Chapter 8).

(vii) The increased production of IL-9 observed in TB patients led to further investigations into the specific helper T-cell subsets that produce IL-9 using flow cytometry. It was recently discovered that Th9 cells were the predominant producers of IL-9 in allergic inflammation [49, 50] but these cells have been poorly characterized in pulmonary TB. Elevated levels of IL-9 were produced by CD4+ and CD8+ cells of TB patients compared to LTBI controls in both compartments (lungs and blood), and these cells exhibited a specific Th9 and Tc9 phenotype, respectively (Chapter 9).

1.2 Origins and history of TB

The early progenitor of *M. tuberculosis* (*M.tb*) is thought to have originated in East Africa about 3 million years ago where it infected early hominids [51]. However, there is less agreement regarding the emergence of the modern members of the *M.tb* complex and how it spread to the new world. Whole genome sequencing and phylogenetic analyses suggest these modern strains originated about 70,000 years ago and spread together with modern humans migrating out of Africa [52]. However, a more recent study proposed a much later emergence, around 6,000 years ago, and may have been carried to the New World by migrating seals which then transmitted the disease to humans in the coastal regions of South America [53].

TB has plagued human civilizations since ancient times. The earliest evidence dates back 5,000 years to ancient Egypt; archeological evidence of spinal tuberculosis (Pott‟s disease) was found in Egyptian mummies [54], which was later confirmed using DNA analysis [55]. Similar evidence has been found in Peruvian mummies [56]. Written records of TB have been described in early texts from India and China, the Old Testament of the Bible and by the ancient Greeks where it was known as *phthisis* [57]. The disease was endemic in Europe throughout the Middle Ages and peaked in the 18^{th} and 19^{th} centuries where the lack of treatment, overcrowding and poor sanitation likely encouraged its spread and high mortality rate [57]. Initial attempts to treat TB involved isolation of patients in sanatoria and alleviation of symptoms by pneumothorax but the exact cause of the disease remained unknown [57]. It was not until 1882 that Robert Koch identified *Mycobacterium tuberculosis* as the disease causing pathogen [58]. This discovery was followed by the development of the BCG vaccine in 1921 [59], the use of streptomycin as the first effective chemotherapy against TB in 1943, then isoniazid and the rifamycins a few years later [57]. However, despite improvements in sanitation and the availability of effective therapies, TB remains a major cause of morbidity and mortality today.

1.3 TB epidemiology

The epidemic of TB is greater today than at any point in history. It is the second greatest cause of death from a single infectious agent, behind only HIV/AIDS. According to the WHO, there were 9 million new cases of TB and accounted for 1.5 million deaths in 2013 [1]. The 2013 global TB incidence rates are shown in Figure 1.1. While significant developments have been made in reducing the burden of disease, progress remains slow and many countries are unlikely to achieve the 2015 United Nations Millennium Development Goals of reducing TB prevalence and mortality by 50% compared to 1990 levels [1]. This is most evident in the African region which accounts for over a quarter of the global TB cases (2.6 million new cases) [1]. Eight of the ten countries with the highest TB incidence rate in the world are located in Sub-Saharan Africa and this region has, by far, the highest mortality rate due to TB [1]. The HIV epidemic is the primary driving force behind the high TB prevalence and mortality in the African region.

South Africa is a particular „hot-bed" of TB activity in this region, having the 5th highest number of TB cases worldwide, behind much larger and densely populated countries such as India and China [1]. Moreover, it has the highest incidence rate per capita (excluding Swaziland and Lesotho, which are „land-locked" by South Africa) in the world [1], equating to ~1 person out of 100 that develops TB every year. In this country, the disease is largely driven by the high prevalence of HIV (61% of TB cases are HIV infected), factors associated with a low economic status (poverty, malnutrition), an overstrained primary healthcare system and the growing burden of drug resistance.

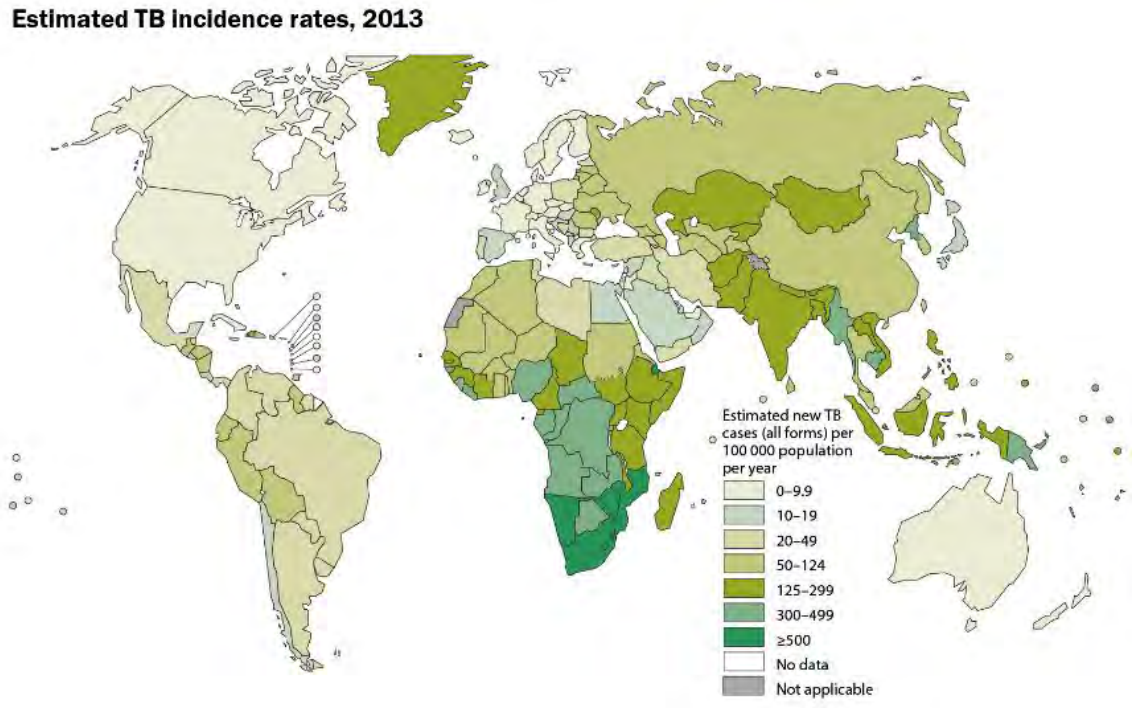


Figure 1.1. Global estimates of TB incidence rates in 2013 as reported by the World Health Organization [1].

1.4 Pathogenesis, transmission and spectrum of TB infection

Mycobacterium tuberculosis (*M.tb*) can infect any organ within the body but infection of the lungs is the most common accounting for ~85% of cases in high burden countries [60]. The bacteria are transmitted via droplet nuclei, approximately 1-5µm in size, aerosolized in the cough of patients with pulmonary tuberculosis [60]. The risk of infection depends on the bacilliary load present in the cough aerosol of the index case, the duration and intensity of exposure, *M.tb* strain-specific virulence factors and the immune status of the contact [5]. Upon inhalation, the droplet nuclei travel through the respiratory tract, escaping the mucosal barriers in the bronchi, and eventually end up in the terminal alveoli where recruited and resident phagocytes, such as macrophages, dendritic cells (DCs) and neutrophils engulf the bacteria [61-64]. During the early stages of infection, *M.tb* can evade the initial host innate immune mechanisms within infected cells and undergo intracellular replication [65]. Infected DCs and macrophages also travel to the local lymph where T-cells are primed and clonally expand in response to *M.tb* antigens. These T-cells then migrate back to the site of infection

thus signaling initiation of the cell mediated immune response, which can take 2-6 weeks after initial infection [66, 67]. The subsequent interaction of macrophages, T-cells and other cell types at the site of disease results in the formation of a granuloma.

In the vast majority (~95%) of infected individuals, the bacilli are effectively contained within the granuloma in a persistent non-replicating state, known as latent TB infection (LTBI) [68]. These individuals show no clinical symptoms but may display *M.tb* specific T-cell responses, as indicated by a positive tuberculin skin test (TST) and/or IFN-γ release assay (IGRA) [10, 69]. However, evidence suggests that the LTBI phenotype may be more heterogeneous than previously assumed [6]. For instance, a proportion of exposed individuals (may be up to 50%) show no immuno-diagnostic evidence of T-cell sensitization (consistently TST and IGRA negative) [70]. In a South African study 20% of highly exposed persons remained TST negative [71, 72]. Such individuals may have a „sterilizing immunity" phenotype where the innate response is sufficient to eliminate infection without engaging adaptive immunity, but this remains unproven [5]. Alternatively, the immune response may be highly localized to the lung without any detectable systemic responses [8]. Studies have also identified individuals who revert their TST and IGRA status weeks to months after becoming initially infected and may perhaps indicate acute resolving infection, but this is also unclear [73-75]. It is difficult to definitively ascertain these various LTBI phenotypes given the suboptimal performance of the currently available immunodiagnostic tests (TST and IGRA) [10].

In approximately 5-10% of exposed individuals, the immune system is not able to effectively contain the bacteria and results in primary active TB [5]. Furthermore, up to 5% of latently infected individuals may develop active TB, termed reactivation TB, if their immune system becomes compromised (HIV, malnutrition, etc.) [5]. Similar to LTBI, active pulmonary TB can present as an array of clinical phenotypes ranging from subclinical disease to cavitatory disease with extensive lung tissue destruction [7, 8]. These individuals represent the primary source of disease transmission. Molecular epidemiology studies have shown that the majority of TB cases in high burden countries emerge as a result of recent transmission rather than reactivation [76-78], even in HIV infected individuals [79].

Thus, *M.tb* infection can result in a spectrum of disease phenotypes characterized by different immunodiagnostic, clinical and radiological profiles (Figure 1.2). But a fundamental question still remains: why do some people exposed to TB progress to active disease despite being immunocompetent whereas others remain healthy? Host immunity undoubtedly plays an important role in the host-pathogen interaction to determine the outcome of infection but what facets of the immune response control the infection or contribute to the development of active TB remains poorly understood.

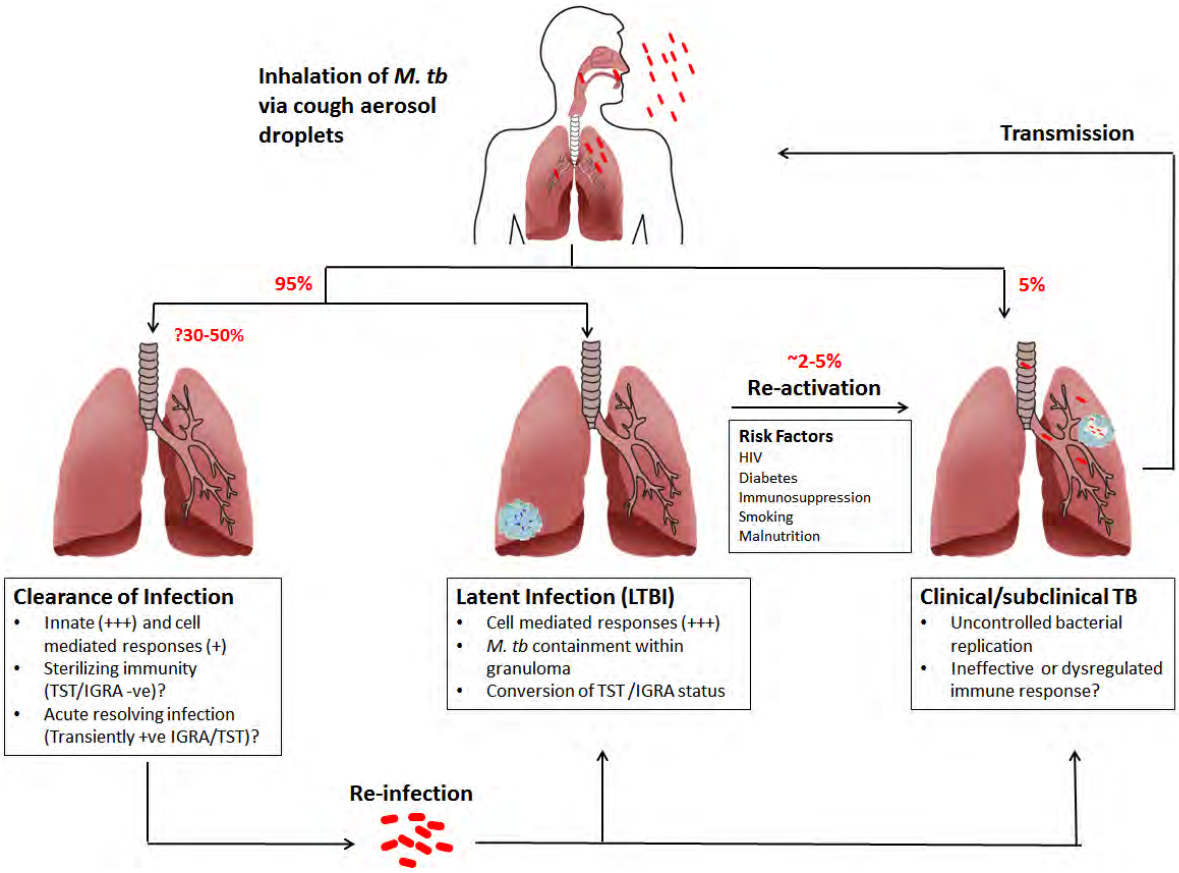


Figure 1.2. The transmission cycle of *M. tuberculosis* and the spectrum of *M. tuberculosis* infection.

1.5 Diagnosis of TB

1.5.1 Active TB

TB detection is one of the key indicators of the MDG framework but remains a challenging aspect of TB control particularly in high burden and resource limited settings. In 2013, over 3 million cases were missed globally either because they were undiagnosed or not notified to National TB programs [1].

TB diagnosis is still heavily dependent on older tools such as smear microscopy and culture. Sputum smear microscopy is the primary diagnostic tool in most high burden settings but suffers from suboptimal sensitivity, particularly in HIV infected individuals [80]. Sputum culture, using automated liquid culture systems, is considered the gold standard for TB diagnosis due to its superior sensitivity [81]. However, the test is still relatively costly and prone to contamination. Furthermore, detection can take weeks resulting in significant delays in treatment initiation [82].

Major advancements in TB diagnosis have been made recently, mainly exploiting nucleic acid amplification technology (NAATs). The most noteworthy being the Gene Xpert MTB/RIF assay, a cartridge based real-time PCR system that can detect *M.tb* DNA and genotypic rifampicin resistance in ~2 hours [83]. The performance of Xpert has been extensively evaluated and, in a recent systematic review, showed a pooled sensitivity and specificity of 88% and 98% in TB suspects, respectively and 68% sensitivity in smear negative cases [84]. Similar performance characteristics were shown in HIV infected cohorts [84]. In a large African multi-centre randomised control trial, Xpert MTB/RIF, in place of smear microscopy, reduced the time to diagnosis and treatment initiation but failed to reduce morbidity and mortality [85]. The test has also been shown to be cost-effective in TB endemic settings [86, 87]. As such, the Xpert MTB/RIF has been recommended by the WHO as the frontline diagnostic test for TB [88], and has undergone widespread rollout across all TB laboratories in South Africa [89].

1.5.2 Latent TB infection

With over one third of the world‟s population infected with TB, it is important to identify latently infected individuals who may progress to active TB, such as those who are HIV infected. Diagnosis of LTBI is difficult because it is based on the presence of an effector memory T-cell response to *M.tb* antigens rather than the direct assessment of mycobacterial load [8]. The tuberculin skin test, in use for over 100 years, elicits a delayed type hypersensitivity immune reaction at the site of injection. However, the test suffers from poor specificity due to previous BCG vaccination and exposure to environmental mycobacteria [90]. More recently, IFN-γ release assays (IGRAs) have been developed that measure systemic IFN-γ T-cell responses to the *M.tb* specific antigens early-secretory-antigen-target-6 (ESAT-6) and culture-filtrate-protein-10 (CFP-10). Two commercial assays are available; the Quantiferon Gold In-Tube ELISA assay (QFT-GIT; Qiagen) and the T-SPOT.*TB* ELISPOT assay (Oxford Immunotec). They show similar sensitivities but higher specificities compared to TST, particularly in prior BCG vaccinated individuals [91]. Developed countries with a low TB burden have recommended these tests for LTBI diagnosis in certain high risk populations either as a replacement to the TST or in conjunction with the TST [92-94]. However, in low income TB endemic areas where the incidence of LTBI is high, their utility is limited because the test is costly and it is unable to distinguish between active TB, previous TB, LTBI and previous exposure to *M.tb* or environmental mycobacteria [10]. Furthermore, the IGRA and TST are unable to identify individuals with LTBI that have a high risk of progression to active TB, which is an important priority in TB research.

1.6 Drug resistant TB

The emergence of drug resistance is one of the most concerning aspects of the global TB epidemic. In 2013, the WHO reported an estimated 480,000 multi-drug resistant TB cases (MDR-TB; defined as resistance to the two first line TB drugs isoniazid and rifampicin) and an estimated 9% of these are suspected of having extensively drug resistant TB (XDR-TB; defined as MDR-TB with additional resistance to an aminoglycoside and at least one fluoroquinolone) [1]. Even more worrying is the existence of strains that are resistant to all known TB drugs, termed totally drug resistant TB (TDR-TB) [95, 96]. Most drug resistant

TB (DR-TB) cases are concentrated in India, China and Eastern Europe but South Africa is not far behind having the 3rd highest number of notified DR-TB cases among 27 high burden countries [1].

Rapid and reliable diagnosis of drug resistant TB, particularly XDR-TB, remains elusive. Genotypic first-line drug susceptibility testing (DST) (Xpert MTB/RIF and Hain MDRTB*plus* Line Probe assay) for RIF and INH is well established [84, 97] but phenotypic methods, using MGIT liquid culture, are usually required for confirmation and to detect second-line drug resistance. The long waiting periods before availability of MGIT DST results, issues with *in vitro* stability of certain second line drugs and discordant phenotypic and genotypic DST profiles often makes diagnosis and design of effective treatment regimens difficult [98]. Nonthetheless, new genotypic second line DST assays, such as the MDRTB*sl*, has shown adequate performance [99] and is currently being evaluated by the WHO [100].

Of greater concern are the limited treatment options available for DR-TB patients. MDR-TB requires treatment for ~18 months with a combination therapy consisting of 5 or more drugs, which have toxic side effects of varying severity [101, 102]. Despite intensive therapy, prognosis remains poor with less than 50% of MDR-TB patients having successful outcomes [103]. The situation is even worse for XDR-TB patients; additional drugs with more severe side effects are required and favourable treatment outcomes occur in less than 20% of patients [103-105]. XDR-TB mortality rates were 46% after 2 years post-diagnosis but climbed to 73% when patients were followed up for a longer period [106]. The financial burden of DR-TB can also be extensive. In South Africa, DR-TB consumes 30% of the NTP budget despite only comprising 2.2% of TB cases [107]. Current healthcare facilities are ill equipped to address the growing number of newly diagnosed DR-TB patients and care for those patients who have failed treatment. Consequently, these individuals remain in the community and facilitate transmission of the disease [108].

Thus, DR-TB threatens to undermine the advances made in curbing the TB epidemic. Despite the availability of new (bedaquiline, delaminid) and repurposed (linezolid) drugs for

DR-TB treatment, other avenues including immunotherapeutic agents, need to be explored to shorten treatment regimens or to augment the efficacy of existing ones. However, this will require an understanding of the immune response and what constitutes protective immunity against TB.

1.7 Vaccines against TB

Given the slow decline of global incidence rates, the ever increasing burden of drug resistance and the shrinking arsenal of drugs available for treatment, an effective vaccine that offers long-term protection may be the only way to effectively eliminate TB. However, currently available vaccine candidates have failed to sufficiently meet these conditions.

The Bacillus Calmette-Guerin (BCG) vaccine, a live attenuated strain of *M. bovis*, has been in use for over 80 years and has been successful in prevented disseminated disease in children. However, the vaccine has a highly variable efficacy [109] and often fails to protect adults from developing active disease, particularly in TB endemic settings where the high burden of environmental mycobacteria is thought to interfere with vaccine activity [2]. Furthermore, BCG caused disseminated infection in HIV infected infants resulting in WHO recommendations against BCG vaccination in HIV infection [110, 111]. More recently, the efficacy of a Modified-Vaccinia-Ankara (MVA) 85A was assessed in phase III clinical trials where BCG vaccinated infants were boosted with MVA85A and followed up for 3 years. However, even though pre-clinical trial results were promising, the MVA85A vaccine failed to provide any additional protection against TB [3, 112]. Despite the lack of protective efficacy elicited by BCG and MVA85A, a number of prophylactic and therapeutic vaccine candidates that either improve or supplement BCG are at various stages of clinical trials. Nonetheless, such trials are expensive and are further hampered by the lack of field testing sites [113]. Therefore, potential candidates need to be thoroughly vetted to ensure the most promising candidates are selected for large scale trials. Given that these vaccines function by eliciting effective immunity, a thorough understanding of the immune response to TB is required.

1.8 The immune response to TB

A complex immune cascade consisting of various interacting cell types and cytokines is initiated in response to *M.tb* infection. In the majority of individuals, the immune response mounts a successful defense, either by completely eradicating the bacilli (sterilizing immunity) or effectively controlling them in a non-replicative latent state (LTBI) [5]. However, it is unclear which specific constituents of the immune response is the driving force of protective immunity. Invaluable information has been obtained from the numerous studies on TB-associated host immunity in *in vitro* systems and animal models but the applicability of these findings to human TB is somewhat limited. *In vitro* systems represent an artificial environment and fail to fully mimic the *in vivo* immune response. Furthermore, many studies evaluate cellular responses of the systemic circulation, as blood is easily acquired and high cell quantities are obtained, but may not represent the situation at the site of disease [38]. Animal models have been extensively used for studying *in vivo* responses but are often not equivalent to human systems due to dissimilar manifestations of disease pathology. For instance, mice fail to develop cavitatory disease because the granuloma lacks the fibrotic and hypoxic environment often observed in human TB [45, 46]. Furthermore, mice do not exhibit the spectrum of disease phenotypes that humans do [6]. Other animal systems, including guinea pigs, rabbits and non-human primates more closely resemble human TB [114, 115] but the scarcity of appropriate culture reagents and high cost of these systems often limits their feasibility in a research setting [8].

Despite these limitations, such models have provided crucial information on host-pathogen interactions and laid the foundation for our current understanding of TB host immunity. They remain essential to identify other potential immunological pathways involved in TB control and as a screening tool for vaccine candidates and immune-modulating therapies [8]. The following sections describe our current knowledge of the innate and adaptive immune system, including the specific cells and cytokines involved, and how they function to limit or promote TB-associated immunopathology. An overview of the cellular mechanisms involved in the immune response in TB is shown in Figure 1.3.

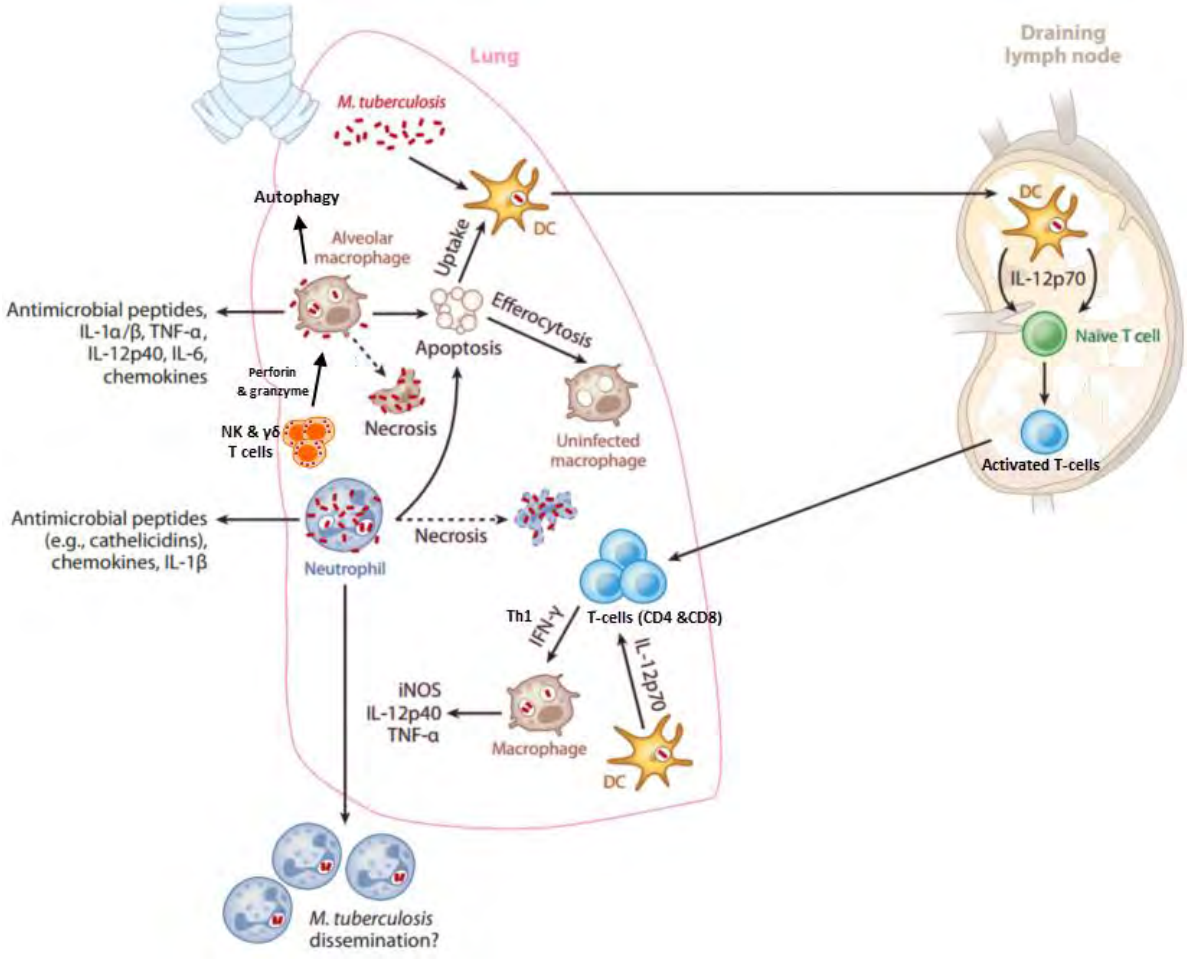


Figure 1.3 Overview of the innate and adaptive cellular immune responses to TB. Figure was adapted and modified from O'Garra *et al* [8].

1.8.1 The innate immune response

1.8.1.1 *M.tb* entry into phagocytic cells

Initiation of the innate immune response occurs immediately after infection. Once the bacilli enter the alveoli, they are internalized by alveolar macrophages, dendritic cells (DCs) and neutrophils [61-64]. Entry into these cells is facilitated by surface-expressing pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (includes mannose receptor (MR), DC-specific intracellular-adhesion-molecule-3 grabbing non-integrin (DC-SIGN)), which recognize various components of *M.tb* (lipoprotein, CpG-

containing DNA and mannose-capped lipoarabinomannan (ManLAM)) [116, 117]. The type of receptor and the specific antigen involved in uptake dictates the nature of the immunological response to *M.tb*. For example, entry via TLR2 activates the NF-κβ pathway and upregulates the vitamin-D receptor resulting in the production of protective proinflammatory cytokines and antimicrobial peptides, respectively [118-120]. Conversely, uptake of *M.tb* via binding of ManLAM to MR and DC-SIGN in macrophages drives production of IL-10 and TGFβ which suppress protective immune responses [118, 121].

1.8.1.2 Granuloma formation

Granuloma formation is a distinct characteristic of TB infection in humans representing a dynamic relationship between host immunity and bacterial persistence. In this regard, the granuloma can effectively contain *M.tb* infection while simultaneously providing the bacilli with a suitable environment to facilitate long term survival [122]. Initial infection of macrophages and neutrophils initiates a cytokine and chemokine cascade that activates microbicidal mechanisms in infected cells and recruits additional macrophages, neutrophils and dendritic cells as well as natural killer and γδ T-cells to the site of infection [123]. Chemokines such as MCP-1, CCL12 and CCL13 recruit macrophages [124] whereas IL-17, produced by γδ T-cells [125], is thought to be responsible for neutrophil recruitment [126]. TNFα and IFN-γ production by infected macrophages is responsible for activating phagocytic cells and maintaining the granuloma structure [127, 128]. These cells accumulate around the infected cells while activated CD4+ and CD8+ T-cells arriving from the local lymph nodes, further surround the primary foci and mount a Th1 response resulting in additional cell recruitment and activation [123]. The resulting structure consists of a mass of infected and uninfected macrophages and neutrophils at the centre surrounded by a layer of lymphocytes and fibroblasts [129]. Within the granuloma, macrophages may fuse to form multinucleated giant cells or differentiate into foamy macrophages or epitheliodal cells [129]. In most cases, the *M.tb* bacilli are contained within the centre of the granuloma and are controlled by the surrounding phagocytic cells and lymphocytes. Usually, a necrotic caseum forms at the centre, a consequence of macrophage death, which is hypoxic and unfavourable for *M.tb* proliferation [130]. However, a dysregulated immune response may cause *M.tb*

containment to fail resulting in exposure of the pathogen to an oxygenated environment and a subsequent increase in bacterial proliferation [6, 131]. The bacilli are released into the environment and disseminate resulting in active disease. The typical structure of a caseous gramuloma is shown in Figure 1.4.

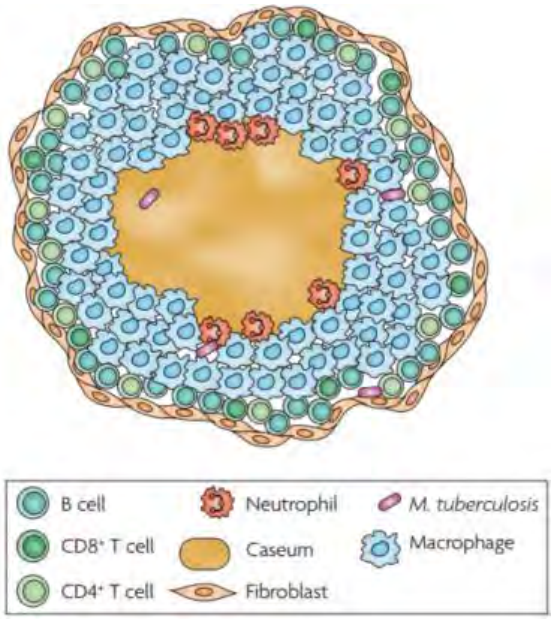


Figure 1.4. The typical structure of a caseous granuloma. Taken from Barry *et al.* [6].

1.8.1.3 Innate cellular immune mechanisms and evasion by *M.tb*

1.8.1.3.1 Macrophages

Macrophages, activated by cytokines IL-12, IFN-γ and TNFα, possess multiple anti-mycobacterial mechanisms and represent the first line of defense against *M.tb* [132, 133]. Once phagocytosed, bacilli are enveloped within phagosomes that become acidified which eventually undergo lysosomal fusion and subsequent proteolytic degradation [134, 135]. Similarly, the production of reactive nitrogen and oxygen intermediates, such as nitric oxide (NO), exert toxic effects on *M.tb* [136]. However, *M.tb* has developed mechanisms to evade these intracellular killing pathways; various *M.tb* components, such as ManLAM, PKnG and lipid phosphatase can either delay or inhibit phagosome maturation or fusion with lysosomes

[60, 134]. *M.tb* prevents recruitment of inducible nitric oxide synthase (iNOS) to the phagosome and the lipid rich envelope of the bacilli is particularly resistant to oxidants [137, 138].

1.8.1.3.2 Apoptosis

Programmed cell death of infected macrophages by apoptosis is thought to be protective in TB infection as it limits growth and prevents dissemination of *M.tb* [139]. The process is facilitated mainly by TNFα but other mechanisms have also been shown to activate pathways of cell death (Fas-FasL interaction, ATP, H_2O_2) [140-143]. Studies in both mice and humans have shown that TNFα-induced apoptosis reduced *M.tb* viability in macrophages [140, 144, 145]. Additionally, apoptotic vesicles containing *M.tb* antigens may be taken up by dendritic cells for more effective antigen presentation and activation of CD8 T-cells during the adaptive immune response [146].

There is convincing evidence that *M.tb* inhibits apoptotic mechanisms. Virulent *M.tb* strains induce very little apoptosis allowing for uncontrolled intracellular growth [147-149]. Specific *M.tb* components drive this reduction in apoptosis. For instance, LAM inhibits apoptosis in a Ca^{2+} dependent manner [150]. Inactivation of anti-apoptitic *M.tb* genes *secA* and *nuoG*, that encode a virulence-related secretion system involved in controlling ROIs and NADH dehydrogenase respectively, resulted in enhanced apoptosis and antigen specific CD8+ T cell priming [151, 152]. Inhibition of apoptosis by *M.tb* skews the infected cells to cell death by necrosis, allowing *M.tb* to disseminate and infect surrounding cells [153, 154].

1.8.1.3.3 Autophagy

Autophagy is an innate mechanism responsible for encapsulating and trafficking cytosolic materials, in autophagosomes, to lysosomes for degradation [155]. In macrophages, autophagy is an important cell survival mechanism during starvation but also plays a vital role in eliminating intracellular pathogens and has been suggested as one of the mechanisms by which the host cell overcomes the *M.tb* evasion tactic of inhibiting phagosomal

maturation [156, 157]. Autophagy effectively kills *M.tb* through lysosomal fusion with the autophagosome and can be induced by IFN-γ and TNFα through the immunity-related GTPase M (IRGM1) pathway [158, 159], TLR signaling [160, 161] or by cathelicidin via Vitamin D metabolism [162]. It also assists with antigen processing by delivering *M.tb* antigens, present in the autophagosomes, for MHC I and II presentation to CD8+ and CD4+ T-cells [163, 164]. However, autophagy induction is influenced by the effects of Th1/Th2 polarization [44, 158, 159]. While Th1 cytokines induce autophagy, Th2 cytokines such as IL-4 and IL-13 are inhibitory and act via the starvation induced Akt signaling pathway or IFN-γ induced STAT-6 pathway [44]. Given its impact on promoting intracellular killing of *M.tb in vitro*, Cheallaigh *et al* suggested targeting autophagy, by using autophagy-inducing compounds or suppressing autophagy-inhibiting immune responses, may be a useful immunotherapeutic strategy for TB treatment [157].

1.8.1.3.4 Efferocytosis

Efferocytosis is a recently described mechanism of antimicrobial control inherently associated with apoptosis [145]. Apoptotic macrophages containing *M.tb* are engulfed by non-infected surrounding phagocytes, through recognition of „eat me" signals present on the surface of apoptotic cells such as phosphatidyl serine [165] and calreticulin [166], to form a double membrane efferosome. The contents of the efferosome, including *M.tb*, are subsequently destroyed through lysosomal fusion or oxidative killing [145]. It is thought that efferocytosis is the ultimate bactericidal effect of apoptosis as the contents of apoptotic cells would otherwise be released in the extracellular environment leading to secondary necrosis and inflammation [145, 167]. However, in *L. major* infection, efferocytosis of infected neutrophils is thought to enhance spread of the parasite to macrophages [168]. Whether this may also occur in *M.tb* is not clear and much research still needs to be performed to determine the significance of this mechanism in TB.

1.8.1.3.5 Vitamin D and cathelicidin

Vitamin D also plays a role in suppressing *M.tb* growth within macrophages. Evidence comes from patients with vitamin D deficiency or polymorphisms in the vitamin D receptor that show increased susceptibility to TB [169, 170]. Vitamin D is metabolized within granulomatous tissue to its active form, 1,25-dihydroxyvitamin D, resulting in the production of the antimicrobial peptide cathelicidin, which restricts *M.tb* growth directly [120] or by induction of autophagy [162]. However, 1,25-dihydroxyvitamin D can also downregulate Th1 responses which are protective in TB [171].

1.8.1.3.6 NK and γδ T-cells

NK and γδ T-cell subsets are considered part of the innate response as they are partially activated, respond rapidly following infection and modulate other cell types. NK T-cells in the innate pathway do not require MHC presentation but rather recognize antigens, mainly glycolipids, through the CD1 family of antigen presenting surface molecules. These cells exert their mycobactericidal activity through production of IFN-γ and release of cytotoxic granules [172]. They also induce DC maturation and recruit other inflammatory cells to the site of infection [173, 174]. In mice, NK T-cells directly kill *M.tb* and confer protection against aerosolized infection [175, 176]. TB patients exhibited reduced frequency and functionality of NK cells in peripheral blood [177]. However, a subset of NK T-cells that produce IL-4 were found to be increased in TB patients [178], and may contribute to a subversion of protective innate responses.

Similar to NK T-cells, γδ T-cells respond to non-peptide phosphoantigens without the requirement of activation via MHC presentation. Both human and animal studies suggest a crucial role in *M.tb* immunity [179]. Vγ9Vδ2 T-cells, the predominant form found is humans, are rapidly recruited to site of *M.tb* infection and directly kill infected cells via release of cytotoxic molecules such as granulysin and perforin [180].These cells can also produce large amounts of IFN-γ and TNFα and cooperate with other immune cells [181, 182] to elicit a protective response.

1.8.1.3.7 Neutrophils

Neutrophils, together with macrophages, represent the first line defense against *M.tb* infection. However, their role in TB is somewhat controversial [183]. Neutrophils are the predominant *M.tb* infected phagocytic cell type [61] and are recruited early to the site of infection [183] where they internalize *M.tb* via TLRs binding [184] or opsonisation [185]. The mycobactericidal effect of neutrophils are employed in a number of ways; they kill *M.tb* directly through antimicrobial peptides such α-defensins (human neutrophil peptides) and cathelicidin [186, 187], or indirectly by cooperating with macrophages to remove apoptotic neutrophils [188, 189], enhancing DC activity [190] and playing a role in granuloma formation [191]. In contrast, evidence suggests that neutrophils can actually contribute to the pathology associated with chronic TB. In mice, neutrophil deletion leads to enhanced protection [192, 193] and in humans, systemic neutrophilia is associated with poor prognosis and slow sputum conversion [187, 194]. Additionally, virulent *M.tb* strains can inhibit neutrophil apoptosis and delay T-cell responses in a similar way to macrophages [195]. A failure to eliminate *M.tb* means that neutrophils are a potential reservoir of bacilli which facilitate dissemination of disease [196-198]. Furthermore, neutrophil necrosis releases cytotoxic contents into the microenvironment and can lead to granuloma liquefaction, excessive inflammation and immunopathology [61, 198].

1.8.1.3.8 Dendritic cells (DCs)

Dendritic cells are the primary antigen presenting cell responsible for initiating adaptive T-cell responses [199] and evidence suggests they enhance the immune response to TB [200, 201]. In DC depleted mice, CD4+ T-cell responses were insufficient to control bacterial replication [202]. After initial infection, large numbers of DCs congregate in the lungs [203, 204] and internalize *M.tb* via the TLRs and DC-SIGN [205]. Activated DCs then travel to the lymph nodes and facilitate T-cell priming by upregulating surface expression of MHC and other co-stimulatory molecules for antigen processing and presentation. They also interact with infected macrophages and neutrophils undergoing apoptosis resulting in enhanced DC migration to lymph nodes and more rapid T-cell priming [206, 207]. *M.tb* can impair DC maturation, delay DC trafficking to the lymph nodes and inhibit their ability to expand T-cell

population [208, 209]. For example, infected DCs were shown to be poor stimulators of antigen-specific CD4+ T-cells despite high levels of MHC expression [64]. DC maturation is also dependent on the type of receptor engaged during its interaction with *M.tb*. Entry of *M.tb* via TLRs induces IL-12 secretion and efficient DC activation whereas entry via DC-SIGN inhibits maturation by blocking NFκβ activation and subsequent IL-10 secretion [210]. Finally, *M.tb* can also influence DCs to downregulate protective responses by directing Th2 differentiation or regulatory T-cell (Treg) expansion via induction of IL-1β or PD1, respectively [211, 212].

1.8.2 Adaptive Immunity

Initiation of the adaptive immune response is typically delayed, occurring ~2-6 weeks after initial infection [66]. Infected DCs and macrophages travel to the lymph nodes where they activate CD8+ and CD4+ T-cells via the MHC I and MHC II antigen presentation pathways, respectively. NK and γδ T-cells can also be activated in the lymph nodes via CD1 restricted pathways, as previously discussed. Tregs serve to modulate the immune response and have been described in both the CD4+ and CD8+ populations. The cells of the adaptive immune response are shown in figure 1.5.

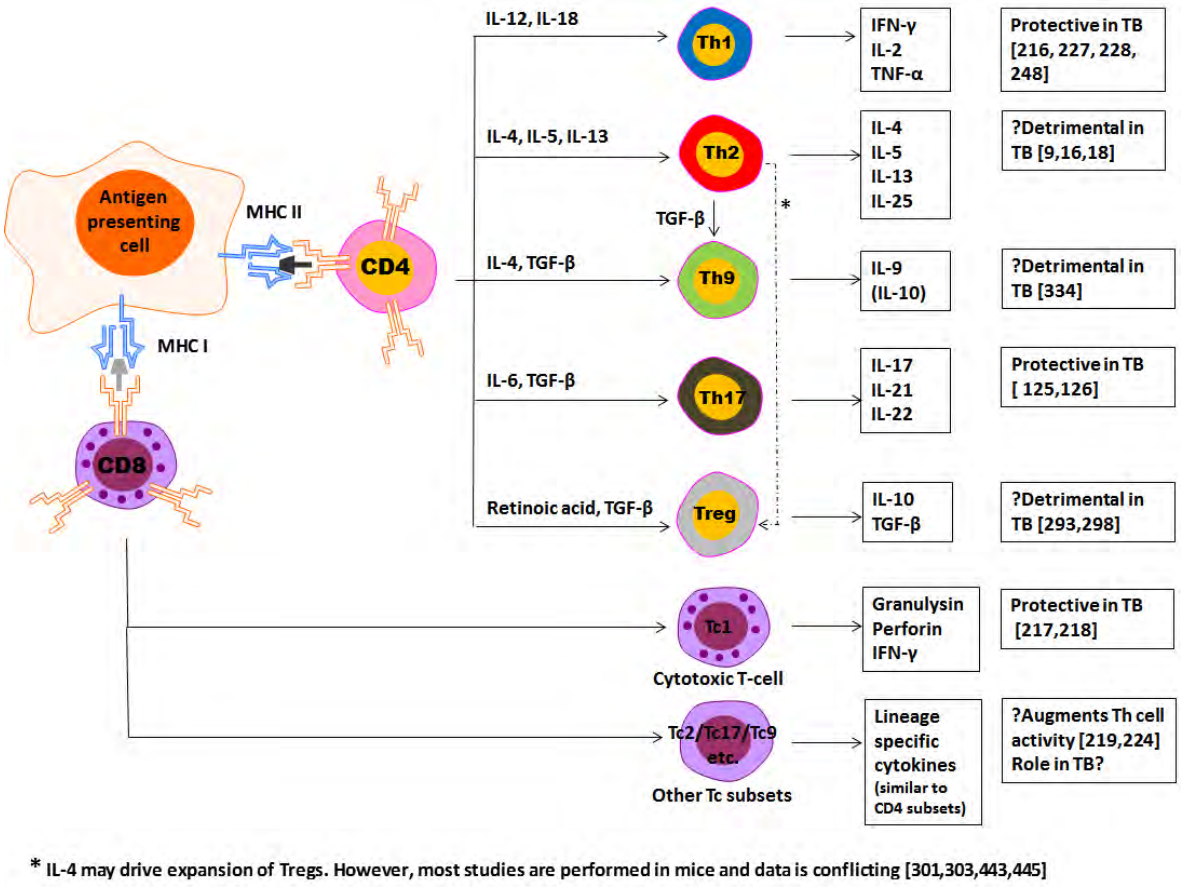


Figure 1.5. The various CD4 and CD8 T-cell lineages of the adaptive immune response and their role in TB immunity.

1.8.2.1 CD8+ T-cells

Murine studies have indicated a role for CD8+ T cells in the immune response to TB. Mice with defective MHC I processing pathways are more susceptible to TB than wild type mice [213, 214]. Studies in humans support similar conclusions [215, 216]. CD8+ cytotoxic T-cells (CTLs) exert their antimycobacterial activity directly by killing *M.tb* infected cells through the production of perforin and granulysin [217], or indirectly by producing IFN-γ and TNFα that recruits and activates macrophages [218]. However, CD8+ T-cells may also differentiate into distinct effector phenotypes based on transcription factor expression and cytokine secretion patterns, in a similar way to CD4+ helper T-cells [219]. In addition to the traditional IFN-γ producing CTLs (known as Tc1 cells), other „non-classical" CD8+ subtypes

producing IL-4 (Tc2), IL-17 (Tc17) and IL-9 (Tc9) have been described and their induction is likely dependent on the cytokine microenvironment [220-223]. These subtypes have mainly been described in tumour immunity but less is known about their role in TB. It has been suggested that these cells support CD4+ T-cell mediated responses rather than promote cytotoxicity [224].

1.8.2.2 CD4+ T-cells

CD4+ T-cells are indispensible to TB immunity and play a central role in the induction and maintenance of the cell mediated immune response. CD4+ T-cell deficient mice are unable to control *M.tb* growth and quickly succumb to disease [216]. Also, HIV infected individuals with reduced CD4 lymphocytes are highly susceptible to TB. CD4+ T-cells polarize into different subsets that secrete distinct cytokine patterns which will ultimately determine the outcome of cell mediated immunity in TB. These helper T-cell (Th) subsets include Th1, Th2, Th17, Th9 and Tregs which all have diverse functions that can either enhance or impair a protective response [216]. These subsets and the major cytokines they produce are discussed below.

1.8.2.2.1 Th1 cells

Th1 effectors differentiate under the control of the transcription factor T-bet and their induction is dependent on IL-12 and IL-18 [225, 226]. Th1 cells usually develop in response to intracellular pathogens and are the primary effector cells responsible for protective immune responses to *M.tb* [216, 227]. They are characterized by secreting high levels of IFN-γ and TNFα.

IFN-γ

IFN-γ is the main cytokine required for a protective immune response in TB [228]. In addition to Th1 cells, it is also produced by CD8+ cells, NK cells and macrophages. Its protective role was first exhibited in lepromatous leprosy patients where administration of IFN-γ to lesions resulted in T-cell and monocyte migration and a subsequent decrease in

bacilliary load [229]. Furthermore, humans and mice with defects in the IFN-γ or IFN-γ receptor genes showed increased susceptibility to *M.tb* infection [230-232]. IFN-γ induces multiple protective effects; it activates macrophages to eliminate intracellular *M.tb*, it augments antigen presentation by inducing MHC I and II expression and subsequent recruitment of CD4+ and CD8+ T-cells, upregulates expression of ROIs and RNIs in phagocytic cells, induces innate mechanisms of autophagy and apoptosis and prevents T-cell exhaustion [60, 158, 233-236]. Its protective effect is so apparent that aerosolized IFN-γ has been used as an immunotherapeutic agent to treat MDR-TB patients with some success [237]. Given these data, it would not be remiss to assume that active TB is associated with a depressed IFN-γ response. However, the extensive number of studies that have investigated the levels of this cytokine have produced conflicting results; some studies, using *ex vivo* or antigen stimulated cells, have reported higher levels of IFN-γ in TB patients compared to controls [9, 11, 238-242] whereas other have reported lower levels [33, 243-247]. Furthermore, studies that examined the site of disease found extremely high levels of IFN-γ in TB patients [9-15]. Thus, while it is undoubtedly important for protection, IFN-γ alone is likely not enough to prevent active disease.

TNFα

The crucial role of TNFα in TB immunity has been demonstrated in both mice and humans. TNFα and TNFα receptor deficient mice showed increased susceptibility to TB infection [248]. Similarly, LTBI subjects receiving anti-TNF treatment for autoimmune diseases exhibited a five-fold increase in TB reactivation rates [249, 250]. TNFα, is produced by T-cells, macrophages, DCs and neutrophils, activates macrophages, and, like IFN-γ, has multiple anti-mycobacterial effects; it increases iNOS expression and activates macrophages in conjunction with IFN-γ [136, 233], induces apoptosis [140, 144, 145], produces chemokines (IL-8, MCP-1 and RANTES) which recruit immune cells to the infection site [124] and plays a role in granuloma maintenance [233, 251]. In contrast, TNFα may also cause the immunopathology often observed in active TB. In mice with progressive disease, treatment with TNFα enhances inflammation and tissue damage causing increased mortality [252]. TNFα is also associated with fever and weight loss and contributes to

immunopathology in human TB [253-255]. IL-4 may also be involved in the toxicity of TNFα as the addition of recombinant IL-4 to mixed Th1/Th2 pulmonary lesions in mice infected with TB exacerbated TNFα mediated inflammation [256].

1.8.2.2.2 Th17 cells

Th17 cells have been recently described as a distinct helper T-cell subset that develops in the presence of IL-6 and TGFβ and characterized by the production of IL-17, IL-21 and IL-22 [257]. They induce protective immune responses in a variety of infections, including *M.tb* [125, 126, 258], but are also involved in promoting inflammation and tissue damage, particularly in autoimmune diseases [259].

IL-17

IL-17 is the signature cytokine secreted by Th17 cells, although other cells may also produce IL-17, including γδ T cells, NK cells and neutrophils [260-262]. γδ T-cells are the primary source of IL-17 early in *M.tb* infection [125]. The foremost role of IL-17 in TB immunity is the recruitment and activation of neutrophils which contributed to early granuloma formation [126]. In IL-17 deficient mice, there was a decrease in neutrophil recruitment and increased disease susceptibility [263, 264]. Furthermore, there is evidence that IL-17 may also enhance the Th1 response in BCG infection [125]. However, excessive production of IL-17 can also be detrimental by causing an influx of neutrophils and subsequently providing an accommodating environment for *M.tb* replication [61]. Indeed, an accumulation of neutrophils is commonly observed in genetically susceptible mice [192, 193]. Furthermore, extensive exposure of neutrophils to IL-17 can alter their phenotype making them more inclined to cause immunopathology [265]. IFN-γ seems to regulate Th17 cells by limiting excessive production of IL-17 and improving disease outcome [266].

1.8.2.2.3 Regulatory T-cells

Regulatory T-cells (Tregs) modulate the immune response by suppressing Th and other cell responses to prevent pathogen-mediated immunopathology, autoimmunity and to maintain

self-tolerance [267]. Tregs have been described in most lymphocyte populations including CD4+ [268, 269], CD8+ [270], NK T- and B-lymphocytes [271]. Within the CD4+ T-cell population, two main subsets have been described: natural CD4+CD25+FoxP3+ Tregs (nTregs), which develop in the thymus and are anergic to T-cell receptor stimulation [272] and inducible Tregs (iTregs), which differentiate in response to TGFβ and retinoic acid from effector T-cells and natural Tregs [4, 273], and exhibit less stable expression of FoxP3 [274]. Furthermore iTregs, which include Tr1 and Th3 cells, are thought to mediate immunosppresive effects partly through the production of immunomodulatory cytokines such as IL-10 and TGFβ (discussed below) [272, 275] but the extent of their suppressive capacity, at least *in vitro*, compared to nTregs remains contentious [276, 277]. In addition to immunosuppressive cytokine production, Tregs may also exert their effects by preventing DC maturation and subsequent T-cell activation [278] via the CLTA-4 pathway [279, 280] or by inducing apoptotic mechanisms via IL-2 sequestration [281, 282] or perforin/granzyme production [283]. However, the precise characterisation of these Treg subsets and their interaction to facilitate immune suppression has not yet been fully elucidated [284, 285].

Since the first report of Tregs in TB almost a decade ago [286], there have been several subsequent studies showing increased Treg levels in the peripheral blood [287-289], pleural effusions [290], broncho-alveolar lavage [291-293] and lymph nodes [294] of TB patients compared to healthy controls. Tregs in the peripheral circulation migrate to the site of disease as demonstrated in a macaque model of TB [295]. The target of Treg suppression appears to be the Th1 response; reports in TB patients have demonstrated an inverse relationship between Treg expression and IFN-γ production and functional depletion of Tregs also resulted in increased IFN-γ levels [286, 287, 292-294]. Similarly, blocking of TNFα resulted in increased Treg activity and subsequently increased susceptibility to disease [296-298]. An *in vitro* model of human *M.tb* infection demonstrated that Tregs attenuate the ability of effector cells to contain *M.tb* growth, likely by downregulating protective immune mechanisms [293, 298]. Evidence suggests that Tregs mediate suppression in TB via IL-10 [292] or cell-contact dependent [299] mechanisms. While Tregs may be required to limit excessive inflammation, their early expansion may delay the initiation of adaptive immunity

allowing for unrestricted bacterial proliferation [300]. A Th2 response can drive Treg expansion [301-303] but it is not known if this is occurring in TB as well.

IL-10

In addition to Tregs, the anti-inflammatory cytokine, IL-10 is produced by most Th subsets as well as neutrophils, macrophages and DCs [304]. In TB, IL-10 downregulates the production of proinflammatory cytokines such as TNFα and IL-12 [305, 306], inhibits phagocytosis and IFN-γ mediated production of ROIs and RNIs, which are all pivotal protective mechanisms against TB [304, 307, 308]. Furthermore, in addition to being produced by iTregs, IL-10 also enhances differentiation of iTregs [309]. IL-10 deficient mice exhibited reduced bacterial loads, enhanced resistance and a concurrent increase in IFN-γ secretion [310, 311]. Similarly in humans, neutralization of IL-10 resulted in increased T-cell proliferation and IFN-γ production [312, 313]. These data suggest that IL-10 prevents bacterial clearance early in disease by downregulating protective responses [310].

TGFβ

Like IL-10, TGFβ has immunosuppressive effects on protective host immunity to TB; it decreases IFN-γ induced MHC II expression [314] and macrophage activation [315] and has modulatory effects on T-cell proliferation [316]. Increased levels of TGFβ are often observed in PBMCs and granulomas of TB patients [317]. TGFβ has been shown to enhance intracellular *M.tb* proliferation [318] and inhibit the protective effects of TNFα [319]. Furthermore, TGFβ is also associated with tissue damage and fibrosis [320, 321]. Existing data suggests that a Th2 response may drive TGFβ leading to subversion of the protective immune response [16].

1.8.2.2.4 Th9 cells

Th9 is a newly described T-cell subset that produces large amounts of IL-9 but not cytokines from other Th lineages [49, 50]. Th9 cells may also produce IL-10 in mice but not in humans [322, 323]. They develop from naïve T-cells in the presence of IL-4 and TGFβ or from Th2

cells in the presence of TGFβ [49, 50, 322, 323]. Despite the distinct cytokine secretion pattern and developmental requirements, the identification of a single lineage-defining transcription factor remains elusive [324]. Given the need for IL-4 in their development, a number of Th2 related transcription factors, such as STAT-6 and GATA-3 have been shown to be involved in Th9 development indirectly by downregulating other IL-9 inhibiting transcription factors [49, 50, 325]. Convincing evidence has emerged that interferon regulatory factor 4 (IRF4) and the ETS transcription factor PU.1 are essential for development and maintenance of Th9 cells [326, 327]. Both bind directly to the *Il9* promotor and mice deficient in either transcription factor show reduced IL-9 production and IL-9-dependent allergic pulmonary inflammation [326, 327]. Similar results have been found in humans [326-328]. However, these transcription factors are also involved in the development of other Th subsets, including Th2 and Th17 [329, 330]. Further investigations are required to identify a Th9 specific transcription factor.

Th9 cells play significant pro-inflammatory roles in asthma [326, 331], allergic airway inflammation [324, 327, 332] and autoimmune disease [49, 333]. However there is a paucity of data regarding their role in TB. Ye *et al* characterized Th9 cells in pleural TB patients where they differentiated in response to antigen presentation by pleural mesothelial cells (PMCs), stimulated PMCs and inhibited PMC apoptosis [334]. Given that these cells have similar functions to Th2 cells, Th9 cells may also contribute to the hypothesis that a dyregulatory Th2 response may be undermining protective immunity to TB.

IL-9

IL-9 was originally considered a Th2 cytokine but has been shown to be produced by other Th subsets (Th9, Th17, Tregs), CD8+, NK and mast cells [335]. The effects of IL-9 are equally diverse having both pro- and anti-inflammatory roles. IL-9 contributes to allergic inflammation by driving mast cell expansion and IL-13 production, stimulating mucus production and airway hyper-responsiveness [336, 337]. Its effect on mast cells also contributes to development of autoimmune disease as shown in mouse models of experimental autoimmune encephalomyelitis (EAE) and colitis [333, 338, 339]. IL-9

promotes parasite clearance by inducing eosinophila, mucus production and increased intestinal muscle contraction [340, 341]. In contrast, IL-9 has also been shown to promote Treg suppression in transplantation tolerance [342]. In APCs, IL-9 induces TGFβ secretion resulting in decreased production of TNFα and ROIs [343].

Very few studies have investigated IL-9 in TB [11, 334, 344, 345] but evidence suggests that IL-9 impairs protective Th1 responses. IL-9 reduced IFN-γ expression in PBMCs of pulmonary TB patients [344] and inhibited IFN-γ induced apoptosis of pleural mesothelial cells in TB pleuritis patients [334]. These data suggest a detrimental role for IL-9 in TB as it seems to undermine protective Th1 responses.

1.8.2.2.5 Th2 cells

As already stated, a Th1 response, while necessary for protection against *M.tb* infection, is not sufficient to prevent disease progression and other mechanisms are likely involved in disrupting protective immunity. This thesis postulates that one of these subversive mechanisms is an underlying Th2 response. Th2 cells are characterized by the secretion of IL-4, IL-5 and IL-13 and drive expansion and differentiation of B-cells, eosinophils and mast cells [346]. They have important roles in clearance of intestinal parasitic infections and inducing inflammation in allergic diseases and asthma [346]. There is a significant body of evidence suggesting a subversive role for Th2 cells in TB (discussed later) but many studies have been observational and it is unclear if a Th2 response is the cause of reduced protection and immunopathology or an inconsequential by-product of active disease. IL-4 is the signature cytokine that facilitates Th2 functions. Another Th2 cytokine, IL-13, has similar properties to IL-4 and bind to receptors that share the IL-4 receptor α chain [44]. IL-13 is upregulated in active TB patients and correlates with IL-4 levels [18]. Furthermore, IL-13 can also downregulate Th1 responses and protective mechanisms such as autophagy [44].

The following sections describe the properties of IL-4 and the current evidence investigating the presence and role of IL-4 in TB, including the putative mechanisms by which IL-4 may subverts protective responses.

1.9 IL-4 and IL-4δ2

1.9.1 Biological functions of IL-4

IL-4 is a pleiotropic cytokine which exert their functions on multiple cellular targets as IL-4 receptors are widely distributed on these cells. IL-4 is a potent inducer of T-cell proliferation [47] and drives Th2 differentiation from naïve T-cells [347]. Due to the reciprocal relationship of Th1 and Th2 cell activation, IL-4 inhibits Th1 responses in T-cells including downregulation of IL-2 receptor expression [348] and IFN-γ production. IL-4 can also inactivate macrophages by decreasing CD14 and Fcγ receptor expression [349, 350] or polarize macrophages toward an alternatively activated form characterized by increased IL-10 and TGFβ expression [351]. In LPS induced monocytes, IL-4 inhibits PGE2 production [47], a potent inducer of apoptosis in innate immune cells [352]. These functions suggest that IL-4 can downregulate protective mechanisms against TB. In addition, IL-4 stimulates and recruits eosinophils, mast cells and basophils to the site of infection or in response to allergens thus promoting mucus production and airway hyper-responsiveness [347]. In B-cells, IL-4 enhances MHC class II expression, induces CD23 (low affinity IgE receptor) and, together with IL-13, IgE class switching [347].

1.9.2 Sources of IL-4 and Th2 differentiation

It was previously thought that only IL-4 was able to induce the development of a Th2 response via IL-4Rα binding and STAT-6 (signal transducer and activator of transcription-6) activation. However, the initial production of IL-4 by non T-cell (granulocytes) or specialized T-cell (NK, γδ T-cells) populations was not essential for induction of CD4+ specific IL-4 responses [353]. Indeed, Th2 differentiation can occur through pathways independent of IL-4. For example, Th cell polarization is dependent on the interaction of pathogen specific PAMPS and PRRs expressed on DCs [354]. *S. mansoni* protein extract induced the development of Th2 cell-promoting effector DCs that drive Th2 development by an OX40-ligand dependent mechanism [355]. *M.tb* and its various components are also capable of polarizing DC to induce Th2 responses. Dendritic cells derived from BCG-infected precursors are able to promote a Th2 response [356]. Similarly, the 30- and 38-kDa *M.tb* antigens partially mature dendritic cells to stimulate development of IL-4 producing

CD4+ T-cells [357]. RD1 antigens and virulent *M.tb*, but not BCG, induce IL-1β production in DCs and subsequently drive the development of Th2 cells [211]. However it is likely a number of other factors may also influence the outcome of Th polarization including tissue specific factors, physical interaction with other cell types and length of TCR engagement [358]. Furthermore, IL-4/STAT-6 independent pathways can also drive Th2 differentiation (discussed below) but whether *M.tb* is able to induce these pathways is unclear.

1.9.3 IL4 receptors and Th2-inducing signaling pathways

1.9.3.1 Membrane bound IL4 receptors

The IL-4 receptor (IL-4R) is widely distributed on various cell types and exists in two forms. The type 1 receptor is a heterodimer complex consisting of IL4Rα and the cytokine receptor common γ chain, γc. This receptor is predominantly found on T-cells and mast cells and, as a result, these cells respond strongly to IL-4 rather than IL-13 [359]. In contrast, the type 2 receptor, commonly found on both haemopoeitic and non-haemopoeitic cells such as macrophages, is comprised of IL-4Rα with IL-13Rα1 and binds both IL-4 and IL-13 [359]. The IL-13Rα2 alone can bind IL-13 and may act as a „decoy" receptor or activate IL-13-mediated fibrosis pathways [360].

1.9.3.2 Soluble IL-4 receptors

The soluble extracellular form of the IL-4R (sIL-4R) is produced by proteolytic cleavage of the membrane bound IL-4Rα [361] and appears to have opposing effects at different IL-4 concentrations. At high IL-4 concentrations, sIL-4R has a neutralizing effect by blocking binding of IL-4 to membrane bound receptors. In contrast, sIL-4R can prolong the half-life and enhance the bioactivity of IL-4 at low IL-4 concentrations, which has been convincingly shown in *in vitro* T-cell culture [362]. Given that sIL-4R has a lower affinity for IL-4 compared to its membrane bound counterpart, circulating IL-4 is protected from degradation and can be released in a delayed fashion [362]. Elevated sIL-4R has been observed in patients with leishmaniasis and malaria [363, 364]. Surprisingly, the opposite is observed in asthmatic children [365] and patients with allergic rhinitis [366] or pulmonary TB [367],

where sIL-4R levels were much lower compared to healthy controls. As such, further studies into the function of sIL-4R and its relevance in TB disease is required.

1.9.3.3 IL-4R dependent and independent signaling pathways

The most well characterized signaling cascade for inducing a Th2 response is the IL4R/STAT6 pathway [368]. STAT-6, bound to the intracellular domain of IL4Rα, becomes activated by Janus kinase (JAK1 and 3) mediated phosphorylation. As a result, STAT6 dimerizes and translocates to the nucleus where it binds directly to the Th2 transcription factor gene, GATA-3 thus facilitating IL-4 and IL-13 expression and subsequent Th2 differentiation [369]. Alternatively, IL-4/IL4R binding can initiate the insulin receptor substrate pathway (IRS; most commonly IRS-2) mediated by the phosphatidylinositol-3-kinase (PI3K) cascade. Murine studies suggest that both pathways work in conjunction to promote IL-4 induced proliferative responses [370].

Studies have shown that $STAT6^{-/-}$ mice are still able to induce Th2 responses [371] suggesting that alternative signaling pathways independent of IL4R/STAT6 can also contribute to Th2 differentiation. The Notch signaling pathway involves phosphorylation-mediated activation of Notch proteins (expressed by CD4+ cells) and subsequent upregulation of IL-4 and GATA3 genes, via RBPJ (recombination-signal-binding protein for immunoglobulin-κ J region) proteins [369]. Mice deficient in Notch 1, Notch 2 or RBPJ exhibited impaired Th2 responses to parasite antigens [372]. The mammalian target of rapamycin complex 2 (MTORC2) forms part of the IRS pathway and acts by downregulating suppressor of cytokine signaling 5 (SOCS5), an inhibitor of STAT6 activation [369, 373]. Finally, Th2 responses can be induced via an IL-2-induced STAT5 activation pathway [374], which is independent of GATA-3 and STAT-6 but requires support from IL-33 [375].

1.9.4 IL-4δ2

1.9.4.1 Alternative splicing

Alternative splicing is an important mechanism for regulating gene expression and promoting functional diversity within the eukaryotic proteome by the generation of multiple mRNA transcripts from a single gene [376]. The subsequent protein isoforms which are produced differ from their native counterparts in terms of structural and biological properties, including binding affinity, enzymatic activity, intracellular localization, stability and bioactivity [376]. In humans, over 90% of multi-exon genes are alternatively spliced. However, the splice variant isoforms are normally expressed at much lower levels (~7 times lower) compared to the full-length protein [377, 378]. In addition to IL-4, alternatively spliced variants of IL-2 [379], IL-6 [380], IL-7 [381], IL-15 [382] and IL-33 [383] have been described and, in some cases, function antagonistically to the wild type isoform [384].

1.9.4.2 IL-4δ2 structure and function

IL-4δ2 is a naturally occurring splice variant of IL-4 generated by deletion of exon 2, a 48 base pair region encoding a stretch of 12 amino acids (positions 22 to 37). The resulting protein structure lacks a double stranded anti-parallel β-sheet and a disulfide bond, due to deletion of a cysteine residue, but still retains the majority of the native IL-4 structure [385, 386]. The modification in structure means that IL-4δ2 only binds to the IL-4Rα chain and subsequently has a reduced binding affinity for the IL-4R [385].

In addition to humans, IL-4δ2 mRNA transcripts have been detected in non-human primates, rabbits, woodchucks, mice and cattle [387, 388] and was found to be expressed in various cell types and tissues [389-391]. However, data regarding IL-4δ2 protein is limited because assays using commercially available antibodies are unable to discriminate between IL-4 and IL-4δ2. Nonetheless, recombinant IL-4δ2 has been successfully expressed in yeast [47] and mammalian cell systems [32] and functional characterization has shown that it is an IL-4 antagonist suppressing IL-4-driven responses *in vitro*. IL-4δ2 inhibits IL-4-induced T-cell proliferation, CD23 and IgE expression in B-cells and blocks the IL-4-mediated inhibitory effects on COX-2 expression and PGE2 production in monocytes [47, 48, 392]. IL-4δ2 also

stimulated production of IFN-γ, IL-6, IL-10, MCP-1 and TNFα in cultured T-lymphocytes [31]. The *in vivo* effects of IL-4δ2 have been demonstrated in a murine gene delivery model where it induced inflammation independent of IL-4 causing T- and B-cell recruitment, collagen deposition and induction of IFN-γ suggesting IL-4δ2 was associated with a Th1 response [32, 393]. Luzina *et al* recently developed antibodies which were able to effectively discriminate between IL-4 and IL-4δ2 and demonstrated that IL-4δ2 was produced naturally as a functional protein by activated T-cells [31]. However, the predominant Th subset (Th1, Th2 or Th17) that produced the majority of IL-4δ2 could not be definitively identified [31].

1.9.4.3 IL-4δ2 in disease

IL-4δ2 has been investigated in systemic sclerosis [394], asthma [395], gastritis [389], transplantation [396] and tuberculosis [397], mostly at the transcriptional level. IL-4δ2 mRNA was upregulated in systemic sclerosis patients along with IL-4 probably because both IL-4 and IL-4δ2 have stimulatory effects on fibrosis [394], a characteristic feature of this disease. Asthma patients expressed significantly elevated levels of IL-4 mRNA whereas IL-4δ2 increased only slightly [395]. IL-4δ2 protein was also found to be increased in PMA stimulated T-cells of asthma patients compared to healthy controls [31]. Studies investigating IL-4δ2 in tuberculosis are discussed later.

1.9.4.4 Other IL-4 splice variants

In addition to IL-4δ2, other IL-4 splice variants have been described in humans and other mammalian species. IL4δ3, where exon 3 is deleted, has been described in rabbits [398] and cattle infected with *Fasciola hepatica* [388] or *M. bovis* [399]. IL4δ3 was found to be associated with protection in BCG vaccinated cattle infected with *M. bovis* [399]. Yuan *et al* demonstrated another splice variant, termed VIL-4, in cynomolgus macaques that induced expansion of Vγ2Vδ2 T-cells [400]. However, these variants have never been investigated in humans. Interestingly an IL-4 mRNA variant, IL-4alt3, was identified in human mononuclear cells where exon 3 was partially deleted but the function and significance of this splice variant in unknown [401].

1.9.5 Challenges in measuring of IL-4 and IL-4δ2

The biological properties of IL-4 often make its detection in biological samples difficult at the transcript and protein levels. IL-4 is active at very low concentrations and is commonly expressed ~3 logs below that of IFN-γ at the mRNA level [24, 25]. It also has a very short half-life (~60 minutes) [402] so any delays in sample processing, which is often the case under field conditions, can result in undetectable levels [26]. Furthermore, IL-4 protein is rapidly internalized via membrane bound receptors [403, 404] or sequestered by soluble receptors [405]. For these reasons, immunoassays such as ELISA and flow cytometry lack the sensitivity to detect levels of biologically active IL-4 [27] and cells often require mitogenic pre-stimulation, which is Th1 biased and may downregulate IL-4 responses [406]. These issues extend to the measurement of IL-4δ2 as well, which tends to be expressed at even lower levels compared to IL-4. In any case, IL-4 and IL-4δ2 cannot be distinguished using currently available immunoassays due to the lack of antibodies specific to each isoform. These factors may be the reason why earlier TB studies, which did not measure IL-4δ2, failed to observe differences in IL-4 expression or detect IL-4 altogether (discussed below). Currently, the most reliable and sensitive method involves quantitative PCR using specific primers that can amplify the mRNA sequences of each isoform separately.

1.10 IL-4 and IL-4δ2 in tuberculosis infection

1.10.1 Studies in active TB using blood

The literature relating to the measurement of IL-4 in human TB is substantial but results have been conflicting so the issue remains contentious. Early reports of increased IL-4 in TB came from studies conducted in the USA and UK [241, 407, 408]. IL-4 mRNA was detected in a proportion of TB patients by conventional RT-PCR [407, 408]. Surcel *et al* reported strong IL-4 ELISPOT responses in *M.tb* antigen stimulated PBMCs of pulmonary TB patients compared to controls [241]. Given the difficulty in measuring IL-4 (discussed in 1.9.5), it was interesting to find that a number of studies, mostly in developing countries, observed very high IL-4 levels in TB patients compared to controls, to the extent that it was detectable in the serum by ELISA [17, 409, 410]. In similar studies performed in India [243], Indonesia [21], The Gambia [411], South Africa [412] and Mexico [413], researchers observed that TB

patients expressed a higher proportion of IL-4 secreting cells in response to *M.tb* or non-specific antigen stimulation when measured by flow cytometry, which has a similar sensitivity to ELISA [414].

In later studies, the notion that a Th2 response may be related to TB became more apparent with the use of PCR techniques that were more sensitive and were also able to effectively distinguish between IL4 and IL-4δ2. In a UK study, Seah *et al* used a nested semi-quantitiative PCR assay to demonstrate that both IL4 and IL-4δ2 were increased in TB patients compared to TST positive controls [18]. Another UK study performed by Dheda *et al*, using quantitative PCR, also found that both IL-4 and IL-4δ2 was increased in TB patients compared to controls and in HIV infected TB patients compared HIV uninfected patients which correlated with disease severity [9, 415]. Similar results were reported in Ethiopia [28, 416] and The Gambia [29] where increased IL-4 and IL-4δ2 mRNA levels in TB patients were strongly associated with responses to the *M.tb* specific antigen, ESAT-6. Demissie *et al* also found that Ethiopians subjects expressed higher IL-4 compared to Danish subjects [28]. Subsequent studies from other research groups in high TB burden settings found a distinct pattern of increased IL-4 and IL-4δ2 which correlated with more severe disease and changed in response to anti-TB treatment [30, 288, 417, 418].

In contrast, some studies, mostly performed before the year 2000, failed to detect any differences in IL-4 expression between TB patients and healthy controls. Studies in the USA, Portugal and The Netherlands did not observe any changes in IL-4 levels when measured by RT-PCR [12, 37, 419], immunohistochemistry [34] or ELISA [35]. Similar findings were obtained in studies of TB patients from Mexico [33], China [420] and South Africa [36]. These conflicting results may be attributed to the technical difficulties in measuring IL-4 (discussed earlier). Furthermore, studies which used conventional PCR did not use primers to distinguish between IL-4 and IL-4δ2.

1.10.2 Studies in active TB using broncho-alveolar lavage

Systemic responses in pulmonary TB can differ significantly from those at the site of disease. For instance, the presence of specialized tissue specific cells with distinct morphological and functional characteristics and the migration of *M.tb*-specific immune cells to the site of infection results in a compartmentalized immune response that is not normally present in the peripheral circulation [38]. However, most studies tend to use peripheral blood because of its ease of acquisition. Consequently, only a handful of studies in pulmonary TB patients have investigated Th2 cytokine responses in the lungs [9, 11, 14, 15, 19, 20, 253, 421, 422] and even fewer have examined both compartments (blood and lungs) [9, 11, 15, 19]. Some studies failed to find differences in IL-4 in BAL samples by in situ hybridization [14], immunohistochemistry [422] or ELISA [253]. Schwander *et al* measured IL-4 protein in PPD stimulated PBMCs and alveolar lavage cells but failed to find differences between TB patients and healthy controls [15]. In contrast, other studies found significantly increased levels of IL-4 in the BAL of TB patients when measured by Luminex [11, 19] and qPCR [9]. Herrera *et al* observed increased IL-4 in *M.tb* supernatants of alveolar cells but not PBMCs [11]. Surprisingly, only one study demonstrated increased mRNA expression of IL-4 and IL-4δ2 in BAL and blood [9]. This study was performed in the UK and none, where both compartments were investigated for expression of the two isoforms, have been performed in a high TB burden setting.

1.10.3 Disease extent and response to therapy

In some studies, increased IL-4 levels were often observed in patients with more severe pulmonary TB, based on radiological and microbiological correlates of disease extent such as the presence and degree of cavitation [21, 421], unilateral *vs* bilateral disease [409], AFB sputum positivity [20], conversion of smear status [17] or a combination of these factors [9, 18, 19]. A higher proportion of IL-4 secreting cells were associated with cavitatory disease compared to non-cavitatory disease [21, 421]. Studies conducted in the UK [9, 18] and Ethiopia [19], using a chest X-ray scoring system which graded the extent of radiological involvement, found that increased IL-4 strongly correlated with disease extent. Finally,

Nolan *et al* observed that higher IL-4 levels in the BAL was found in smear positive compared to smear negative TB patients [20].

Researchers have also reported that IL-4 and IL-4δ2 levels change in response to anti-TB treatment. Most studies have found that IL-4 was significantly decreased at the end of treatment compared to the levels at diagnosis [9, 30, 288, 412, 417]. Siawaya *et al* showed that this decrease can occur as soon as one week after treatment initiation [30]. Studies which also measured IL-4δ2 (using quantitative/semi-quantitative PCR) found that expression of this isoform increased with treatment [9, 30]. In contrast, others found IL-4δ2 levels were unchanged [288] and even decreased following successful TB chemotherapy [417].

1.10.4 Contacts of TB patients and LTBI

As discussed previously, latent TB infection represents the majority of *M.tb* exposed individuals where the infection is established but contained by the host immune system and is characterized by IFN-γ responses to *M.tb* specific antigens such as ESAT-6. Expression of IL-4δ2 mRNA was increased in LTBI donors from Ethiopia and The Gambia but not TB patients suggesting that IL-4δ2 may be associated with a protective phenotype in *M.tb* infection [28, 29]. On the other hand, evidence suggests that IL-4 may also be involved in disease progression. A study in Portugal found that healthcare workers with pre-existing IL-4 responses to *M.tb* antigens *in vitro* went on to develop TB within 2–4 years [23]. Similarly, a Pakistani study found that increased IL-4 responses were associated with household contacts of index cases that developed disease compared to those that remained disease free within the same household [22]. It has also been suggested that the IL-4/IL-4δ2 ratio may serve as an indicator of disease progression [16].

1.10.5 What do increased IL4 and/or IL-4δ2 indicate?

A number of conclusions can be made from the literature presented above. Firstly, there is convincing evidence that IL-4 is upregulated in TB and that it correlates with disease susceptibility and immunopathology whereas the IL-4 splice variant, IL-4δ2 is associated

with protective responses. Secondly, IL-4 levels are raised to a greater extent in TB patients from countries close to the equator, which may be partly due to the Th2-priming adjuvant effect of helminth infection or exposure to environmental mycobacteria [423]. However, it is still unclear if IL-4 causes the immunopathology in TB or is merely a by-product of excessive inflammation. Indeed, evidence of a causal relationship has been demonstrated in a mouse model where adoptive transfer of Th2 cells in infected mice resulted in increased immunopathology [424]. However, whether this is also occurring in humans is unclear. A number of immune mechanisms known to be protective in TB are downregulated by Th2 responses and these are discussed in more detail in the next section.

1.11 Putative effects of a Th2 response on host immunity in TB

1.11.1 Induction of Th2 response by *M.tb*

M.tb has evolved a number of mechanisms by which it manipulates the host immune response to its benefit, allowing the bacteria to survive and, in some cases, undergo unrestricted growth. One such *M.tb* evasion mechanism may involve the induction of a Th2 response. There is ample evidence that IL-4 is increased in TB patients and certain *M.tb* specific components, such as ESAT-6, tend to drive IL-4 expression [28, 29]. Dwivedi *et al* demonstrated that the virulent H37*Rv* strain induces both Th1 and Th2 responses in murine DCs whereas BCG preferentially induces Th1 responses. Furthermore this effect was driven mainly by the RD1 proteins ESAT-6 and CFP-10 [211]. Induction of IL-4 by *M.tb* appears to be strain specific [425] and some studies suggest that virulent *M.tb* strains are strong inducers of IL-4 expression [426, 427]. BALB/c mice infected with highly virulent Beijing strains induced significantly higher IL-4 expression compared to less virulent strains [426]. Infection of human monocytes *in vitro* with Beijing isolates preferentially induced IL-4 and IL-13, through cellular interaction with various *M.tb* lipid components [427]. Additionally, the 16kDa heat shock protein of *M.tb* contains specific epitopes that are recognized by Th2 cells in TB patients [428].

1.11.2 Putative effects of IL-4 on the immune response to *M.tb*

A number of putative Th2 inducing mechanisms have been instigated in downregulating antimycobacterial mechanisms of protection and promoting immunopathology. Some of these mechanisms are discussed below and shown in figure 1.6.

1.11.2.1 Downregulation of a Th1 response

The protective role of a Th1 response, through the various effects of IFN-γ and TNFα on macrophage activation and other innate mechanisms, in TB is unquestionable. However, the relationship between a Th1 and Th2 response is antagonistic, in that these subsets cross-regulate each other so that one predominates over another [429]. For example, IFN-γ (Th1) inhibits murine Th2 cell proliferation whereas Th2 cells inhibit cytokine synthesis of Th1 cells [430]. Thus, increased IL-4 may very well lead to an undermining of Th1 immunity in TB by downregulating IFN-γ mediated responses. In leprosy, ineffective immunity is characterized by a Th2 profile whereas protective immunity is associated with a Th1 profile [431]. Indeed, the downregulatory effects of IL-4 on apoptosis, iNOS production and macrophage activation have been observed in TB models (discussed below). However, the caveat to this notion is that a Th1 response is usually dominant in TB and very high levels of IFN-γ are often observed in the lungs of patients despite exhibiting increased IL-4 responses. Thus, it is unlikely that a simple two way regulation occurs but rather that an IL-4 response is undermining host immunity within the prevailing Th1 environment [16].

1.11.2.2 Alternative activation of macrophages

Macrophages become polarized depending on the cytokine environment in much the same way as T-cells. Th1 cytokines, IFN-γ in particular, drive classical activation of macrophages (termed M1) resulting in increased pro-inflammatory responses and mycobactericidal activity through the production of iNOS, TNFα, IL-6 and IL-12. In contrast, IL-4 and IL-13 drive alternative activation of macrophages (termed M2) which have anti-inflammatory functions, characterized by increased expression of surface receptors MR and DC-SIGN as well as production of IL-4 and IL-10 [351]. The M2 phenotype has been shown to modulate immune

mechanisms that promote intracellular *M.tb* survival [432]. For instance, M2 macrophages increase the intracellular availability of iron, a key requirement for *M.tb* proliferation [433], through the upregulation of transferrin receptor expression [39]. Furthermore, downregulation of iNOS, a well described mechanism of intracellular *M.tb* killing in M1 macrophages, have been demonstrated in M2 macrophage of murine infection models [39, 40]. Kahnert *et al* found that IL-4 activated macrophages exhibited reduced iNOS expression but also increased arginase-1 expression which competes for L-arginine, the key substrate required by iNOS to produce NO [39]. Redente *et al* found that macrophages in *M.tb* infected mice underwent a switch from M1 to M2 polarization late in infection, characterized by high arginase-1 and low iNOS production [40]. In a similar murine model, Ito *et al* showed that the switch to M2 polarization was TLR-9 dependent [434].

1.11.2.3 Inhibition of autophagy and apoptosis

In addition to iNOS production, alternatively activated macrophages also have downregulatory effects on innate mechanisms of apoptosis and autophagy. TNFα induced apoptosis of infected macrophages is inhibited by virulent *M.tb* strains through the secretion of high levels of soluble TNF receptors which form inactive TNFα-TNF receptor complexes [147, 435]. IL-4 is thought to enhance this effect because it downregulates TNFα production in human monocytes and macrophages [41, 42] and increases the expression of soluble TNF receptors [41]. Indeed, infection of THP-1 cells with the virulent Erdmann strain of *M.tb* resulted in reduced expression of TNFα and the surface receptor TNFR1 when IL-4 was added exogenously [436]. Apoptosis inhibition may also occur by upregulation of an anti-apoptotic molecule Bcl-2, via the IL-4/STAT6 signalling pathway [370]. Additionally, Abebe and colleagues found that *M.tb* infected THP1 cells, in the presence of IL-4, induced significantly more necrosis than apoptosis compared to cultures without IL-4, suggesting that IL-4 may not simply inhibit apoptosis but rather preferentially promote necrotic cell death [436]. Indeed, virulent strains of TB can alter the balance of the lipid mediator eiconasoids in infected cells by inducing lipoxin A4 (LXA4; pronecrotic) and inhibiting prostaglandin E2 (PGE2; proapoptotic) resulting in reduced apoptosis and increased necrosis [207, 437]. Interestingly, IL-4 is known to downregulate PGE2 production in phagocytic cells [43, 438]

and this may represent another pathway by which IL-4 impairs apoptosis of *M.tb* infected cells.

Autophagy is considered a rescue mechanism initiated by the host immune response to counteract the *M.tb* mediated inhibition of phagolysosomal fusion [439]. However, evidence suggests that Th2 cytokines (IL-4 and IL-13) are able to block autophagy in infected macrophages via 2 separate pathways; IFN-γ induced autophagy is inhibited via the IL-4/STAT6 pathway while starvation induced autophagy is blocked via the IL-4/IRS-1 pathway [44]. In addition to blocking apoptosis, STAT-6 signaling induction of Bcl-2 also leads to sequestration and subsequent inactivation of Bcl-1, which is essential for autophagy induction [440, 441]. IL-4 induction of IRS-1 and Akt upregulates mTOR which inhibits autophagy induced by starvation [44, 442]

1.11.2.4 Promotion of Treg development

There is increasing evidence that Tregs can play a detrimental role in TB (discussed in 1.8.2.2.3). However, whether IL-4 is involved in driving the expansion and maintenance of Tregs is unclear. Evidence in both humans and mice suggests that Th2 cells are more resistant to Treg suppressive functions and IL-4 can promote the development of Tregs but data is conflicting. Pace *et al* showed that IL-4 preserves murine CD4+ T-cell proliferation in the presence of CD4+CD25+ Tregs and drives their proliferation via IL-4Rα signaling [443]. In humans, CD4+ and CD8+ Tregs generated from thymocytes abrogated Th1 proliferation but had a much lower suppressive effect on Th2 proliferation, which was reduced even further with the addition of IL-4 [444]. IL-4 also induced the generation of Tregs in an IL-10 and TGFβ independent but antigen-specific stimulation-dependent manner [303]. In contrast, Wei *et al* found that TGFβ induced Foxp3+ Treg development from naïve T-cells is inhibited by Th1/Th2 polarizing cytokines [445]. Similarly, the addition of IL-4 inhibited Treg mediated expression in an adoptive transfer murine model of allergic inflammation [302]. Despite these finding, there have been no studies investigating the link between IL-4 and Tregs in a model of tuberculosis.

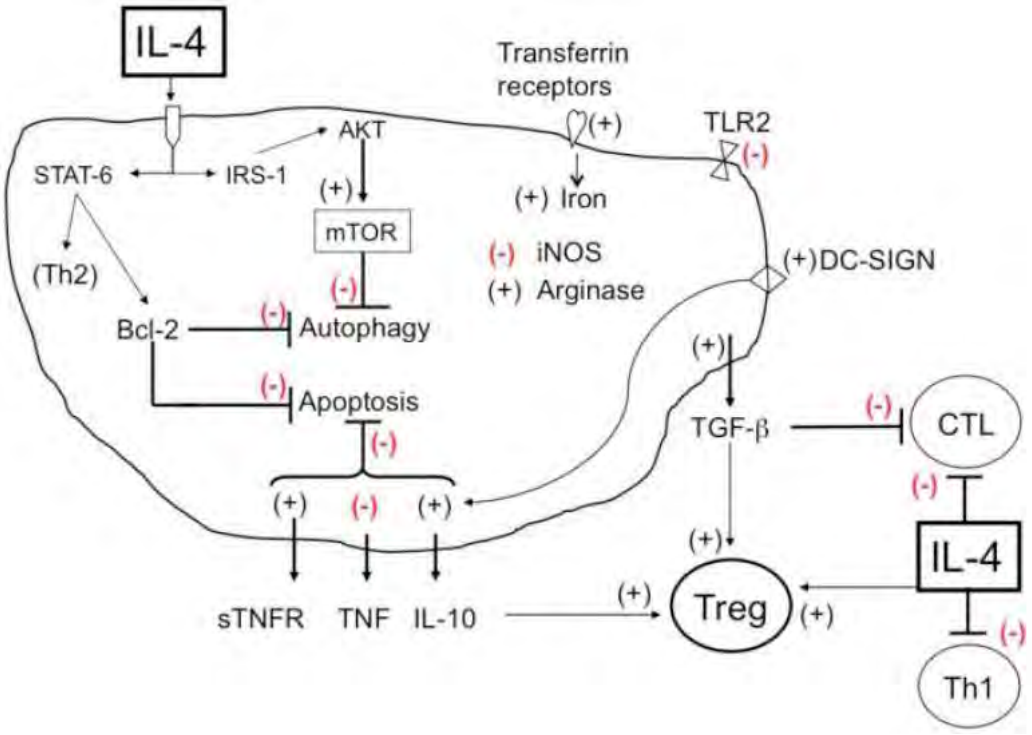


Figure 1.6. Possible pathways and mechanisms by which IL-4 subverts the protective host immune response to *M. tuberculosis*. Taken from Rook [439].

1.12 The implication of Th2 responses in TB vaccines

1.12.1 Inefficacy of BCG

BCG efficacy exhibits wide variability depending on the geographical location but its failure to confer protection against TB is most apparent in high TB burden developing countries where it is greatly needed [446]. Although there is not a definitive explanation for this phenomenon, individuals from these countries tend to have a background Th2 response against mycobacterial antigens. For example, Malawian BCG vaccinated infants induced significantly more IL-4, IL-13, IL-5 and IL-9 in PPD-stimulated blood cells, compared to matched BCG-vaccinated UK infants [447]. This notion is also supported by the observation that IL-4 is easily detectable in biological samples of TB patients from equatorial regions whereas more sensitive techniques and the use of prestimulation protocols are often required to observe measurable IL-4 levels in TB patients from developed countries [423]. It has been suggested that pre-exposure to non-tuberculous mycobacteria (NTM) and chronic helminth

infection may contribute to the reduced efficacy of BCG, which are common in these developing countries. Exposure to NTM may boost background levels of immunity which either blocks or masks the ability of BCG to induce a protective response [2, 448]. This may occur through the induction of a Th2 or regulatory responses. Vaccinated mice exposed to live *M. avium* via the oral route exhibited increased IL-4 levels and reduced BCG efficacy [449]. Also, pre-exposure to NTM reduced BCG efficacy in Gambian neonates via a mycobacterial-specific Treg mechanism and subsequent reduction in Th1 and Th17 responses [450]. A similar effect is likely to occur as a result of chronic helminth infections which are potent inducers of Th2 cytokines and regulatory T-cell responses. Helminth-infected Ethiopian subjects showed reduced immune responses to BCG compared to those treated with anti-helminthics [451]. Indeed, helminth infection can downregulate protective responses to *M.tb*. Filariasis patients showed exaggerated IL-4-specific memory responses and reduced IGRA responses compared to uninfected patients [452] and mice infected with *N. brasiliensis* exhibited impaired resistance to *M.tb* infection due to increased M2 macrophage polarization [453]. However, it should be noted these organisms can induce a Th1 response as well [454] and it may be the Th1/Th2 balance that is the crucial factor in determining efficacy.

These data suggest that failure of BCG in developing countries is due to the priming of a Th1+IL-4 mixed response by NTM exposure or the presence of helminth infections. The immunity gained through these environmental influences likely impairs the ability of BCG to replicate and mount a sufficient protective response. This background response may also explain why a high dose of *M.tb* is required to cause disease. Laboratory mice from developing countries which have previously been exposed to environmental mycobacteria, required ~10^5-10^6 organisms to overcome the background immunity and cause disease whereas pathogen free mice from countries such as the USA, develop disease at a much lower dose [455, 456]. In humans from these countries where a background mixed Th1+Th2 response exists, this partial immunity is able to control the low dose infection. In contrast, infection with a large dose or virulent IL-4-inducing *M.tb* strains may be initially controlled but eventually, the preexisting IL-4 (Th2) response will increase to a point where it can subvert Th1 mediated protective immunity and cause progressive disease [16, 256, 439].

1.12.2 Vaccine design in the context of a Th2 response

There are a large number of vaccine candidates currently in various stages of development and clinical trial phases which use preparations of inactivated whole cell extracts, *M.tb* genes in viral vectors, fusion proteins and live recombinant BCG or attenuated *M.tb* [457]. It is not feasible to test every candidate in large scale efficacy trials so it is of paramount importance to select the most promising ones at preclinical development or an early stage of clinical testing. Firstly, vaccine preparations should be tested in animal models within the context of helminth infection or NTM exposure, using a high dose challenge so as to mimic the situation in developing countries where a new vaccine is most needed [439]. Secondly, if IL-4 is indeed causing impairment of protective immunity, then it may be more appropriate to screen vaccines based on their ability to downregulate Th2 responses rather than just induce more Th1. Indeed, MVA85A induced strong IFN-γ ELISPOT responses but failed to be efficacious [3]. Such preparations may be useful as a prophylactic vaccine to prevent infection pre-exposure or as a therapeutic vaccine to supplement chemotherapy after development of active disease (Figure 1.7). Two vaccines candidates have been shown to downregulate Th2 responses with promising results in preclinical and clinical trials. The hsp65 DNA vaccine was found to be protective as a therapeutic vaccine in mice where it downregulated IL-4 and promoted CD8+ CTL and Th1 production [458]. In another murine model, the vaccine showed strong therapeutic effects against MDR- and XDR-TB [459]. Similarly, heat killed *M. vaccae* drives regulatory T-cells that target Th2 cells while simultaneously inducing CTL responses [460]. It has shown protective efficacy as a therapeutic vaccine in mice [461, 462] but its effect was more variable in human trials. It had limited or no additional benefit to standard therapy in trials performed in Southern Africa [463, 464]. In contrast, single or multiple doses were associated with bacteriological and radiological improvement compared to the placebo group in two other trials [465, 466].It has also shown some benefit as a multiple dose prophylactic vaccine in Tanzania [467].

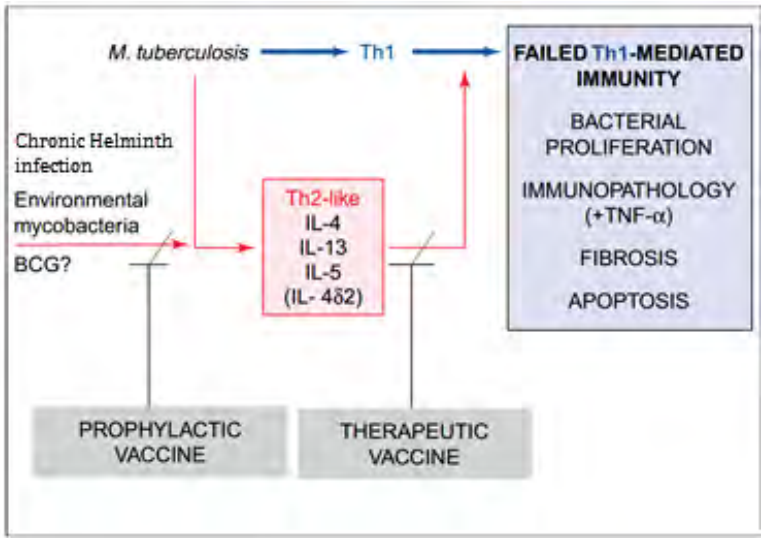


Figure 1.7. The role of the subversive Th-2-like response in progressive TB and its implications of vaccine design. Taken from Rook *et al*. [468].

1.13 Conclusion

The data outlined in Chapter 1 provides an outline of the immunological events during TB infection, the possible mechanisms of disease progression, biological attributes of IL-4 and IL-4δ2, a review of the literature regarding the role of IL-4 and IL-4δ2 in TB and how it relates to vaccine design.

However, there are still some issues regarding the IL-4 hypothesis that remain unclear. Indeed, the foremost question is whether IL-4 is causative or a consequence of TB disease in humans. The role of IL-4δ2 within this context also needs further clarification. Furthermore, the compartment specific expression of human Th1 and Th2 cytokines (lung and blood) in human TB, particularly in a high burden setting, and the interaction with other T-cells subsets has not been fully elucidated. The experiments performed in this thesis will attempt to clarify some of these topics.

2. CHAPTER 2: General methods and demographic data

2.1 Study Design and ethical considerations

The design of the study was cross sectional. The study was approved by the University of Cape Town Research Ethics Committee (UCT REC). Samples were obtained from participants undergoing bronchoscopy and venepuncture as part of previous and existing ethically approved studies being conducted at the Lung Infection and Immunity Unit, University of Cape Town. Informed consent was obtained from all study participants in accordance with UCT REC guidelines.

2.2 Participant selection and recruitment

2.2.1 Pulmonary tuberculosis patients

Pulmonary tuberculosis (TB) patients were recruited at part of ongoing studies from primary care clinics in and around the Cape Town area including Langa Clinic, Chapel Street Clinic, Gugulethu Clinic and Vanguard Clinic. All recruited patients were HIV uninfected, more than 18 years old and were not pregnant. TB diagnosis was microbiologically confirmed by sputum smear microscopy and/or culture at the time of recruitment. Auramine fluorescence microscopy and Bactec MGIT 960 liquid culture were performed by the National Health Laboratory Service as part of routine diagnostic testing. All patients were receiving anti-TB treatment for less than 2 weeks at the time of recruitment. Any patients with chronic immunosuppressive diseases, Th2-related conditions or receiving immunosuppressive medications were excluded from the study, as determined by a review of medical records and interviews conducted by the recruiting nurse. All inclusion criteria are listed in table 2.1.

2.2.2 Presumed latently infected controls

Presumed latently infected (LTBI) controls were defined as asymptomatic individuals with no previous history of TB and were both tuberculin skin test (TST) and interferon-gamma release assay (IGRA) positive. There is no reliable method to determine latent infection and,

in our opinion, these criteria best represented exposure to *M.tb* and subsequent control of infection. LTBI controls were close contacts of TB index cases, healthcare workers recruited from ongoing studies and laboratory personnel. All LTBI controls were HIV uninfected, more than 18 years old and not pregnant. As with TB patients, LTBI controls were excluded if they had any chronic immunosuppressing or Th2-related conditions. Inclusion criteria for LTBI controls are listed in table 2.1.

Table 2.1: Inclusion criteria for the recruitment of study participants.

Pulmonary tuberculosis patients (TB)	**Latent TB Infected controls (LTBI)**
• HIV uninfected • ≥18 years old • Not pregnant • Microbiologically confirmed diagnosis (sputum smear and/or TB culture positive) • ≤ 2 weeks of anti-TB chemotherapy • No concurrent immunosuppressive conditions (diabetes, malignancy) and not currently on immunosuppressive medications • No concurrent Th2 -related conditions (asthma, allergic rhinitis, autoimmune disorders, worm infestation	• HIV uninfected • ≥18 years old • Not pregnant • Asymptomatic • No previous radiological evidence of active TB • Tuberculin Skin Test (TST) positive • IGRA (Quantiferon Gold In-Tube) positive • No concurrent immunosuppressive conditions (diabetes, malignancy) and not currently on immunosuppressive medications • No current Th2-related conditions (asthma, allergic rhinitis, autoimmune disorders, worm infestation)

2.3 Clinical procedures

2.3.1 Venipuncture

Venipuncture was performed by a qualified research nurse. A maximum of 45ml whole blood was collected in 9 ml sodium heparin vacutainer tubes (Lasec) for PBMC isolation and other immunological assays. 2.5ml of whole blood was also collected directly into PAXgene blood RNA tubes (Qiagen) to preserve the mRNA profile for qPCR analysis (chapter 3).

2.3.2 Tuberculin skin test (TST)

The TST was performed on all potential LTBI controls according to standard protocols. Briefly, 0.1ml of tuberculin PPD RT 23 (Statens Serum Institute) was intradermally administered into the participant‟s forearm by a qualified research nurse. The induration size was measured 48-72 hours post-injection. The TST was considered positive if the induration size was >10mm.

2.3.3 Bronchoscopy

Bronchoscopy was performed on TB patients and LTBI controls as part on on-going immunological studies being conducted at the Lung Infection and Immunity Unit. Informed consent was obtained from all participants prior to participation in the study and were compensated according to local ethical policy. Prescreening blood tests were performed to identify any possible bleeding tendencies and a CXR were performed to exclude participants with other respiratory illnesses. Bronchoscopy was not performed if participants had any of the following: asthma, uncontrolled hypertension, ischaemic heart disease, diabetes, pregnancy and immunosuppression.

An experienced pulmonologist performed all bronchoscopy procedures. The participants‟ medical history, blood test results and CXR were reviewed to evaluate their suitability to undergo the procedure. Bronco-alveolar lavage (BAL) fluid was collected according to standard procedures and proper safety protocols were strictly followed. Briefly, after administration of lignocaine anesthaetic, the bronchoscope was passed through the airway

and the tip was wedged into the right middle lobe bronchus. A 300ml lavage using sterile saline, in 60 ml aliquots was performed under low suction (<20cm H_20). The lavage fluid was aspirated into a sterile glass container and then transported on ice directly to the laboratory for processing as described in section 2.4.3.

2.4 Laboratory Methods

2.4.1 Quantiferon Gold In-tube

A Quantiferon-Gold-In-Tube IGRA assay (Qiagen) was performed on all potential LTBI participants according to the manufacturer‟s instructions. Briefly, 3ml of blood was collected directly into Quantiferon blood tubes containing (i) no antigen (Nil control), (ii) *M. tb* specific antigens ESAT-6, CFP-10 and TB7.7 (TB antigen) and (iii) PHA (Mitogen control) and incubated at 37°C for 16 hours. After centrifugation of the tubes, the plasma was collected and used to determine the levels of IFN-γ (IU/ml) by ELISA, according to the manufacturer‟s instructions. An IFN-γ detection level of > 0.35 IU/ml was considered a positive result.

2.4.2 PBMC isolation

PBMCs were separated from whole blood by density gradient centrifugation as per standard protocols. Briefly, whole blood was centrifuged at 1800 rpm for 10 minutes and the plasma was removed. The uppermost layer of the remaining cells, which contained leucocytes („buffy coat“), was collected and mixed with 1xPBS (Lonza) in a 1:1 ratio. The entire suspension was layered on Ficoll Histopaque (Sigma-Aldrich) at a 3:1 ratio (cell suspension: ficoll) and centrifuged in a swing bucket rotor at 1800 rpm for 25 minutes with the centrifuge brakes turned off. The subsequent mononuclear cell layer was harvested, washed twice in 1x PBS, and re-suspended in growth media [RPMI 1640 containing glutamine (Lonza) and 10 % human AB serum (Western Province Blood Transfusion Services), 100U/ml penicillin/ streptomycin (Sigma-Aldrich) and 0.1% Fungin (Invivogen)]. Cells used in the mycobacterial containment assay were resuspended in growth media without penicillin/streptomycin. An aliquot of cells were stained using Turks solution and counted on

a Neubauer haemocytometer counting chamber. Cells were then seeded in tissue culture plates for various immunological assays (chapter 5-9).

2.4.3 BAL cell isolation

After the bronchoscopy procedure in the respiratory clinic (section 2.3.3), the BAL fluid was immediately transported on ice to the laboratory. The BAL fluid was filtered through sterile 2-ply gauze to remove any mucus and particulate debris. The filtered BAL fluid was then transferred to a 50ml tube and centrifuged at 1200rpm for 10 minutes. The BAL supernatant was removed and the resulting pellets were combined, washed twice in 1xPBS and resuspended in growth media. Cell viability was determined by Trypan blue exclusion staining and cells were counted using a Neubauer haemocytometer counting chamber. Cells were either stored in RLT lysis buffer (Qiagen) to preserve the RNA profile (qPCR analysis in chapter 3) or seeded in tissue culture plates and stimulated with TB antigens for downstream Luminex (chapter 8) or flow cytometry (chapter 9) assays.

2.4.4 Generation of monocyte derived macrophages

Monocyte derived macrophages were generated from PBMCs as previous described [293, 469]. PBMCs (isolated as described in section 2.4.2) were seeded at $2x10^6$ cells/well in a 96 well flat bottom plate or at $6x10^6$ cells/well in a 24 well plate for CFU counting and flow cytometric analysis in the mycobacterial stasis assay, respectively. The cells were incubated at 37°C with 5% CO_2 for 5 days to allow adherent monocytes to differentiate into macrophages. On day 3, wells were replenished with growth media. Non adherent cells were removed by washing with warm RPMI after 5 days. Adherent monocytes were presumed to be at $2x10^5$/well in the 96 well plate and $6x10^5$/well in the 24 well plate as ~10% of PBMCs are composed of monocytes [293, 470, 471].

2.4.5 Preparation of H37*Rv* stocks

H37*Rv* (donated by State Laboratory) was grown in Middlebrook 7H9 broth (Difco) supplemented with 10 % OADC (oleate-albumin-dextrose-catalase) enrichment media (BD

Biosciences) and 0.02% glycerol (Merck). Cultures were grown in a tissue culture flask (Corning) at 37°C and 5% CO_2 in a humidified incubator and shaken daily. Growth was assessed periodically until cultures reached mid-log phase. 1ml aliquots of culture were then snap frozen in 10% glycerol using liquid nitrogen and stored at -80°C.

Colony forming units (CFU)/ml, were determined using three randomly selected frozen H37Rv tubes. The bacterial suspension was homogenised by aspiration using a 1ml insulin syringe. 10-fold serial dilutions were made from the stock and the three lowest dilutions (10^2, 10^1, 10^0 CFUs) were plated in replicates of 6 onto Middlebrook 7H10 gridded agar plates (BD Biosciences) supplemented with 10% OADC. Plates were incubated in a 37^{o}C oven for 10-16 days or until visible colonies were formed. Colonies were counted using an inverted microscope and the CFU/ml for each tube was calculated using the flowing formula:

$$\left(\text{Number of colonies (Avg of 6 replicates)} \times 100 \times \text{Dilution factor}\right) = \text{CFU/ml}$$

2.4.6 Immunofluorescence staining and flow cytometry

Cells were stained with fluorescently labelled antibodies for analysis by flow cytometry (chapter 5, 7 and 9). Cells were harvested into 15x75mm 5ml polystyrene tubes (BD Biosciences), washed in 2ml FACS buffer (0.01% sodium azide, 10% human AB serum, 1xPBS) and centrifuged at 1200rpm for 5 minutes. The supernatants were removed and the cell pellet was resuspended in 100ul of FACS buffer. Appropriate fluorescently conjugated antibodies for cell surface markers were added at previously titrated volumes to the cells and incubated in the dark for 15 minutes at room temperature. Cells were then washed to remove unbound antibodies and fixed in 100µl of BD Cell Fix diluted 1:10 in H_2O (BD Biosciences) for 15 minutes in the dark. After another wash step, cells were permeabilized in 500ul PERM II solution diluted 1:10 (BD Biosciences) for 10 minutes in the dark. In panels measuring FoxP3 (chapter 7), fixation and permeabilization was performed using Human FoxP3 buffer set (BD Biosciences), according to the manufacturer‟s instructions. Appropriate fluorescently

conjugated antibodies for intracellular markers was added to each tube at previously titrated volumes and incubated for 30 minutes in the dark. The cells were then washed to remove unbound antibodies and 100ul of 4% paraformaldehyde solution was added to each tube for at least 30 minutes. All antibodies used in flow cytometry experiments are listed in table 2.2.

Flow cytometric analysis was performed using a BD LSRII Flow Cytometer (BD Biosciences). Fluorescence compensation was performed prior to data acquisition to eliminate any spectral overlap that may occur between fluorophores with similar emission spectra. BD compensation beads (BD Biosciences) were used to make compensation controls where the appropriate beads were stained with a single fluorescently conjugated antibody, according to the manufacturer‟s instructions. Voltages for each fluorescent channel was adjusted until the mean fluorescent intensity of the different cell population were equal.

Following data acquisition, the numbers of cells expressing the appropriate makers were determined using FACSDiva software (BD Biosciences) and expressed as a percentage of the parent population.

Table 2.2. List of fluorescently labeled monoclonal antibodies used for flow cytometry experiments.

Monoclonal antibody	Fluorescent conjugate	Source	Catalogue No	Used in
CD3	Alexa fluor 700	BD Pharmingen	561027	Used in all flow cytometry panels (Chapters 5,7,9)
CD4	APC H7	BD Pharmingen	560837	
CD8	PeCy5	Biolegend	300914	
CD19	PE	eBiosciences	12-0199-42	CD23 expression assay (Chapter 5)
CD23	APC	eBiosciences	17-0238-42	
CD14	PE Cy7	Biolegend	301814	Mechanisms of IL-4 mediated subversion of mycobacterial containment (chapter 7)
CD16	PE Cy7	Biolegend	302016	
CD25	PE	BD Pharmingen	557138	
IFNγ	PE	Biolegend	506507	
IFNγ	PeCy7	eBiosciences	505826	
TNFα	APC	BD Pharmingen	5513841	
DC-SIGN	PerCP	Biolegend	330110	
LC3B	Alexa 488	Cell Signalling Technologies	3868S	
FOXP3	Alexa fluor 647	BD Pharmingen	560045	
IL10	Alexa fluor 488	Biolegend	505013	
IL-9	PE	BD Pharmingen	560807	Expression of IL-9 in T-cells (chapter 9)
IL-17A	PerCP	eBiosciences	45-7179-42	
IL-13	FITC	R&D Systems Inc.	IC2131F	
PU.1	Alexa 647	Cell Signalling Technologies	2240S	

2.5 Statistical analysis

Data sets were evaluated for normality using the Shapiro-Wilk test. The Mann Whitney test was used to determine differences between participant groups and between the different biological compartments that were sampled. The Wilcoxon matched-pairs signed rank test was used to determine differences in samples before and after addition of recombinant IL-4. In all tests, a p-value of <0.05 was considered significant. Statistical analyses were performed using GraphPad Prim version 5.0 (GraphPad) and Microsoft Excel (Microsoft).

2.6 Demographics and clinical characteristics of study participants

Patient demographics and clinical characteristics of all participants are shown in table 2.3

Table 2.3. Demographic and clinical characteristics of study participants.

	Characteristics	Active pulmonary TB	Presumed LTBI	All Participants
	Number of participants (n)	51	45	96
Demographics	Median age in years (IQR)	35.0 (27.5-44.5)	39.5 (34.5-50.3)	39.0 (29.0-46.5)
	Sex (%)			
	Male	39 (79.6)	15 (33.3)	54 (57.4)
	Female	10 (20.4)	30 (66.7)	40 (42.6)
	Ethnicity (%)			
	Black African	40 (78.4)	27 (60.0)	67 (69.8)
	Mixed Ancestry	11 (21.6)	14 (31.1)	25 (26.0)
	European Descent	0 (0.0)	4 (8.9)	4 (4.2)
Clinical characteristics	Smear status (%)			
	Smear positive	36 (70.6)	-	-
	3+	10 (19.6)	-	-
	2+	6 (11.8)	-	-
	1+	20 (39.2)	-	-
	Smear negative	12 (23.5)	-	-
	Unavailable	3 (5.9)	-	-
	TB sputum culture (%)	51 (100%)	-	-
	≥1 previous episode of TB (%)	16 (31)	-	-
	Median length of time from start of treatment to recruitment in days (IQR)	5 (1-10)	-	-

3. CHAPTER 3: Expression of mRNA encoding IFN-γ, IL-4, and IL-4δ2 using cells from peripheral blood and the lungs

3.1 Introduction

Protective immunity to tuberculosis (TB) is known to require a Th1 response. Strong evidence comes from genetic studies in mice where mutations in the IFN-γ gene resulted in granuloma formation and disseminated growth of bacilli [228, 472]. In humans, genetic defects in the IFN-γ receptor 1 gene is associated with increased susceptibility to tuberculosis [473]. Despite strong Th1 responses in TB patients, particularly at the site of disease [9, 11-15], some exposed individuals still progress to active disease. Moreover, BCG and newer TB vaccine candidates, including MVA85A, have poor efficacy in developing countries despite inducing strong IFN-γ responses [112]. These observations suggest that this Th1 response is only part of the answer and there is some other underlying mechanism which, even in the presence of a potent Th1 response, is undermining TB immunity.

One hypothesis is that an underlying Th2 immune mechanism is subverting the protective Th1 response, leading to progressive TB. A number of studies have shown that the levels of IL-4, the principal Th2 cytokine, are elevated in TB and correlate with disease severity [9, 17, 18, 21]. In one study, increased IL-4 levels in healthcare workers were able to predict progression to active disease [23]. Furthermore, IL-4 has been shown to have deleterious effects on protective host response mechanisms in TB, including the downregulation of iNOS production [39, 40], autophagy [44] and apoptosis [41, 436] in macrophages as well as contributing to tissue damage and fibrosis [256].

The study of IL-4 is not meaningful without studying IL-4δ2, a naturally occurring alternatively spliced variant of IL-4 where exon 2 is deleted. IL-4δ2 is a functional antagonist of IL-4 and dampens the effects of IL-4 on T cell proliferation, CD23 expression on B cells and LPS-induced cycloxygenase-2 expression and prostaglandin E2 secretion in monocytes *in vitro* [47, 48]. IL-4δ2 mRNA has been detected in asthma [474] and is increased in scleroderma [394] suggesting it may have an inflammatory role. Only recently, it was found

that IL-4δ2 is not only expressed on a transcriptional level but is also produced as a protein *in vivo,* and is associated with the production of pro-inflammatory cytokines by T-lymphocytes including IFN-γ, IL-6 and TNF-α [31, 393]. Current data suggests that it is associated with a protective phenotype in TB as IL-4δ2 levels are increased in healthy controls compared to TB patients[28, 29, 416] and also increase following successful anti-TB chemotherapy [9, 30]. However, its precise role in relation to IL-4, in the host immune response to TB is not clear.

Only one study has investigated IL-4 and IL-4δ2 simultaneously in the peripheral blood and site of disease [9] but not in a TB endemic setting. In pulmonary TB, the study of cytokine expression in the lungs is important as it accurately reflects the innate and adaptive immune response to host-pathogen interactions [38]. Furthermore, accumulation and activation of immune cells at the site of infection can be significantly different from the peripheral circulation (reviewed in [38]). Thus, analysis of both compartments can provide clinically relevant insights into cytokine expression patterns.

The aim of this chapter was to determine the mRNA expression levels of IFN-γ, IL-4 and IL-4δ2 in cells obtained from peripheral blood and broncho-alveolar lavage (BAL) in TB patients and presumed LTBI controls using a validated quantitative PCR (qPCR) assay. qPCR was chosen for cytokine determination as it is more sensitive, compared to other conventional assay methods (ELISA), for measurement of low expressing cytokines. Furthermore, it is the only available method to distinguish IL-4 and IL-4δ2. Nonetheless, soluble levels of IL-4 and other cytokines were assessed in these samples and will be discussed in chapter 8.

3.2 Methods

3.2.1 Sample collection and processing

Whole blood and broncho-alveolar lavage (BAL) fluid was obtained from TB patients and presumed LTBI controls as described in sections 2.3.1 and 2.3.3, respectively. Approximately 2.5ml of whole blood was obtained from participants and immediately

transferred to a PreAnalytiX PAXgene Blood RNA tube (Qiagen) in order to preserve the mRNA profile. PAXgene tubes were incubated at room temperature for 16-24 hours to ensure complete lysis of blood cells, followed by storage at -80^{o}C. BAL cells were isolated from BAL fluid as described in section 2.4.3. A total of 350µl RLT lysis buffer (supplemented with β-mercapto-ethanol) from the RNeasy Plus Mini kit (Qiagen) was added to ~1-2x10^{6} BAL cells followed by storage at -80 ^{o}C.

3.2.2 Choice of primers, hydrolysis probes and amplification targets used for quantitative PCR

The primer and hydrolysis probes sequences used in this study were taken from published sequences in the literature [9, 288] and are shown in Table 3.1. The primers were designed to span exon-exon boundaries so as not to amplify genomic DNA and thus increase assay specificity. Furthermore, the design of intro-spanning primers was an absolute requirement to differentiate between the IL-4 and IL-4δ2 transcripts. Primer binding sites and amplification target size and locations are shown in Figure 3.1. Primers and probes were synthesized by IDT (Integrated DNA Technologies Ltd.) on a scale of 25nM and 100nM, respectively. Primers were purified by standard desalting whereas probes underwent HPLC purification. A fluorophore (FAM) and two fluorescent quencher (FQ) molecules (an internal ZEN FQ and IOWA BLACK FQ at 3‟-end) were incorporated into the hydrolysis probes. Primer sequence specificity was confirmed by melt curve analysis on the Rotor-Gene 6000 (Qiagen) using the Rotor-Gene SYBER Green PCR kit (Qiagen) assay, according to the manufacturer‟s instructions (Appendix; Figure A1).

Table 3.1: Primer and probe sequences, taken from the literature [9], to determine cytokine mRNA levels.

Gene of Interest (NCBI Accession numbers)	**Sequence** ○ Forward primer 5‟ - 3‟ ● Reverse primer 5 ‟- 3‟ ◊ Probe 5‟- 6-FAM/ZEN/IOWA BLACK FQ - 3‟	**Tm** (^{o}C)	**Product size** (bp)
HuPO	○ GCTTCCTGGAGGGTGTCC	58.0	
(NM_001002.3)	● GGACTCGTTTGTACCCGTTG	55.9	106
	◊ TGCCAGTGT/ZEN/CTGTCTGCAGATTGG	61.3	
IFN-γ	○ TTCAGCTCTGCATCGTTTTG	53.7	
(NM_000619.2)	● TCCGCTACATCTGAATGACCT	55.6	112
	◊ TGGCTGTTA/ZEN/CTGCCAGGACCCA	63.0	
IL-4	○ GCTGCCTCCAAGAACACAAC	56.8	
(NM_000589.3)	● TGTAGAACTGCCGGAGCAC	57.3	71
	◊ AAACCTTCT/ZEN/GCAGGGCTGCGAC	62.5	
IL-4δ2	○ CCTCACAGAGCAGAAGAACAC	55.4	
(NM_172348.2)	● TGTAGAACTGCCGGAGCAC	57.3	74
	◊ AAACCTTCT/ZEN/GCAGGGCTGCGAC	62.5	

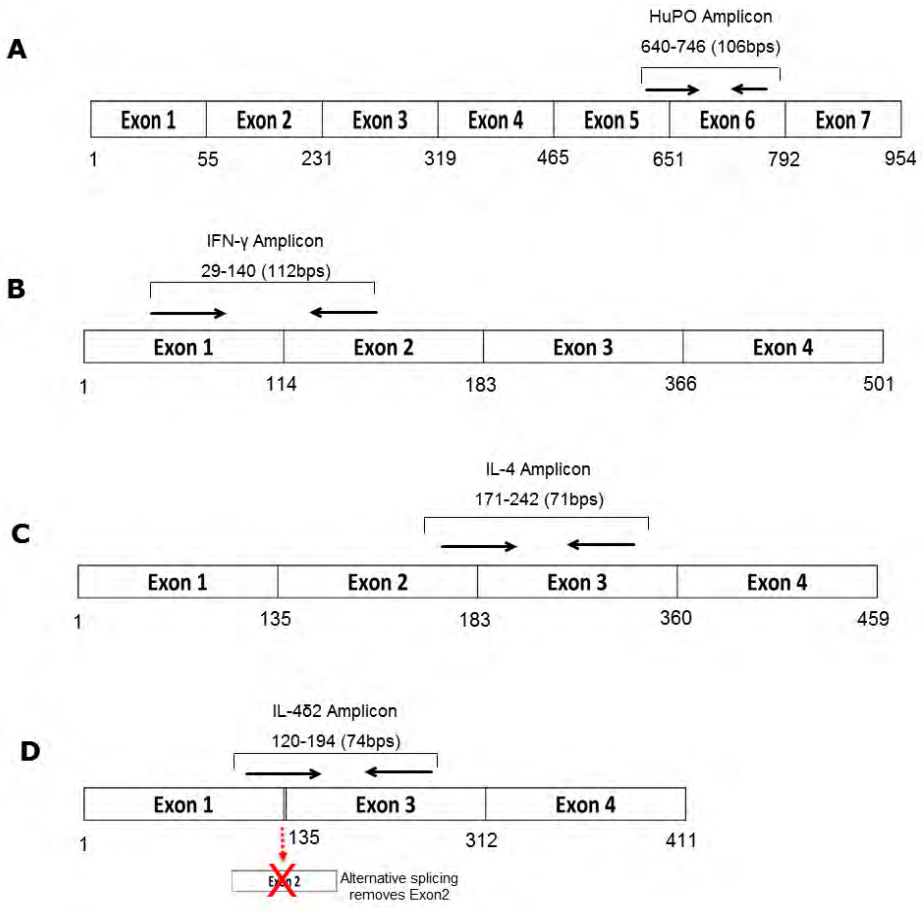


Figure 3.1 Primer binding sites and amplicon location and sizes for (A) HuPO (B) IFN-γ (C) IL-4 and (D) IL-4δ2. In (A), (C) and (D), the forward (sense) primer spans the exon-exon boundary. The numbers indicate the base pair position of the exon-exon boundary. The size of the amplicon is indicated in parentheses. In (C), the forward primer crosses the exon 2-exon 3 boundary for detection of full length IL-4. In (D), an alternative splicing event removes exon 2 in IL-4δ2 so the forward primer is designed to cross the exon1-exon3 boundary.

3.2.3 RNA extraction and assessment of RNA quality

RNA was extracted from whole blood in PAXgene tubes using the PreAnalytiX PAXgene Blood RNA kit (Qiagen), according to manufacturer‟s instructions. DNaseI treatment was performed to remove genomic DNA. RNA extraction from BAL cells was performed using the RNeasy Plus Mini Kit (Qiagen) according to manufacturer‟s instructions. Genomic DNA was removed using a gDNA eliminator column. All standard protocols for cleaning, handling

and storage during RNA isolation procedures were strictly followed. Quality (and quantity) assessment of isolated RNA was performed on an Agilent Bioanalyzer 2100 (Agilent) using the Agilent RNA 6000 Nano kit, according to manufacturer's instructions. Total RNA obtained ranged from 0.5-5µg. Samples were only used in reverse transcription reactions if an RNA Integrity Number (RIN) of ≥7.5 was obtained.

3.2.4 Reverse Transcription and qPCR

The iScript cDNA synthesis kit (BioRad) was used to convert RNA samples to cDNA, according to manufacturer's instructions. The kit uses a modified MMLV-derived RNase H+ transcriptase. RNA quantity limited the amount of input RNA to 500ng per reaction. A 20µl reaction was set up containing 500ng of sample RNA, reverse transcriptase, nuclease free water and iScript mastermix (containing a proprietary mixture of oligo(dT), random hexamers and RNase inhibitor). The reaction was performed in a G-Storm thermal cycler (G-Storm) using following conditions: 5 minutes at 25ºC; 30 minutes at 42ºC; 5 minutes at 85ºC. Appropriate control reactions containing no reverse transcriptase enzyme were also included.

Amplification of cDNA was performed on a Rotor-Gene Q real-time PCR machine (Qiagen). Reactions were set up manually using the Rotor-Gene probe PCR kit, according to the manufacturer's instructions. The 2x Rotor-Gene probe PCR Master Mix contained HotStar Taq *Plus* DNA Polymerase, dNTP mix and Rotor-Gene Probe PCR buffer (containing a proprietary mixture of Tris-Cl, KCl, NH_4Cl, $MgCl_2$ and Q-Bond). Primers were used at pre-validated concentrations of 500nM for HuPO and IFN-γ and 250nM for IL-4 and IL-4δ2. Probes were used at concentrations of 250nM for all analytes, as previously described [9]. 2µl of sample cDNA and 5µl of 10-fold serial dilutions of linearized plasmid standards (described in section 3.2.5) were added to the appropriate sample and standard tubes, respectively. The final PCR reaction volume was 20µl. The amplification conditions were as follows: initial step of 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Standards were run in triplicate and samples were run in duplicate. Appropriate no template controls (NTC) and no reverse transcriptase controls were includes in each qPCR run.

3.2.5 Method of mRNA quantification

Absolute quantification, where the mRNA copy number in a sample is expressed in relation to a standard curve containing known numbers of nucleic acid copies, was chosen as the method of mRNA quantification. It allows for greater control of inter-assay variation in PCR efficiency and experimental reproducibility. This is beneficial especially when large sample numbers are assayed over a period of time from different study sites [475]. As such, an external standard curve using recombinant DNA inserted into a plasmid was chosen because of its long term stability, high reproducibility and its ability to more appropriately mimic the length of native mRNA. Furthermore it shows higher sensitivity compared to recombinant RNA standards [476].

3.2.5.1 Generation of cDNA targets of interest

PBMCs from a healthy control were isolated (described in section 2.4.2) and $1x10^6$ cells were stimulated for 16 hours with 10µg/ml of phytohaemmaglutinin (PHA) in a 96-well round bottom plate. Total RNA was extracted and reverse transcribed using 1µg of RNA, as described in section 3.2.3 and 3.2.4. cDNA was amplified using the Kappa Taq PCR kit (Kappa Biosystems) and cloning primers (listed in Table A1 of the Appendix), according to the manufacturer‟s instructions. The cloning primers were designed to amplify a region of the cDNA containing the target qPCR sequence for IFN-γ and HuPO. The PCR reaction conditions were as follows: initial step of 95°C for 2 minutes, followed by 30 cycles of 95°C for 1 minute 30 seconds, 50°C for 1 minute, 72°C for 1 minute and a final extension step of 72°C for 10 minutes. The amplified PCR product was run on a 1% Agarose gel at 200V for 1 hour (described in section 4.2.1.3). The bands of interest were excised and purified using Zymoclean Gel DNA Recovery Kit (Zymo Research), according to manufacturer‟s instructions. IL-4 and IL-4δ2 cDNA containing plasmids were generated during cloning procedures to express their recombinant proteins (chapter 4).

3.2.5.2 Sub-cloning of cDNA into pGEM-T Easy plasmid

Purified cDNA was ligated into the pGEM-T Easy plasmid (Promega) at a vector:insert ratio of 3:1, transformed in JM109 chemically competent *E. coli* cells (Promega) and purified using the GeneJet Plasmid Miniprep kit (Thermo Scientific), according to the manufacturer‟s instructions (more detailed description in chapter 4). Plasmids were sequenced by Inqaba Biotech Ltd using an ABI 3500XL DNA sequencer to confirm the successful insertion of the target DNA into the plasmid.

3.2.5.3 Linearization and serial dilutions of the plasmid standards

The HuPO, IFN-γ, IL-4 and IL-4δ2 plasmids were linearized using *SacI* FastDigest enzyme (Thermo Scientific), according to the manufacturer‟s instructions. The plasmids were linearized because circular plasmid standards tend to overestimate copy numbers and reduce PCR efficiency [477]. Each plasmid was identified to have a single *SacI* restriction site outside the target cDNA sequence. Linearized plasmid concentrations were re-measured and the exact copy number per tube was determined using the following formula:

$$\left(\frac{\text{Amount of plasmid (ng) x Avogaro's constant}}{\text{length of plasmid x } 1*10^{9} \text{ x } 655}\right) = \textbf{copy number}$$

10-fold serial dilutions containing down to 10^1 copies were made in nuclease-free H_2O and added to appropriate PCR tubes (Qiagen) for qPCR amplification reactions.

3.2.6 Data Analysis and Normalization strategy

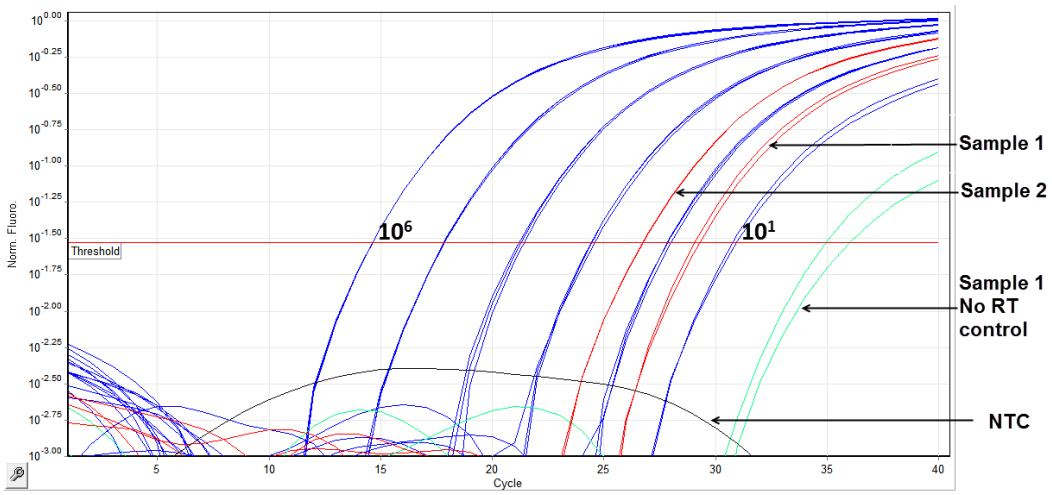
Data analysis was performed using the Rotor-Gene Q series software 2.0 (Qiagen). The cycle threshold (Ct) was automatically determined by the software for each qPCR run. Samples that were not reproducible (technical replicates with variations in Ct values >0.5 were considered outliers) or that were below the detection limit of the assay were given an arbitrary value of 1 copy in the final analysis, as previously described [9]. Copy numbers

were determined by absolute quantification and normalized using HuPO as a reference gene. The suitability of HuPO as a reference gene in pulmonary tuberculosis gene expression studies has been pre-validated [478] and used in similar study settings [30, 288]. Gene of interest copy numbers are expressed per million copies of HuPO. Coefficient of variance was used to assess inter- and intra-assay variation. Inter-group and inter-compartmental differences in cytokine mRNA expression were determined using Mann-Whitney U-test. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc.) and Microsoft Excel (Microsoft).

3.3 Results

3.3.1 Validation of qPCR assay

qPCR assay validation was performed on plasmid standards prior to analysis of samples. The limit of detection of the assay was determined to be 10 copies as the replicate Ct values below 10 copies were very variable (difference in Ct replicates >0.5). In most experiments, PCR efficiency approached 100% and R^2 value was >0.99. No transcripts were detectable in the „no template" controls (NTC) or in the „no reverse transcriptase" controls (no RT controls) indicating a lack of genomic DNA contamination. The coefficient of variance between technical replicates within a single assay (intra-assay variation) was <~20% at each concentration. Figure 3.2 shows an example of IL-4 copy number determination by absolute quantification in a validated qPCR assay. PCR efficiency, copy numbers and Ct values were measured in 5 separate experiments and compared in order to assess inter-assay variation. The mean PCR efficiency was 100% (95% CI; 96.1-103.9) and the coefficient of variance at each dilution was <10% (Figure 3.3A). Inter-assay variations in Ct values are shown in figure 3.3B.



Name	Type	Ct	Given Conc	Calc Conc	% Var
10^6	Standard	14.6	1,000,000	1,020,782	2.10%
10^6	Standard	14.62	1,000,000	1,010,603	1.10%
10^6	Standard	14.61	1,000,000	1,011,025	1.10%
10^5	Standard	17.83	100,000	105,806	5.80%
10^5	Standard	17.85	100,000	104,097	4.10%
10^5	Standard	17.84	100,000	105,145	5.10%
10^4	Standard	21.18	10,000	10,097	1.00%
10^4	Standard	21.26	10,000	9,527	4.70%
10^4	Standard	21.4	10,000	8,669	13.30%
10^3	Standard	24.42	1,000	1,037	3.70%
10^3	Standard	24.57	1,000	934	6.60%
10^3	Standard	24.41	1,000	1,048	4.80%
10^2	Standard	27.83	100	95	5.00%
10^2	Standard	28	100	85	15.50%
10^2	Standard	27.84	100	94	6.10%
10^1	Standard	30.94	10	11	7.20%
10^1	Standard	30.76	10	12	21.00%
Sample 1	Unknown	29.28		34	
Sample 1	Unknown	29.05		40	
Sample 2	Unknown	26.73		205	
Sample 2	Unknown	26.75		203	
sample1 noRT	Unknown	35.98		0	
sample1 noRT	Unknown	34.93		1	
NTC	NTC				

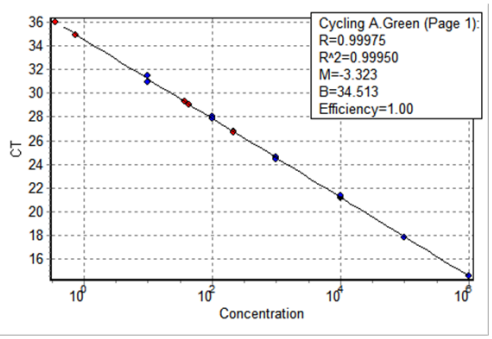


Figure 3.2. Generation of IL-4 standard curve to determine unknown values by absolute quantification method using Rotor-Gene Q software. Serial 10-fold dilutions of IL-4 plasmids standard (blue) and 2 unknown samples (red) were amplified in a qPCR assay. No reverse transcriptase (no RT; green) controls and no template controls (NTC; black) and were also included. The fluorescence plot (top) was used to generate the standard curve (bottom right). PCR efficiency was 100% and R^2 value was 0.9995 for this assay. The calculated copy numbers (Calc conc) for the samples (red), no RT control (Green) and NTC (black) derived from the standard curve are shown in the adjacent table. The variance (%Var) for individual standards was <22% and coefficient of variance (intra-assay variation) for each dilution was <20%. Both controls did not have any detectable copies.

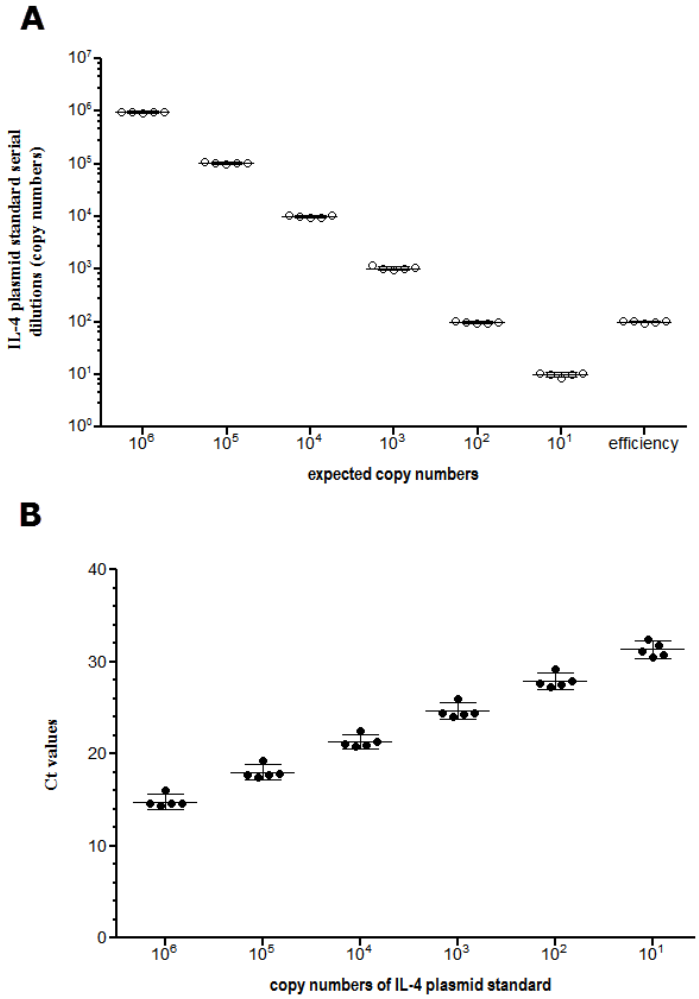


Figure 3.3. Inter-assay variability in (A) copy number and (B) Ct value of quantitative PCR (qPCR) assay to determine IL-4 plasmid standard copy number. qPCR amplification was performed on serial 10-fold dilution of linearized IL-4 plasmid standard in five separate runs. Each circle represents a single experiment. The x-axis show the expected copy numbers and the y-axis show the expected copy numbers in (A) and Ct values in (B). Lines and error bars represent mean values and 95% confidence intervals, respectively. In (A), the coefficient of variance at each dilution and for PCR efficiency (mean 100.0; 95% CI 96.1-103.9) was <10%.

3.3.2 mRNA expression levels of IFN-γ, IL-4 and IL-4δ2 in TB patients compared to presumed LTBI controls in BAL and peripheral blood

Messenger RNA (mRNA) levels in peripheral blood were assessed in 23 tuberculosis (TB) patients and 22 presumed latently infected (LTBI) controls, after RNA samples with low RIN values were excluded. BAL cells (BAL) were obtained from 8 TB patients and 7 presumed LTBI controls.

There were no significant differences in IFN-γ mRNA expression levels (per 10^6 copies of HuPO) between TB patients compared to LTBI controls in the peripheral blood or in BAL (Figure 3.4A). However, median IFN-γ expression levels in BAL was ~6-fold higher compared to peripheral blood in both TB patients (6783 vs. 1721, respectively; p=0.005) and LTBI controls (6281 vs. 1871, respectively; p=0.02).

TB patients had significantly higher peripheral blood IL-4 mRNA expression levels compared to LTBI controls (126 vs. 42; p=0.02) but these differences were not observed in BAL (Figure 3.4B). In contrast to the observed IFN-γ expression profile, median IL-4 mRNA expression levels were significantly reduced in BAL compared to the blood in both TB patients (126 vs. 10, respectively; p=0.003) and LTBI controls (42 *vs*. 3, respectively; p=0.005).

There were no observed differences in IL-4δ2 expression between TB patients or LTBI controls either in BAL or peripheral blood (Figure 3.4C). TB patients did have slightly higher IL-4δ2 levels in the blood compared to BAL (6 *vs*. 1, respectively; p=0.04) but no other differences in IL-4δ2 expression levels were observed between the two different compartments. Median expression levels of IL-4δ2 were generally low and expression in some samples, particularly from BAL, was below the detection limit of the assay. Median cytokine expression levels are shown in Table 3.2.

When TB patients were stratified by smear grade, there were no significant differences in IFN-γ or IL-4 levels (IL-4δ2 not analyzed due to low expression) between smear negative

and the smear positive samples (+1 to +3) in peripheral blood (Appendix; Figure A2). Gene expression in BAL was not analyzed by smear grade due to the small sample numbers.

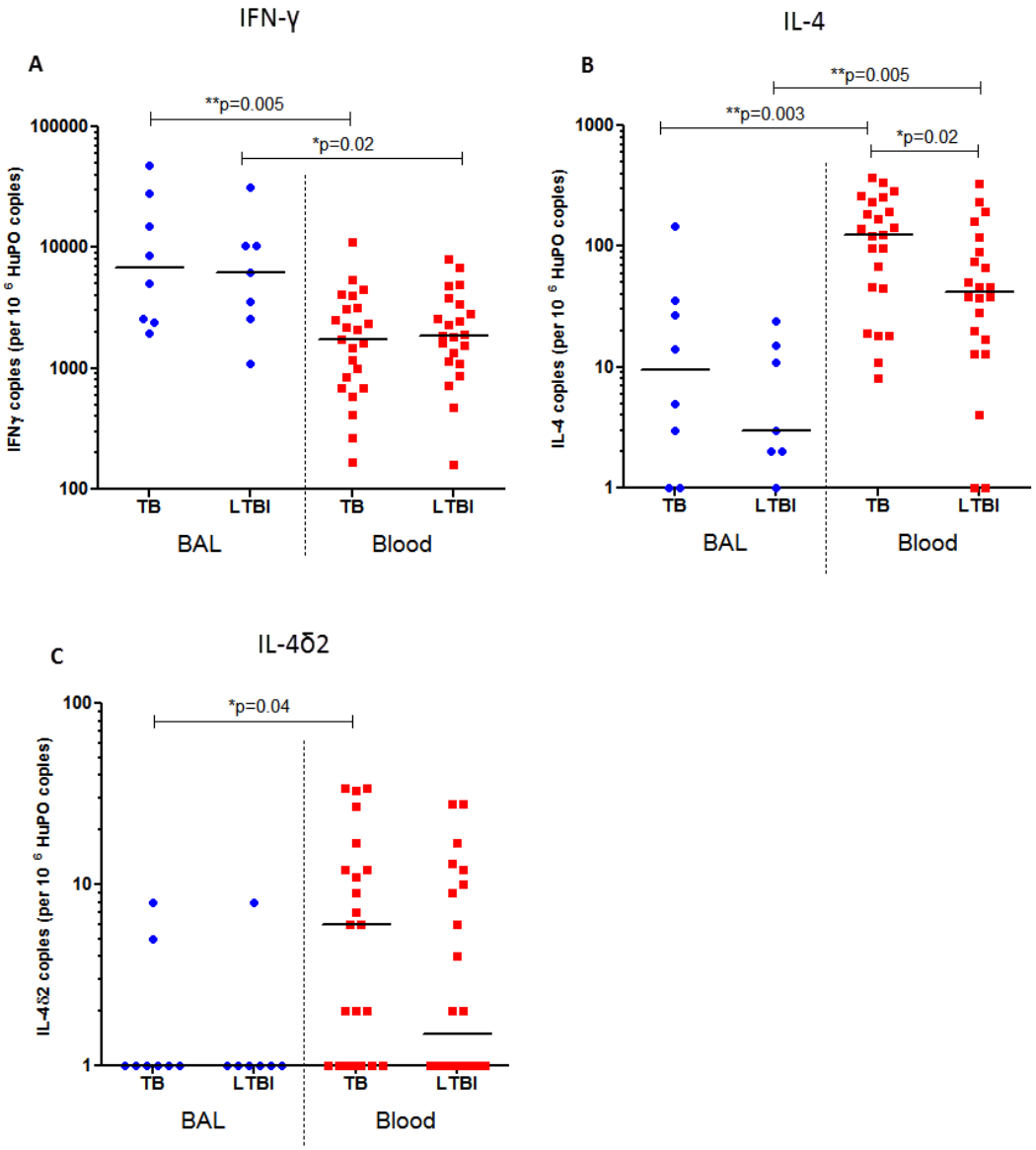


Figure 3.4. mRNA expression levels of (A) IFN-γ (B) IL-4 and (C) IL-4δ2 in cells of broncho-alveolar lavage (BAL; blue circles) and peripheral whole blood (Blood; red squares) from patients with pulmonary tuberculosis (TB; BAL n=8, Blood n=23) and presumed latently infected controls (LTBI; BAL=7, Blood n=22) as measured by quantitative real-time PCR. Data is shown on a $\log_{10}$ scale and copy numbers are expressed per million copies of HuPO. Statistical analyses between groups were performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

3.3.3 IFN-γ:IL-4 and IL-4δ2:IL4 expression ratios in BAL and whole blood of TB patients and presumed LTBI controls

The ratio of IFN-γ:IL-4 expression, thought to represent the Th1:Th2 balance, was significantly lower in peripheral blood of TB patients compared to LTBI controls (22 vs. 53; p=0.01). In BAL, there were no significant differences in the IFN-γ:IL-4 between TB patients and LTBI controls (999 vs. 1098, respectively; p=0.78) (figure 3.5A). When the different compartments were compared, the median IFN-γ:IL-4 ratios were significantly higher in BAL compared to peripheral blood of TB patients (p=0.0001) and LTBI controls (p=0.0001), which was mainly attributable to the very high expression levels of IFN-γ in BAL. There were no differences in the IFN-γ:IL-4 ratio when TB patients were stratified by smear grade (Appendix; Figure A2).

There were no significant differences in the IL-4δ2:IL4 ratio in BAL or whole blood of TB patients compared to LTBI controls. Similarly, no differences were detected when the different compartments (BAL *vs*. peripheral blood) were compared in TB patients or LTBI controls.

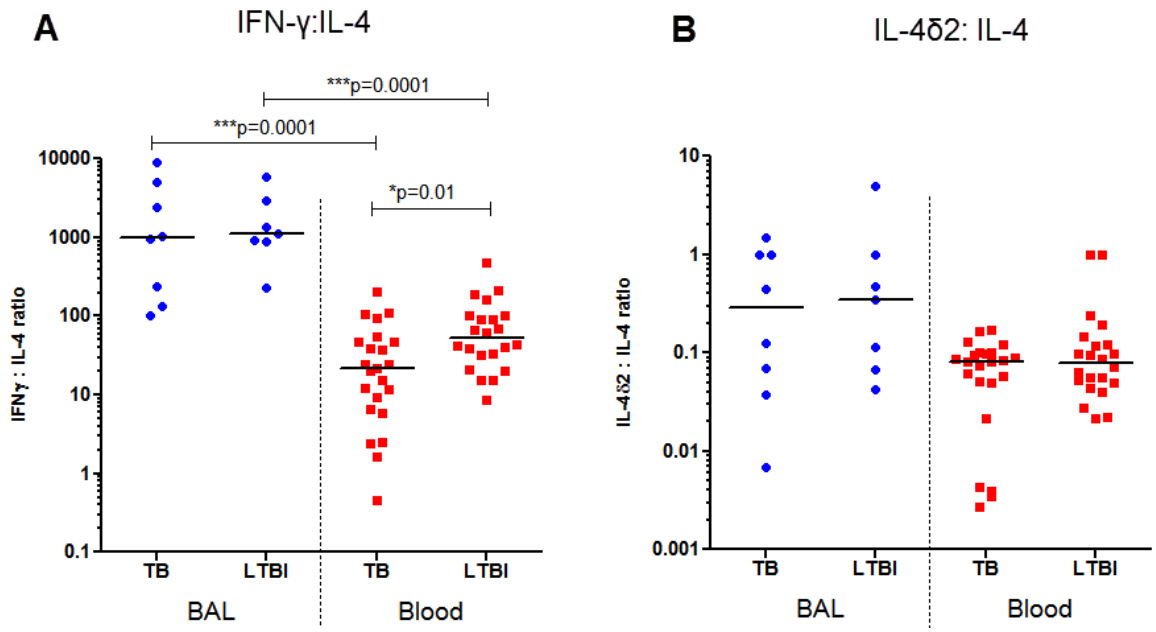


Figure 3.5. Cytokine mRNA expression ratios of (A) IFN-γ:IL-4 and (B) IL-4δ2:IL-4 in cells of broncho-alveolar lavage (BAL; blue circles) and peripheral blood (Blood; red squares) from patients with pulmonary tuberculosis (TB; BAL n=8, Blood n=23) and presumed latently infected controls (LTBI; BAL=7, Blood n=22) as measured by quantitative real-time PCR. Data is shown on a $\log_{10}$ scale. Statistical analysis between groups was performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

Table 3.2. Median cytokine mRNA expression levels and ratios of mRNA levels in broncho-alveolar lavage (BAL) and peripheral blood of tuberculosis (TB) patients and presumed latently infected controls (LTBI) as measured by quantitative real-time PCR. mRNA levels are expressed as median copies per 10^6 copies of HuPO and interquartile range (IQR) in parentheses. Statistical analysis between groups was performed using the Mann-Whitney test and a p-value of <0.05 (bold) was deemed significant.

mRNA Analyte	BAL		Blood		p-value
	TB (n=8)	LTBI (n=7)	TB (n=23)	LTBI (n=22)	
IFN-γ Median copy number (IQR)	6783 (2452-24918)	6281 (2570-10438)	1721 (692-3161)	1871 (1131-3521)	[a]p= 0.87 [b]p= 0.53 **[c]p=0.005** **[d]p=0.02**
IL-4 Median copy number (IQR)	10 (2-34)	3 (2-15)	126 (45-232)	42 (16-98)	[a]p=0.49 **[b]p=0.02** **[c]p=0.003** **[d]p=0.005**
IL-4δ2 Median copy number (IQR)	1 (1-4)	1 (1-1)	6 (1-12)	2 (1-11)	[a]p=0.74 [b]p=0.30 **[c]p=0.04** [d]p=0.10
IFN-γ:IL-4 Median ratio (IQR)	999 (161-4344)	1098 (877-2927)	22 (7-47)	53 (29-101)	[a]p=0.78 **[b]p=0.01** **[c]p=0.0001** **[d]p=0.0001**
IL-4δ2:IL-4 Median ratio (IQR)	0.29 (0.05-1.0)	0.34 (0.07-1.0)	0.08 (0.05-0.1)	0.07 (0.05-0.12)	[a]p=0.86 [b]p=0.54 [c]p=0.09 [d]p=0.08

p-values compare the median copy number/ratio in [a]TB BAL *vs.* LTBI BAL, [b]TB blood *vs*. LTBI blood, [c]TB BAL *vs*. TB Blood, [d]LTBI BAL *vs*. LTBI Blood

3.4 Discussion

The cytokine mRNA expression levels of IFN-γ, IL-4 and IL-4δ2 were determined in the peripheral blood and BAL of TB patients and LTBI controls using a validated qPCR assay. In peripheral blood, IL-4 mRNA levels were significantly higher in TB patients compared to LTBI controls but no differences were observed in IFN-γ or IL-4δ2 expression levels. In BAL, there were no significant differences in the mRNA expression of IFN-γ, IL-4 or IL-4δ2 between TB patients and LTBI controls. When the different compartments within the same group were compared, higher IFN-γ and lower IL-4 was observed in the BAL compared to peripheral blood in both TB patients and LTBI controls. Significantly lower IL-4δ2 expression was also observed in the BAL compared to the blood of TB patients but not in LTBI controls. Overall, IL-4δ2 expression levels were very low. The IFN-γ:IL4 ratio in peripheral blood was significantly lower in TB patients compared to LTBI controls. No differences were observed in the IL-4δ2:IL-4 ratio between the two groups.

3.4.1 Inter-group and compartment-specific differences in cytokine expression

3.4.1.1 IFN-γ

There is extensive evidence confirming the protective role of IFN-γ in TB (discussed in section 3.1). IFN-γ, produced by activated T-cells, natural killer (NK) cells and macrophages, exerts its mycobactericidal activity by activating macrophages and inducing phagosomal maturation, autophagy [158] and the production of reactive nitrogen and oxygen intermediates [235, 479]. It also plays a role, together with TNF-α, in granuloma formation [68].

In the data presented here, there were no differences in IFN-γ mRNA expression levels between TB patients and presumed LTBI controls either in the peripheral blood or BAL, which is consistent with other gene expression studies using unstimulated cells [9, 18, 417, 480]. Conversely, other studies have found either lower [33, 243-247] or higher IFN-γ levels [9, 11, 238-240, 242] in TB patients compared to controls. These discrepancies most likely reflect differences in the study geographical location or the experimental design, including the use of cell pre-stimulation protocols and method of detection (qPCR vs. ELISA or flow

cytometry). Indeed, recall responses of T-cells to *M.tb* antigens shows a decrease in IFN-γ levels in TB patients compared to healthy controls ([33, 246, 247] and results reported in chapter 8). Disease severity may also play a role as patients with more extensive disease are associated with higher IFN-γ production and levels decrease after anti-TB treatment [35, 240, 481]. Few studies have directly compared IFN-γ expression in the periphery and site of disease in a high burden setting [11, 15, 19]. While I found no inter-group (TB *vs.* LTBI) differences, there was a six-fold increase in IFN-γ expression in the lungs compared to the peripheral blood. Peripheral blood is commonly used to measure cytokine responses because samples can be easily acquired, but may not reflect the immune response at the site of infection [8, 38]. High IFN-γ levels at the disease site is not uncommon and has been previously shown in the BAL [9, 11, 14, 15], pleural fluid [13, 482, 483] and lymph nodes [12] of TB patients. These results, and data presented in this chapter, may reflect the accumulation of IFN-γ-producing effector cells to the site of tissue inflammation, through both active recruitment and local expansion of T-cells. As a result, there is a subsequent decrease in peripheral circulating T-cells [8, 38]. These results suggest that TB patients are still able to produce a potent Th1 response, particularly at the site of disease, and there may be another underlying mechanism that is causing disease progression.

3.4.1.2 IL-4

I found significantly higher IL-4 mRNA expression in peripheral blood of TB patients compared to LTBI controls. IL-4, the prototype Th2 cytokine, is traditionally considered an anti-inflammatory cytokine that can down-regulate the protective Th1 response, but evidence suggests that it may also have immunopathological consequences in disease [16]. For instance, its role in asthma-associated airway inflammation is well described [484-486]. In the last two decades, there has been an increasing body of evidence that a Th2 response is associated with *M.tb* disease. Indeed, despite a strong Th1 response in progressive TB, IL-4 levels are found to be increased compared to healthy controls [9, 29, 487] and correlate with disease severity [9, 418, 488]. Furthermore, these levels decrease following anti-TB treatment [9, 30, 417]. The data presented here is in agreement with a number of studies [9, 30, 288, 417]. However, some earlier studies failed to detect any differences in IL-4 levels

[12, 33, 35, 419]. These failures may reflect the use of insensitive detection methods. IL-4 protein levels, as measured by Luminex, in antigen driven culture supernatants approached the detection limit of the assay (Chapter 8). IL-4 detection is challenging, even in Th2 driven diseases, as it is physiologically active at concentrations 3 logs below that of IFN-γ [24, 25]. Additionally, the short half-life of IL-4 [9, 402, 489, 490], the rapid cellular internalization of IL-4 [403, 404], the sequestration of IL-4 by soluble IL-4 receptors [405] and the use of pre-stimulation protocols that favor a Th1 response [406] also contribute to the difficultly in measuring IL-4. Furthermore, many of these studies failed to distinguish IL-4 and IL-4δ2, which can have significant effects on study conclusions [30]. As such, I chose a probe-based quantitative PCR approach to measure IL-4 because of its superior sensitivity and the fact that it is currently the only reliable method to distinguish IL-4 and IL-4δ2.

Investigation of the lung compartment revealed marginally higher IL-4 levels in the BAL of TB patients compared to LTBI controls but these levels were not significant. However, IL-4 mRNA expression levels were significantly lower (~12-fold) in BAL compared to peripheral blood. Few human studies have investigated IL-4 levels in both the peripheral blood and the site of disease [9, 11, 482]. Barnes *et al* [482] found a comparable relationship in pleural TB, where IL-4 levels were significantly higher in the blood compared to pleural fluid. Conversely, other investigators found similar levels of IL-4 in both peripheral blood and BAL [9, 11]. In addition to reasons discussed previously, these discrepancies in compartment-specific IL-4 expression profiles may be explained by differences in Th1 and Th2 cellular and cytokine trafficking. Indeed, the high IFN-γ levels seen in BAL is likely due to Th1 cellular recruitment to the site of disease [8, 491] and these CD4+T-cells tend to express high levels of the Th1 homing receptors CXCR3 and CCR5 [492]. However, less is known about the extent of Th2 cell migration to the site of infection. One possible explanation may be that the recruitment of Th1 cells to the lungs results in fewer of these cells circulating in the periphery. As a result, this allows for a greater expansion of the IL-4 producing T-cell population in the peripheral blood.

Another reason for these observed differences is the uncertain contribution of other cell types producing IL-4. In pulmonary TB, IL-4 appears to be predominantly expressed by T-cells,

but production by non T-cells can be significant [9]. Alveolar macrophages remain the most abundant cell type at the site of infection [493], despite substantial lymphocytic infiltration into the alveolar space. Macrophages normally polarize to an M1 phenotype (classical activation), and produce IFN-γ [494], early in TB disease [495] but M2 (alternative activation) polarization, driven by IL-4, also occurs in some TB patients [493]. IL-4 can be produced by these M2 macrophages [391] but the frequency of these cells early in disease and the extent to which they produce IL-4 is unclear. Other IL-4 producing cell types, including mast cells, neutrophils and eosinophils, have also been shown to migrate to the lungs in both murine [192, 496] and human TB [183, 497]. However, lung mast cells produce very little IL-4 [498] and the contribution of neutrophils and eosinophils to IL-4 production has not been fully elucidated. Given these data, it is possible that the proportion of cells in the lung lavage, obtained by bronchoscopy, contain fewer IL-4 producing cells compared to the blood and result in the observed lower mRNA expression in BAL.

Other factors that may have played a role in the low IL-4 expression levels in the BAL include the time of recruitment. IL-4 levels tend to be highest at the onset of disease but as little as 1 week of therapy can significantly reduce IL-4 levels [30]. Furthermore, cytokine detection will also be dependent of the level of inflammation and disease extent in the lung section that is lavaged [499].

My findings of elevated IL-4 levels in TB patients compared to controls, in the presence of a potent Th1 response, does favour the hypothesis that a dysregulating Th2 response may be undermining immunity to TB.

3.4.1.3 IL-4δ2

This is the first study to investigate the levels of IL-4δ2 in both the lungs and the peripheral blood in the context of a TB endemic setting. Most TB studies investigated IL-4δ2 in peripheral blood [30, 288, 397, 417] and only one, conducted in the UK, compared IL-4δ2 expression levels in both BAL and blood of pulmonary TB patients and controls [9]. These studies found either increased levels [9, 418] or no difference in expression between the two

groups [288, 417]. Studies, which measured IL-4δ2 mRNA pre- and post-treatment, found these levels to increase after therapy [9, 30, 417]. My study did not find any significant differences in IL-4δ2 between TB and LTBI either in the BAL or blood. However, IL-4δ2 was significantly higher in blood compared to BAL where it was mostly undetectable. These discordant results may be explained by the use of pre-stimulation protocols [418] or differences in the study setting (low *vs.* high burden TB settings) where non-tuberculin reactors were used in the control group [9]. However, even in these studies, IL4δ2 expression was very low. This is not surprising given that splice variant expression can be as little as 15% of the parent cytokine [377, 378].

The role of IL-4δ2 in relation to IL-4 has not been well characterized in the context of TB. Evidence suggests that their relationship is not a simple antagonistic one. Alternative splicing is a versatile regulatory mechanism that produces protein isoforms with differential expression patterns in various tissues and may well have different biological properties compared to the full-length protein [376, 500]. For example, IL-4δ2 mRNA stability is decreased compared to IL-4 mRNA in TB [501]. Recent evidence has shown that IL-4δ2 is produced as a functional protein and can act much like a Th1 cytokine, stimulating lymphocyte recruitment and production of pro-inflammatory cytokines [32, 393, 502].These effects are different and independent of IL-4 [502]. However, its role in tuberculosis is unclear. Some studies have shown increased IL-4δ2 levels post-treatment, which, suggests that it is associated with a protective immune response during TB infection [9]. It may down-regulate the effects of IL-4, have an additive effect on Th1 type cells, including promotion of IFN-γ expression, or both. Conversely, much like IL-4, it also stimulates collagen production in fibroblasts and may contribute to fibrosis seen during TB infection [256]. Low expression levels of IL-4 (mRNA and protein) suggest it is functional at picomolar levels *in vivo* [24, 25, 27], but we do not know the concentrations at which IL-4δ2 is biologically active. In *in vitro* cultures, at least 100 fold higher concentrations of IL-4δ2 compared to IL-4, are required to see an effect on T-cell proliferation (Chapter 5 and [47, 392]). My study and previously published work, have demonstrated that the levels of IL-4δ2 mRNA in samples from TB infected patients, approaches the detection limits of the assay and similar levels are seen in

asthma patients [474]. Thus further studies are required to elucidate the significance and exact role of IL-4δ2 during TB infection.

3.4.2 Significance of the Th1:Th2 balance

3.4.2.1 IFN-γ:IL-4 ratio

I found a lower IFN-γ:IL-4 ratio in peripheral blood, but not BAL, of TB patients compared to LTBI controls. The finding that TB patients have elevated levels of IL-4 but similar IFN-γ levels, compared to LTBI controls, suggest a mixed Th1:Th2 response during infection. It is clear that the Th1 response remains dominant and the Th2 response may be playing a subversive role by not necessarily downregulating the Th1 response but perhaps by contributing to immune dysregulation, leading to the observed immunopathology and subsequent disease progression. The results presented here are consistent to other studies [9, 416-418, 503, 504] that correlate this ratio with disease severity [9, 418] and response to anti-TB treatment [9, 503]. A mixed Th1:Th2 response in TB infection has been shown in murine models of TB where TNF-α toxicity may be causing the observed immunopathology [256]. However, I did not observe differences in this ratio in the BAL which, could be explained by compartment-specific differences in cytokine expression similar to what was observed with IL-4 expression.

3.4.2.2 IL4δ2:IL-4 ratio

I found no difference in the IL4δ2:IL-4 ratio between those with TB and LTBI, either in the BAL or peripheral blood. Similar to the IFN-γ:IL-4 ratio, the IL4δ2:IL-4 ratio has also been associated with protection against active TB and has been reported to increase in response to treatment [9, 30]. Data about the IL4δ2:IL-4 ratio in TB are conflicting; some reports found a lower IL4δ2:IL-4 ratio [29, 417], whereas others found no difference in those with active TB versus LTBI [9, 288]. My findings are consistent with the latter. The discordance may be explained by the variations in the ratio during the course of disease. The IL4δ2:IL4 ratio is likely to be lower during early onset of disease (when IL-4 is highest) and higher during and at the end of treatment (lower IL-4 and higher IL4δ2) [9, 417]. Furthermore, the exact

function of IL4δ2 in TB and its relationship with IL-4 is unknown. The clinical relevance of the ratio in predicting disease progression requires clarification.

3.4.3 Limitations

There were a number of limitations to the study. Unavoidable delays in transporting samples from the clinic to the laboratory sometimes prevented BAL fluid samples from being processed immediately. Delayed sample processing can significantly affect detection of low expressing cytokines [26], due to the presence of intrinsic RNases in biological samples, and could explain the low mRNA expression levels of IL-4 and IL-4δ2 observed in the BAL. This was not an issue with peripheral blood because the RNA profile was immediately fixed when blood was drawn in PAXgene tubes. Nonetheless, only samples with RNA of sufficient quality (RIN>7.5) were included in the study.

The small BAL sample size may have contributed to the lack of significant differences in mRNA expression between the TB and LTBI groups and subsequent interpretation of the data. Recruitment of participants to undergo an invasive bronchoscopy procedure was difficult and limited cell recovery often meant that immune assays needed to be prioritized based on the amount of alveolar cells that were available. Furthermore, the BAL collection procedure and delays in sample processing (discussed above) sometimes resulted in recovery of low quality RNA and warranted exclusion of these samples from qPCR analysis. Nonetheless, additional participants will be recruited from other ongoing studies to increase the number of BAL samples in both the TB and LTBI groups

Resource constraints prevented the collection of radiological data to assess the severity of disease in pulmonary TB patients. A number of previous studies showed that higher IL-4 levels were associated with more severe disease, such as those with more extensive cavitation [9, 18, 19, 21]. However, I used the degree of sputum positivity as an estimate of disease severity, as done in another study [18], but I found no correlation between sputum smear status and cytokine expression levels. Similar constraints and high attrition among patients prevented collection of samples for assessment of cytokine levels post-TB treatment.

The helminth infection and exposure status in the participant groups was not assessed in this study. Concurrent helminth infection can prime Th2 responses resulting in high IL-4 levels [452]. However, the effect of helminths would presumably be equally distributed between the groups as both TB patients and LTBI controls were recruited from the same communities and reside in the same environment. Furthermore, the clinical diagnosis of helminth infection is challenging and requires both serological testing (worm specific IgE) and analysis of faecal samples (presence of eggs) to distinguish between past and current infection. These tests were not performed due to resource and ethical restrictions and, in any case, they are not considered reliable „rule out" tests. Additionally, diagnosis is further complicated as exposure to helminths is not only dependent on time but may require a genetic component which determines the immune response (Professor Gerhard Walzl, personal communication and in [505]). However, I did attempt to determine the exposure status by asking participants and reviewing medical records about any previous or current helminth infection.

3.5 Conclusion

I have shown that IFN-γ and IL4 mRNA expression profiles in TB are compartment-specific. IFN-γ expression in the lungs was higher compared to the peripheral blood but no differences were observed between TB patients and presumed LTBI controls. This suggests that a strong Th1 response exists at the site of disease but this response is not sufficient to contain the infection. Additionally, significantly increased IL-4 levels were observed in the peripheral blood of TB patients compared to controls driving a skewed Th1:Th2 response in TB patients. This was evident by the lower IFN-γ:IL-4 ratio in TB patients compared to controls. However, it is not known if this skewed Th2 response is actually driving the immunopathology associated with TB or is merely a bystander effect due to inflammation. Furthermore, the exact role of IL-4δ2 in TB remains unclear. In order to investigate these effects further, I will attempt to generate recombinant IL-4 and IL-4δ2 protein and directly determine their effects on mycobacterial survival.

4. CHAPTER 4: Cloning & protein expression of recombinant IL-4 and IL-4δ2

4.1 Introduction

Efficient strategies for the production of recombinant proteins remain an important aspect of the medical research, biotechnology and pharmaceutical industries. The choice of the expression system used is essential to produce correctly folded and functional proteins that best mimic their native counterparts. Recombinant cytokines have been expressed in a variety of systems depending on their ultimate use.

E. coli is one of the most versatile and widely used hosts for recombinant protein expression due to its well described physiology and genetic makeup, rapid growth rate, low setup costs and high protein yields [506, 507]. However, the utility of *E. coli* systems is limited in the expression of eukaryotic proteins, particularly mammalian proteins. Proteins expressed in prokaryotic systems lack post translational modifications, such as such as glycosylation and disulfide bond formation (depending on the compartment where folding occurs), which can have important functions in protein stability and bioactivity [507-509]. *E. coli* also lacks appropriate chaperone molecules to stabilize eukaryotic proteins leading to protein misfolding and formation of inclusion bodies. Protein refolding often requires harsh conditions and often results in non-functional proteins [507, 510]. Additionally, high levels of endotoxin inherent in bacterial expression can affect downstream immunological assays [506, 507].

Baculovirus protein expression systems are ideal for recombinant expression of mammalian proteins as it allows for proper protein folding, correct signal peptide cleavage and post translational modifications [506, 511, 512]. The non-reducing environment within the insect cell cytoplasm also allows for disulfide bond formation, which is essential to the conformational structure of IL-4. Furthermore, there is no risk of endotoxin contamination which can affect downstream immunological assays [507, 513]. As such, recombinant proteins produced in this system are much more likely to be functional. However, baculovirus systems do have their drawbacks. The reagents are costly and the process of

producing high titre baculovirus stocks can be lengthy. While high protein expression levels of up to 30% of total cell protein have been reported using this system [506], yield can be considerably lower and usually requires extensive optimization of infection and culture conditions to produce sufficient amounts of protein. Improper protein folding and formation of intracellular aggregates has also been reported to occur in this system, although not to the same extent as in prokaryotic expression systems [514, 515]. Furthermore, glycosylation in baculovirus expressed proteins, such as the addition of sialic acid deficient N-linked glycans, is not equivalent to mammalian glycosylation patterns and can lead to different levels of bioactivity [506, 507]. Nonetheless, functional cytokines from various mammalian species, including humans, have been successfully expressed in a baculovirus system [516-518]. For these reasons and others described in Table 4.1, a baculovirus system was chosen for the expression of IL4 and IL-4δ2.

The IL-4 protein has been extensively studied. The mature protein contains 129 amino acid residues consisting of 4 exons. The protein is glycosylated on Asn_{38} [519] and contains 3 disulfide bonds ($Cys_{3\text{-}127}$, $Cys_{24\text{-}65}$, $Cys_{46\text{-}99}$) [520]. IL-4 has been previously expressed in *E. coli* [521, 522], yeast [47, 523], insect cells [524] and mammalian cells [32, 393] for structural and functional studies. In IL-4δ2, exon 2 is deleted by alternative splicing to produce a protein consisting of 113 amino acid residues. The protein is similar in structure to IL-4 but contains only 2 disulfide bonds ($Cys_{3\text{-}127}$, $Cys_{46\text{-}99}$) and a free cysteine (Cys_{65}) residue as a result of the deletion [385]. Recent evidence suggests that IL-4δ2 is produced *in vivo* [31]. However, few studies have previously attempted to express recombinant IL-4δ2 [47, 393, 525] and none in a baculovirus system.

The aim of this chapter is to clone and express functional recombinant IL-4 and IL-4δ2 in a baculovirus-insect cell expression system. These proteins were assessed for function and used in downstream immunological assays to determine their effect in a human model of TB.

Table 4.1: Advantages and disadvantages of a baculovirus insect cell system and an *E. coli* expression system for recombinant protein expression. Adapted from [506, 507].

	Advantages	Disadvantages
Bacterial *E. coli* expression system	• Simple, fast (3 days) • Inexpensive • Very high protein yields • Well described physiology and genetic make-up.	• Formation of inclusion bodies and may require protein denaturation and refolding leading to non-functional proteins • Endotoxin contamination of proteins • No post-translational modification
Baculovirus Insect cell expression system	• Properly folded and functional proteins usually produced • Eukaryotic post-translational modification • Higher protein yields than other eukaryotic systems	• Complex optimisation of expression conditions • Slow (3-4 weeks) • Relatively expensive • Post-translational modifications may not be equivalent to mammalian systems • Viral proteases present in insect cells can degrade the recombinant protein

4.2 Methods

4.2.1 General recombinant cloning methods

4.2.1.1 IL-4 sequence and primer design

Full length human IL-4 cDNA sequence (Accession No: NM_000589.2) was purchased from Origene (Origene Technologies Ltd) and was used in all subsequent cloning steps to generate the IL-4 and IL-4δ2 constructs described in this chapter.

All primers were designed using DNAMAN and vector NTI Advance 11.0 (Invitrogen) and were synthesized by Integrated DNA Technologies (IDT) (Listed in Appendix section B). Primers incorporated restriction sites in their design to facilitate restriction digestion and subsequent cloning into appropriate vectors. Some primers were designed to add sequences coding decahistidine (10x His) affinity tags or Tobacco Etch Virus (TEV) protease recognition sites to enable affinity purification of expressed proteins and subsequent removal of the affinity tag following purification. Inverse primers were designed to amplify DNA regions flanking a specific sequence to be deleted, such as exons and signal peptides, within the constructed vectors, where necessary. These primers were phosphorylated on their 5‟ end to allow for efficient ligation.

4.2.1.2 PCR amplification

PCR were performed using the Kappa HiFi DNA polymerase kit (Kappa Biosystems), according to the manufacturer‟s instructions. Briefly, a 50µl reaction mixture was made consisting of 1x Kappa HiFi Fidelity buffer (containing 2mM Mg^{2+}), 0.25mM dNTPs, 0.5µM primers, 50U of Kapa HiFi DNA polymerase, 10-50ng of DNA template and nuclease-free H_2O. PCR amplification was performed on a G-Storm thermal cycler (G-Storm). A typical PCR amplification consisted of an initial denaturation step of 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 minute 30 seconds, annealing at 50-60°C for 30 seconds, extension at 72°C for 30seconds/kb and a final extension step at 72°C for 5 minutes. Inverse PCR [526], using appropriate inverse PCR primers, was performed to remove unwanted coding regions from nucleotide sequences. Gradient PCR was performed to identify the

optimal annealing temperature based on the Tm of each primer set and minimize nonspecific binding. 1-5% DMSO was added in reactions to minimize the formation of primer-dimers when necessary. PCR products were pooled and purified using the GeneJet PCR purification kit (Thermo Scientific), according to manufacturer‟s instructions, and subsequently quantified using UV spectroscopy at A_{260} with a Nanodrop spectrophotometer (Thermo Scientific).

4.2.1.3 Agarose gel electrophoresis and DNA extraction

Agarose gel electrophoresis analyses was performed to determine the purity and yield of PCR products or to determine the size of DNA fragments generated by restriction enzyme digestion (described in 4.2.1.4). A 0.8-2% DNA gel was prepared containing 1x GelRed (Biotium Inc.) and run in 1x Tris-acetate buffer (TAE, pH 8.0) at 100-120V for 1-2 hours. Gels were visualized in a Syngene GBOX F3 system (Syngene Inc.). 5µl of Generuler 1kb DNA ladder (Thermo Scientific) was included for size determination of PCR products and restriction fragments. For downstream cloning procedures, DNA bands of interest were excised and purified using the GeneJet Gel Extraction kit (Thermo Scientific).

4.2.1.4 Restriction Enzyme Digestions

PCR products and plasmid vectors, containing the relevant restriction sites, were cleaved with appropriate restriction enzymes (REs) to facilitate ligation of the PCR product and vector. Restriction fragment analysis was also performed on ligated constructs using specific REs as an initial screening step, prior to DNA sequencing, to determine correct insertion of the target DNA into the plasmid vector. For products generated by inverse PCR, parental DNA sequences were eliminated post inverse PCR using *DpnI*. Amplified DNA generated using PCR is not methylated, therefore resistant to *Dpn*I digestion. REs (Thermo Scientific) were used to perform restriction digestions according to the manufacturer‟s instructions.

4.2.1.5 Additional DNA modifications

In cases where self-ligation was to be avoided, such as after RE digestion of plasmid DNA required for ligation with an insert, DNA was treated with FastAP thermosensitive alkaline phosphatase (Thermo Scientific) according to the manufacturer‟s instructions.

4.2.1.6 DNA ligation

T4 DNA ligase (Thermo Scientific) was used to re-ligate an inverse PCR product or to ligate a PCR product and linearized vector according to the manufacturer‟s instructions. 5µl of each reaction was used in subsequent transformations.

4.2.1.7 DNA transformations and plasmid preparations

Chemically competent JM109 *E. coli* cells were obtained from commercial sources (Promega) and used for all DNA transformations, according to manufacturer‟s instructions. Briefly, 5µl of ligation mixture was added to a 100µl aliquot of competent, gently mixed and incubated on ice for 30 minutes. Appropriate negative controls (containing no ligation mixture) were included. The cells were heat shocked at 42°C for 90 seconds and then placed on ice for 2 minutes. The entire mixture was then added to 900µl of pre-warmed Luria broth (LB) media and incubated at 37°C for 1 hour 30 minutes in a shaking incubator. 10µl and 100 µl aliquots of each transformation mixture were plated onto LB agar plates containing 100µg/ml Ampicillin (Sigma Aldrich) and incubated at 37°C overnight. Individual colonies were chosen randomly to inoculate 5 ml LB media containing 100µg/ml Ampicillin and incubated for 16-20 hours at 37°C in a shaker incubator. Plasmids were extracted from the cultures and purified using the GeneJet Plasmid Miniprep kit (Thermo Scientific), according to manufacturer‟s instructions. The insertion or deletion of target sequences was determined by restriction fragment analysis (section 4.2.1.8) and DNA sequencing (section 4.2.1.9).

4.2.1.8 Restriction fragment analysis

Following cloning, plasmids were screened to determine if a specific target sequence was inserted (insertion of PCR product into plasmid) or deleted (by inverse PCR). Vector NTI 11.0 (Invitrogen) was used to identify differences in the presence and number of restriction sites between the recombinant and parent plasmid. The selected restriction enzymes were used to digest both the recombinant and parent plasmids (section 4.2.1.4) and subsequently visualized on an agarose gel (section 4.2.1.3) to determine the sizes of the fragments produced. The final plasmid constructs that generated restriction fragments consistent with insertion or deletion of the target sequence were sent for DNA sequencing.

4.2.1.9 DNA sequencing

Following initial screening of recombinant plasmids by restriction fragment analysis, plasmids were sent for DNA sequencing to Inqaba Biotech Ltd. to verify the sequence of the target. Sequencing was performed using an ABI 3500 XL sequencer. 10 μM of sequencing primers and a minimum of 200ng of plasmid were supplied for each sequencing reaction. Sequence alignment to the DNA sequence of interest was performed using Bioedit v7.2.5 (Ibis Biosciences).

4.2.2 Protein expression methods

Protein expression was performed in a baculovirus system where *Spodoptera frugiperda* (*Sf21*) insect cells were infected with baculovirus. An outline of the steps required for protein expression is described in Figure 4.1.

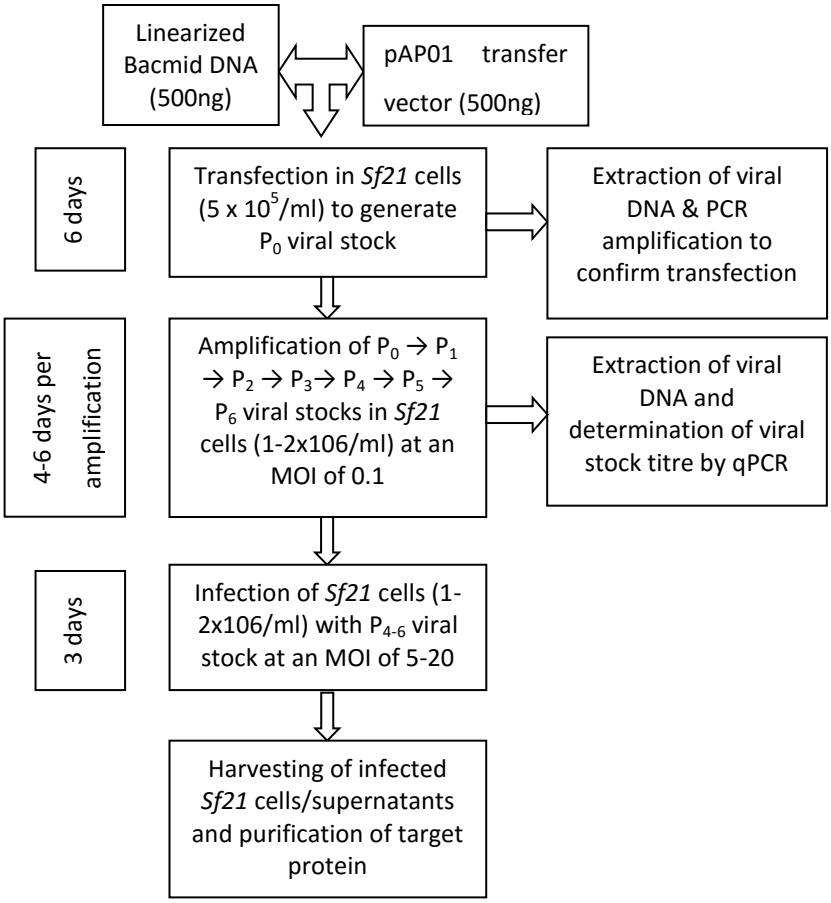


Figure 4.1. Steps illustrating transfection, generation of high viral titre baculovirus stocks and subsequent protein expression

4.2.2.1 Preparation of Linearized Bacmid DNA

H5996 cells containing bacmid pBAC10:KO_{1629} were plated on LB agar for isolation of single colonies. For plasmid maintenance within the host cells, all growth media was supplemented with 10ug/ml kanamycin (Sigma-Aldrich) and 30ug/ml chloramphenicol (Sigma-Aldrich). Colonies were obtained on LB plates after incubation overnight at 37^{o}C. A single colony was used to inoculate 5ml LB broth incubated at 37°C for ~8 hours in a shaking incubator. This culture was subsequently used to re-inoculate 500ml of LB broth and incubated at 37°C for ~16 hours in a shaking incubator. Cells were harvested and centrifuged at 3000rpm for 30 minutes at 4°C and bacmid DNA was subsequently isolated and purified

using the BAC100 Plasmid purification kit (Macherey Nagel). Bacmid DNA was linearized using the *Bsu36I* restriction enzyme (New England Biolabs) in a 500µl reaction containing ~350µl bacmid DNA, 1 x NEB buffer 3, 300 units of *Bsu36I* and nuclease-free H_2O. The reaction was at 37°C for 5 hours followed by heat inactivation at 80°C for 20 minutes. The bacmid DNA was precipitated using isopropanol and centrifugation. The pelleted DNA was resuspended in 100µl TE buffer and quantified on a Nanodrop spectrophotometer. Linearization of bacmid DNA was confirmed by running both undigested and digested bacmid DNA on a 1% agarose gel (section 4.2.1.3). Linearized bacmid DNA was then stored at -20°C.

4.2.2.2 Growth and maintenance of *Sf21* cells

Sf21 cells were grown from frozen glycerol stocks by first thawing an aliquot of cells on ice for 30 minutes. Once thawed, the entire aliquot was added to 20-30ml of Insect-Xpress media supplemented with 2% fetal bovine serum (FBS; Lonza) in a 100ml shaker flask. Cells were then incubated at 28°C in a shaker incubator. Cells were counted regularly by Trypan blue (Sigma-Aldrich) exclusion staining to monitor cell growth rate and viability. Cells were sub-cultured periodically if the cell concentration exceeded $3x10^6$/ml.

4.2.2.3 Transfection of Bacmid and Transfer vector

The baculovirus $pBAC_{10}$:KO_{1629} has been previously described elsewhere [527]. The $pBAC_{10}$:KO_{1629} has the following attributes: an F replicon from *E. coli*, which allows the viral genome to be amplified in *E. coli*; a chloroamphenicol acetyl transferase (*cat*) cassette in the essential ORF1629 and a single *Bsu36I* restriction site to linearize the bacmid DNA. Co-tranfection of the pAP01 transfer vector and linearized bacmid DNA $pBAC_{10}$:KO_{1629} allows for recombination between the homologous DNA regions (603 and 1629) so that the gene of interest between the homologous sites is transferred from the pAP01 vector to the bacmid DNA. The presence of the *cat* cassette ensures that baculovirus will only replicate if a homologous recombination event occurs (Figure 4.2).

A 24µl transfection mixture was made for each plasmid construct, consisting of 500ng of pPR30 vector (containing the gene of interest), 500ng of linearized bacmid DNA, 12ul of Lipofectin (Invitrogen; diluted 2:1 in sterile H_2O) and sterile H_2O. Appropriate lipofectin only and bacmid DNA only controls were included. Additionally, a pPR30 vector containing no insert was also transfected with bacmid DNA. Transfection mixtures were incubated for 30 minutes at room temperature. A 6-well plate was seeded with $1x10^6$ *Sf21* cells in 2ml of serum free Insect-Xpress media (Lonza) and incubated at 28°C for 1 hour to allow cell adherence. After removal of spent media, the transfection mixture was added to the cells and incubated at 28°C overnight. The spent media was again removed and 2ml of Insect-Xpress media supplemented with 2% fetal bovine serum (FBS; Lonza) was added to the cells. The cells were incubated at 28°C for 5 days after which virus-containing supernatant stocks (P_0 viral stocks) were harvested and stored at 4°C. Viral DNA was isolated from 200µl of each P0 viral stock using the High Pure Viral Nucleic Acid Kit (Roche) according to manufacturers‟ instructions. PCR amplification of the viral DNA and subsequent agarose gel electrophoresis was performed to confirm the presence of the gene of interest in the viral DNA.

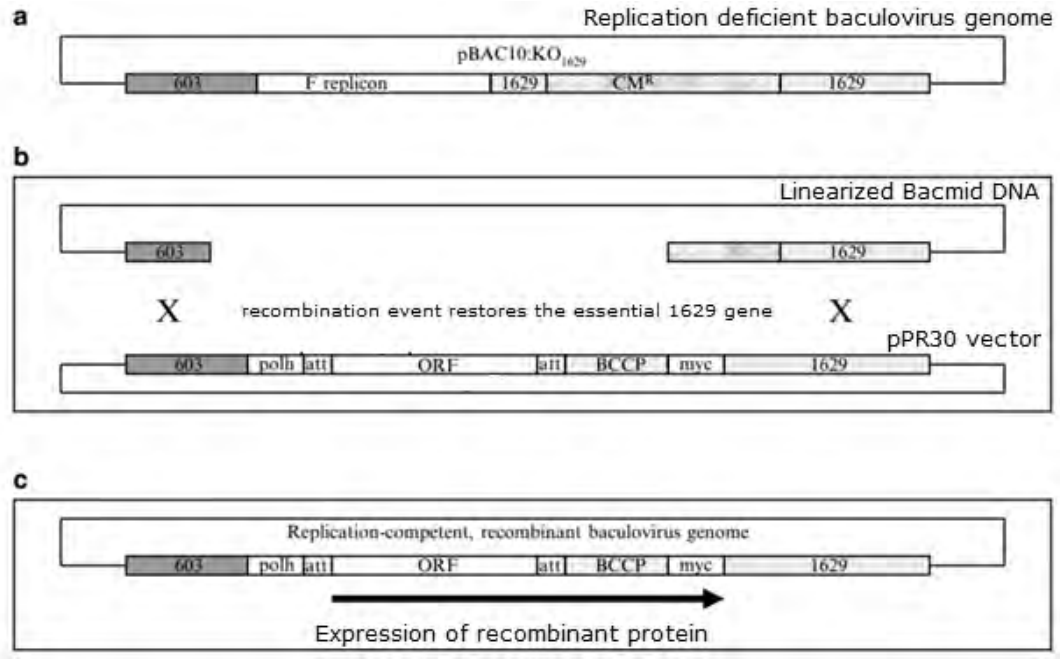


Figure 4.2. Recombination event that occurs during co-transfection of insect cells with baculovirus. Adapted from Blackburn *et al*. [527].

4.2.2.4 Amplification of viral stocks

High-titre viral stocks were required for scale-up of protein expression. A 6-well plate was seeded with $1x10^6$ *Sf21* cells in 2ml of serum free Insect-Xpress media (Lonza) and incubated at 28°C for 1 hour to allow cell adherence. After removal of spent media, 500µl of P_0 virus and FBS supplemented Insect-Xpress media was added and incubated at 28°C for 6 days. Virus-containing supernatants (P_1 viral stocks) were harvested and stored at 4°C. For the production of P_2 and P_3 virus stocks, a T150 tissue culture flask was seeded with 5×10^7 *Sf21* cells, the spent media removed and replaced with 30ml of FBS supplemented media. 500µl of P_1 virus in 3ml of FBS containing media was added to the cells and incubated at 28°C for 6 days. Virus-containing supernatants (P_2 and P_3 viral stocks) were harvested and stored at 4°C. Viral titres of P_3 stocks were determined by a viral qPCR assay (described in section 4.2.2.5). P_4 and P_5 viral stocks were produced in suspension cultures. *Sf21* cells were grown in 500ml flasks to a concentration of $1\text{-}1.5x10^6$/ml. Cells were infected with P_3 viral

stock at an MOI of 0.1 at 28°C for 4-5 days in a shaker incubator. Supernatants were harvested and viral titres were determined as above.

4.2.2.5 Quantitative PCR to determine titres of viral stocks

Determination of titres of viral stocks was performed by the Centre for Proteomic and Genomic Research (CPGR). Viral DNA was purified from viral stocks using the High Pure Viral Nucleic Acid kit (Roche) according to the manufacturer‟s instructions. Viral DNA was quantified in a SybrGreen quantitative PCR assay against a standard curve of DNA obtained from virus with a known titre, as determined by plaque assay. The titres of viral stocks ranged from 10^6-10^8 plaque forming units (pfu)/ml.

4.2.2.6 Protein expression in *Sf21* cells

IL-4 and IL-4δ2 proteins were expressed in *Sf21* suspension cultures using high-titre P_4 and P_5 baculovirus stocks. Similarly, viral stocks produced from empty transfer vector were used as an expression control. Preliminary small scale expression experiments were performed to determine the recombinant protein location (intracellular or secreted), optimal day of harvest and multiplicity of infection (MOI) for each construct. Typically, *Sf21* cells were grown to 1-1.5x10^6/ml in shaker flasks and infected at an MOI of 5-20, depending on the construct used. Cells were then incubated for 48-72 hours at 28°C in a shaker incubator. Cell morphology and viability were periodically assessed by trypan blue exclusion staining and examination under a microscope to confirm infection of cells.

4.2.2.7 Preparation of cell supernatants and clarified cell lysates

Both *Sf21* cells and cell supernatants were harvested to determine if the recombinant protein was secreted into the media or remained intracellular. For secreted proteins, cell supernatants were collected in 50ml tubes followed by the addition of 1% Benzonase (Novagen) and 1x EDTA-free protease inhibitor cocktail (Roche) to each tube. These were stored on ice prior to protein purification. For recombinant proteins expressed intracellularly, infected *Sf21* cells were pelleted by centrifugation at 1000rpm for 10 minutes at 4°C followed by 3 washes in

cold 1xPBS (Lonza). Cells were then re-suspended in lysis buffer containing 50mM sodium phosphate buffer pH7.7, 50mM KCl, 20% glycerol, 2mM $CaCl_2$, 10mM $MgCl_2$, 0.1% Triton X-100, 1mM TCEP, 1% Benzonase, 1x EDTA-free protease inhibitor cocktail and sterile H_2O. Approximately 1ml of lysis buffer was added per $10x10^6$ cells. The cells in lysis buffer were incubated on ice for 30 minutes with gentle shaking followed by vortexing and sonication in a water bath sonicator for 1 minute. Cell lysates were centrifuged at 13000rpm for 30 minutes at 4ºC. The clarified lysates were collected, pooled and placed on ice before protein purification.

4.2.2.8 Protein purification and concentration

His-tagged proteins, as well as the expression control were purified using the His-Spin Miniprep Kit (Zymo Research) according to the manufacturer‟s instructions. However, adjustments were made to the protocol to accommodate for larger sample volumes. ~40ml aliquots of cell supernatants or ~10ml aliquots of clarified lysates were evenly split into 50ml tubes. In order to mimic the buffer conditions of the His-binding buffer, the following components were added to the cells supernatants or clarified cell lysates to a final volume of 50ml: 50mM Sodium phosphate buffer at pH7.7, 300mM NaCl, 0.03% Triton-X and 10mM Imidazole. 300-350µl of His affinity gel, consisting of Ni^{2+} agarose beads, was added to each 50ml tube and placed on a tube rotator at 4ºC for 30-45 minutes to allow binding of the His-tagged proteins to the beads. The samples containing beads were then centrifuged at 800rpm for 1 minute to pellet the beads and the supernatants were removed taking care not to disturb the beads. The beads were then resuspended in His-Binding buffer and carefully transferred to a Zymo-spin P1 column. Beads were then washed 3 times in His-Wash buffer (50mM Sodium phosphate buffer pH7.7, 300mM NaCl, 0.03% Triton-X, 50mM Imidazole) followed by incubation in His elution buffer (50mM Sodium phosphate buffer pH7.7, 300mM NaCl, 0.03% Triton-X, 500mM Imidazole) for 10 minutes and collection of the eluate.

GST-tagged proteins were purified from clarified cell lysates using Glutathione Magnetic Beads (Thermo Scientific), according to the manufacturer‟s instructions. As with His purifications, adjustments were made to the protocol to accommodate for larger sample

volumes. ~10ml aliquots of clarified lysates were evenly split into 50ml tubes. In order to mimic the buffer conditions of the GST-binding buffer, the following components were added to the clarified cell lysates to a final volume of 30ml: 125mM Tris buffer at pH8.0, 150mM NaCl, 0.03% Triton-X. 150µl of Glutathione magnetic Beads, consisting of iron oxide beads attached to reduced glutathione, were added to each tube, placed on a tube rotator at 4ºC for 1-1.5 hours to allow binding of the GST-tagged proteins to the beads. A magnet was applied to the tubes to immobilize the magnetic beads and the supernatants were removed by pipetting. The beads were then carefully transferred to 1.5ml microcentrifuge tubes and washed 4 times in GST-binding buffer using a MagnaBind Magnet (Thermo Scientific). Beads were then incubated in GST Elution buffer (12 5mM Tris buffer at pH8.0, 150mM NaCl, 50mM reduced glutathione) for 5 minutes and the eluates were collected.

Protein eluates, both from His or GST purifications, were concentrated and buffer exchanged using Amicon Ultra 0.5ml centrifugal filters (Millipore) with a molecular weight cut-off of 3kDa. The filters were centrifuged at 13000 rpm at 4°C in 20 minute intervals. All of the eluate was added to the centrifugal filter to concentrate the sample. This was followed by RPMI 1640 (Lonza) for buffer exchange. The final volume of the protein in RPMI ranged from 50-150µl. An aliquot was collected for determination of protein concentration. Aliquots of each fraction in the purification process were collected for SDS-PAGE and Western Blot analysis. Proteins were stored at -80^{o}C in low-bind microcentrifuge tubes (AEC Amersham) in single-use aliquots supplemented with 2% endotoxin-free BSA (Sigma Aldrich).

4.2.2.9 Determination of protein concentration

Protein concentration was determined by the Bradford method using Protein Assay Dye (BioRad), according to the manufacturer‟s instructions. The microassay method was performed in a 96-well flat-bottom plate using a bovine serum albumin (BSA) standard curve ranging from 0-500µg/ml. Samples were diluted as appropriate. The standard curve analysis and concentration determination was performed using GraphPad Prism 5.0 (GraphPad).

4.2.2.10 SDS-PAGE gel electrophoresis and Western Blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were used to analyze the size, purity and immunoreactivity of expressed protein and were performed according to standard guidelines [528]. Either a 12% (GST-tagged proteins) or 15% (His-tagged proteins) polyacrylamide gel were cast and protein sizes were determined using the PageRuler Plus Prestained protein ladder (Thermo Scientific). Gels were typically run at ~100-120V and were either stained overnight using Acqua Stain (Vacutec) or used for Western Blot transfer. The purity of the recombinant protein was estimated from the eluted protein fraction band of the SDS-PAGE gel by densitometry using Image J scientific image processing software (Wayne Rasband) and expressed as a percentage of total protein present in the eluted fraction. Gels for Western Blot were transferred onto Hybond-C nitrocellulose membrane (GE Healthcare). Protein transfer was confirmed by staining with Ponceau S solution (Sigma Aldrich) for 1 minute followed by 3 washes with 1 x TBST buffer (100 ml 10 x TBS [0.5 M Tris, 0.66 M NaCl, pH 7.6], 10 ml 10% Tween-20, 890ml H_2O) to remove the stain. The membranes were then blocked in 5% fat-free milk powder in 1 x TBST buffer for 1 hour and probed overnight at 4°C using the following antibodies: 1:5000 dilution of HRP-conjugated Anti-His antibody (Sigma Aldrich) for His-tagged proteins; 1:1000 dilution of HRP-conjugated Anti-GST antibody (Santa Cruz Biotech) for GST-tagged proteins; 1:2500 dilution of HRP-conjugated Anti-IL-4 antibody (Abcam) for IL-4 and IL-4δ2. Membranes were then washed 3 times with 1xTBST buffer for 5 minutes with shaking. Proteins were detected using the Super Signal West Pico Chemiluminescent HRP Substrate (Thermo Scientific) and visualized using a G:BOX chemiluminescent reader (SynGene).

4.2.2.11 TEV cleavage

In order to remove the protein affinity tag, proteolytic cleavage of the 7 amino acid TEV recognition site within the fusion protein was performed using ProTEV protease (Promega), according to the manufacturer's instructions. A 100µl reaction was set up containing 10 units of ProTEV enzyme, 1mM of DTT, 1x ProTEV buffer, sterile H_2O and up to 20µg of protein. Protein to be cleaved were first buffer exchanged in 1xProTEV buffer, as described in section 4.2.2.8. The reaction mixture was incubated for 1, 4 and 16 hours at 30^{o}C. Fractions

were analysed by SDS-PAGE gel electrophoresis (section 4.2.2.10) and the remaining reaction mixture was purified as described in section 4.2.2.8. Cleaved protein was present in the supernatants after incubation with the beads whereas the ProTEV protease and cleaved His tag remained bound to the beads. The fractions containing the cleaved protein were buffer exchanged in RPMI 1640, their concentrations were measured and stored as described in section 4.2.2.8.

4.3 Results

4.3.1 pPR030 vector characteristics

The pPR030 transfer vector is a modified plasmid derived from the pTriEx 1.1 *E. coli* transfer vector (Figure 4.3). The pPR030 vector has a late phase viral polyhedron promotor, *polh*, which controls protein expression; an attR1 gene which is required for homologous recombination; β-lactamase coding sequence which confers ampicillin resistance; bacmid 603 and 1629 DNA regions that flank the insertion sequence and are homologous to regions in the bacmid genome. The presence of the *cat* cassette in the essential 1629 region in pBAC10:KO_{1629} means that replication competent virus will only be produced if a successful homologous recombination event occurs (described in section 4.2.2.3 and Figure 4.2). The pPR030 construct was originally constructed with a biotin carboxyl carrier protein (BCCP) tag to produce fusion proteins for immobilization applications such as microarray assays [527]. Due to the strong dissociation constant between biotin and streptavidin, the tag is not compatible with affinity purification and was subsequently removed by inverse PCR using primers AP1_Inv1 and AP_Inv2 (Appendix section B). The resulting plasmid was now called pAP01.

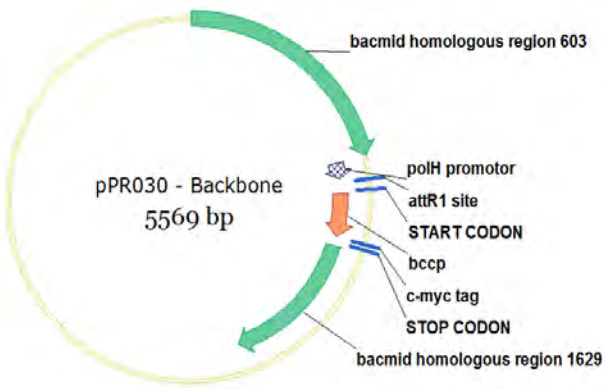


Figure 4.3. pPR030 plasmid backbone used as transfer vector for transfection into *Sf21* insect cells

4.3.2 Cloning and protein expression of IL-4

4.3.2.1 Cloning of His-tagged IL-4 construct

The cloning procedure for the construction of pAP01 containing His-tagged IL-4 construct is described in figure 4.4A and as follows: the IL-4 cDNA was amplified from the Origene construct in 2 PCR reactions; the first PCR reaction, using primers AP2_F and AP2_R1 (Appendix section B), added a *BamH1* restriction site at the 5‟-end of the IL4 cDNA sequence and the codons for TEV cleavage sequence and the first 6 histidine residues at the 3‟- end of the IL-4 sequence. The second PCR, using primers AP2_F and AP2_R2, added codons for the last 4 histidine residues, a stop codon (TAG) and an *AvrII* site at the C-terminal end. The final PCR product (IL-4-TEV-His) was ligated between cohesive restriction enzyme sites *BamHI* and *AvrII* in the parent vectors. pAP01-IL4-TEV-His (nucleotide sequence in section B of Appendix) was the resultant plasmid of this cloning strategy, and was used for transfection and expression of His-tagged IL-4 protein.

The structure and translated amino acid sequence of IL4-TEV-His is shown in Figure 4.4B and contains the following components: the full IL-4 cDNA sequence including the 24 amino acid signal peptide; a Tobacco Etch Virus (TEV) protease cleavage site for removal of the affinity tag after purification; a decahistidine (10x His) tag to facilitate Ni^{2+}-NTA affinity purification.

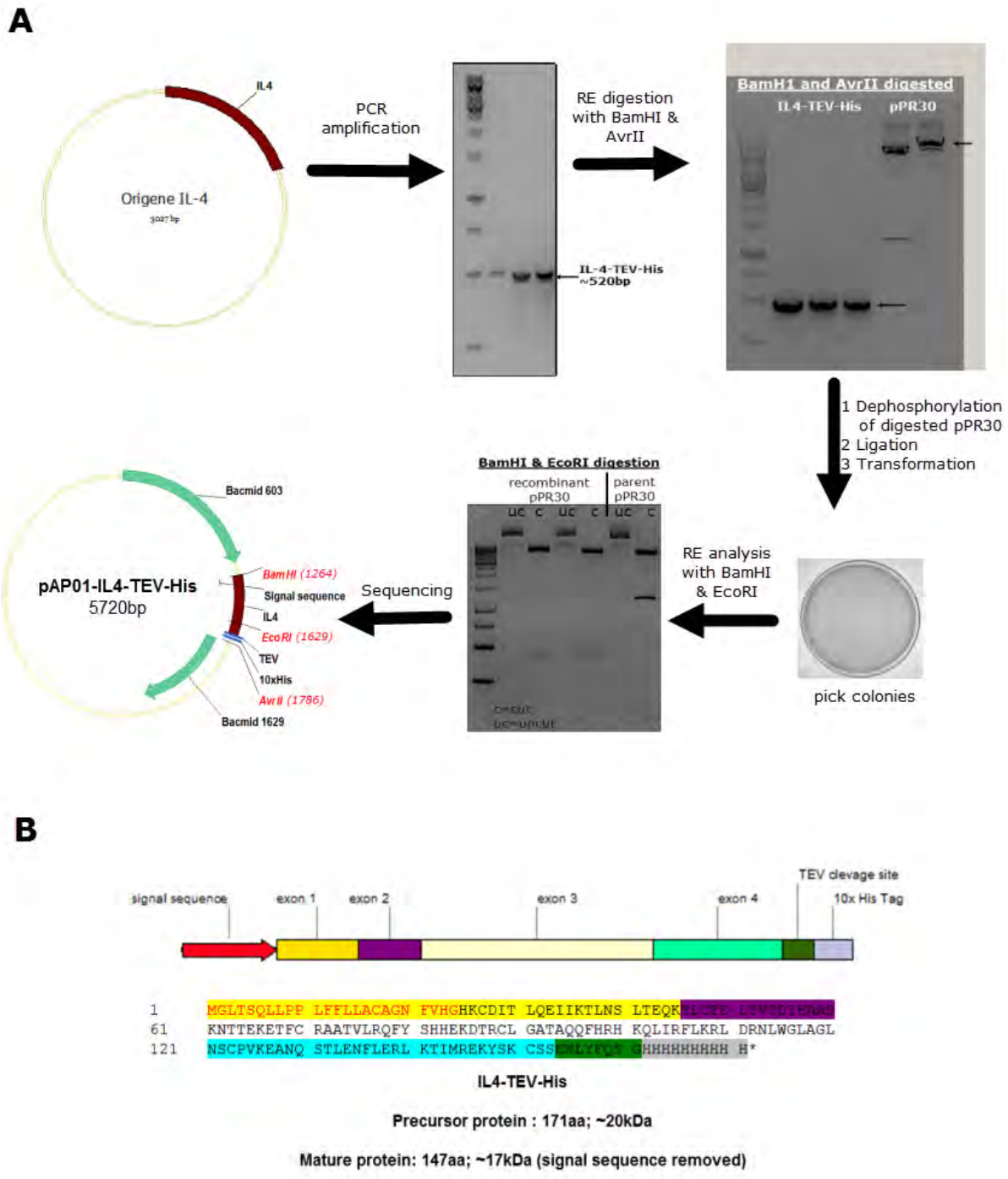


Figure 4.4 (A) The cloning steps to produce the pAP01-IL-4-TEV-His construct and (B) the structure and translated amino acid sequence of IL-4-TEV-His

4.3.2.2 Protein expression & nickel chelate affinity chromatography purification of His-tagged recombinant IL-4 protein

The pAP01-IL4-TEV-His construct was transfected with linearized pBAC10:KO_{1629} bacmid DNA in *Sf21* cells to produce P_0 viral stocks. The presence of the IL-4-TEV-His construct was determined by PCR amplification of P_0 viral DNA and visualization of the amplified inserts by agarose gel electrophoresis. After several rounds of viral amplification, high titre P_5 viral stocks were produced in the range of 10^8 pfu/ml and used for protein expression. Initial small scale expression experiments were performed using an MOI of 5, 10 and 20 and harvesting cells 2 days and 3 days post infection. An MOI of 10 and harvesting 3 days post infection (PI) produced the optimal yield of recombinant IL-4 (rIL-4) protein. Furthermore, the majority of protein was found to be present in the cell supernatants. These conditions were chosen to infect *Sf21* cells in a 500ml shaker flask culture at a cell concentration of $1.5x10^6$/ml. At 3 days PI, cells were elongated and enlarged at ~50-60% viability indicating viral infection of cells. Cell supernatants were harvested and purified as described in section 4.2.2.8. Analysis of the protein fractions by SDS-PAGE gel electrophoresis indicated the presence of 2 bands at ~19kDa and ~16kDa in the purified and concentrated fractions (Figure 4.5A). The expected size of His-tagged rIL-4 is ~17-18kDa and these two bands could represent different glycosylated forms of IL-4, which has also been shown to occur in baculovirus expressed swine rIL-4 [518]. However, this may not be the case given that only a single band was observed in the Western blots (Figure 4.5 B and C). The purity in this fraction was ~85% as determined by band densitometry using Image J software (lanes 5 and 6 of Figure 4.5A). Expression and purification using empty pPR30 vector did not produce equivalent bands of this size. Western blot analysis using anti-His and anti-IL-4 antibodies produced similarly sized bands confirming that this protein was indeed His-tagged rIL-4 (Figure 4.5 B and C). Protein yield from a 500ml culture ranged between 60-120µg. Cleavage of the His tag using ProTEV (described in section 4.2.2.11) was attempted but the majority of protein was lost during the cleavage and subsequent purification process (Figure 4.5 D). Due to the low recovery yield after cleavage, it was decided not to remove the His tag. Characterization and functional assessment of rIL-4 was performed and reported in chapter 5.

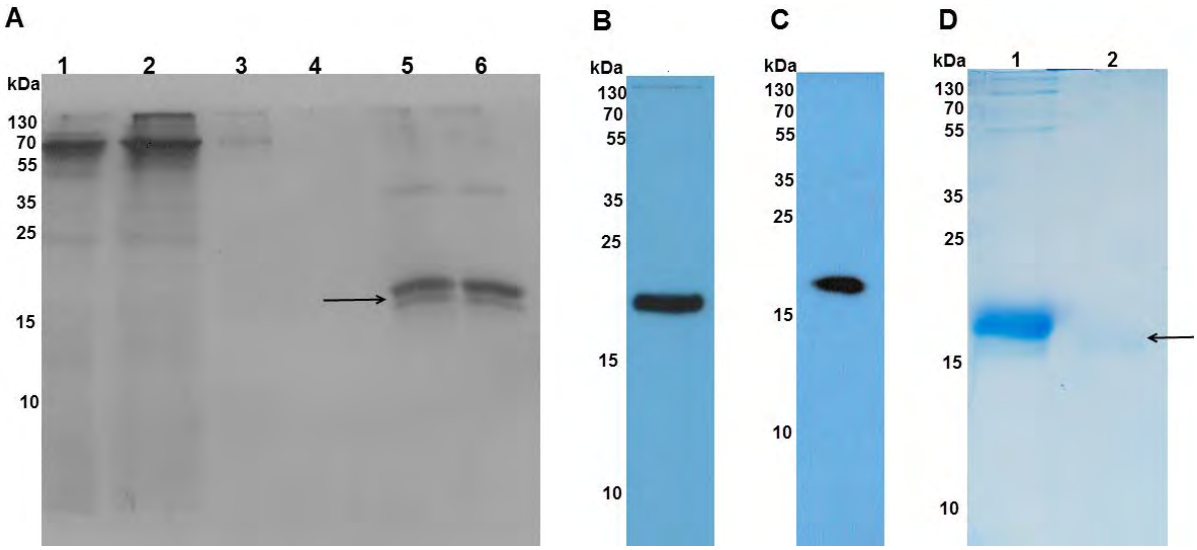


Figure 4.5. Purification of His tagged recombinant IL-4 (rIL-4) using Ni^{+} affinity column and cleavage of His tag using TEV protease.(A) 15% SDS PAGE gel of rIL-4 purification fractions showing (1) post-infection *Sf21* insect cell supernatant (2) flow-through after column binding (3) 1st wash with 50mM imidazole (4) 2nd wash with 50mM imidazole (5) elution of rIL-4 at 500mM imidazole (6) elution of rIL-4 at 750mM imidazole. The eluted protein had a size of ~17kDa (arrow). Western blot analysis of the purified rIL-4 fraction probed with (B) anti-His antibody at 1:5000 and (C) anti-IL4 antibody at 1:2500. (D) 15% SDS-PAGE gel of purified rIL-4 (1) before cleavage with TEV protease and (2) after cleavage with TEV protease. Low protein yield was obtained post cleavage (arrow). Molecular weight markers are shown on the left in kiloDaltons (kDa). 10-15µl of each fraction was loaded onto the gel.

4.3.3 Cloning and protein expression of IL-4δ2

4.3.3.1 Cloning of His-tagged IL-4δ2 construct

In humans, an alternative splicing event results in the deletion of exon 2 from the IL-4 gene to produce the IL4δ2 splice variant. Here, exon 2 within the IL-4 cDNA sequence of the pAP01-IL4-TEV-His construct was deleted by inverse PCR. The inverse PCR was performed using the primers AP3_Inv1 and AP3_Inv2 (Appendix section B), which flanked the exon 2 sequence. Parental DNA was removed using *DpnI* treatment, and the amplified PCR product was re-ligated. The successful deletion of exon 2 is confirmed by the absence of *HincII* restriction site that falls within this deleted nucleotide sequence. The final construct, pAP01-

IL4δ2–TEV-His was used for protein expression. However, based on the results of initial rIL-4δ2 protein expression experiments (reported in section 4.3.3.2), the signal peptide of IL-4δ2 was deleted from the pAP01-IL4δ2-TEV-His construct by inverse PCR using the primers AP4_Inv1 and AP4_Inv2, as described above. This new construct, called pAP01-NSIL4δ2-TEV-His, was used in subsequent protein expression of His-tagged rIL-4δ2. The cloning procedure is described in figure 4.6A. The nucleotide sequence of the pAP01-IL4δ2-TEV-His is shown in section B of the Appendix. The structure and translated amino acid sequence of IL-4δ2-TEV-His is described in Figure 4.6B.

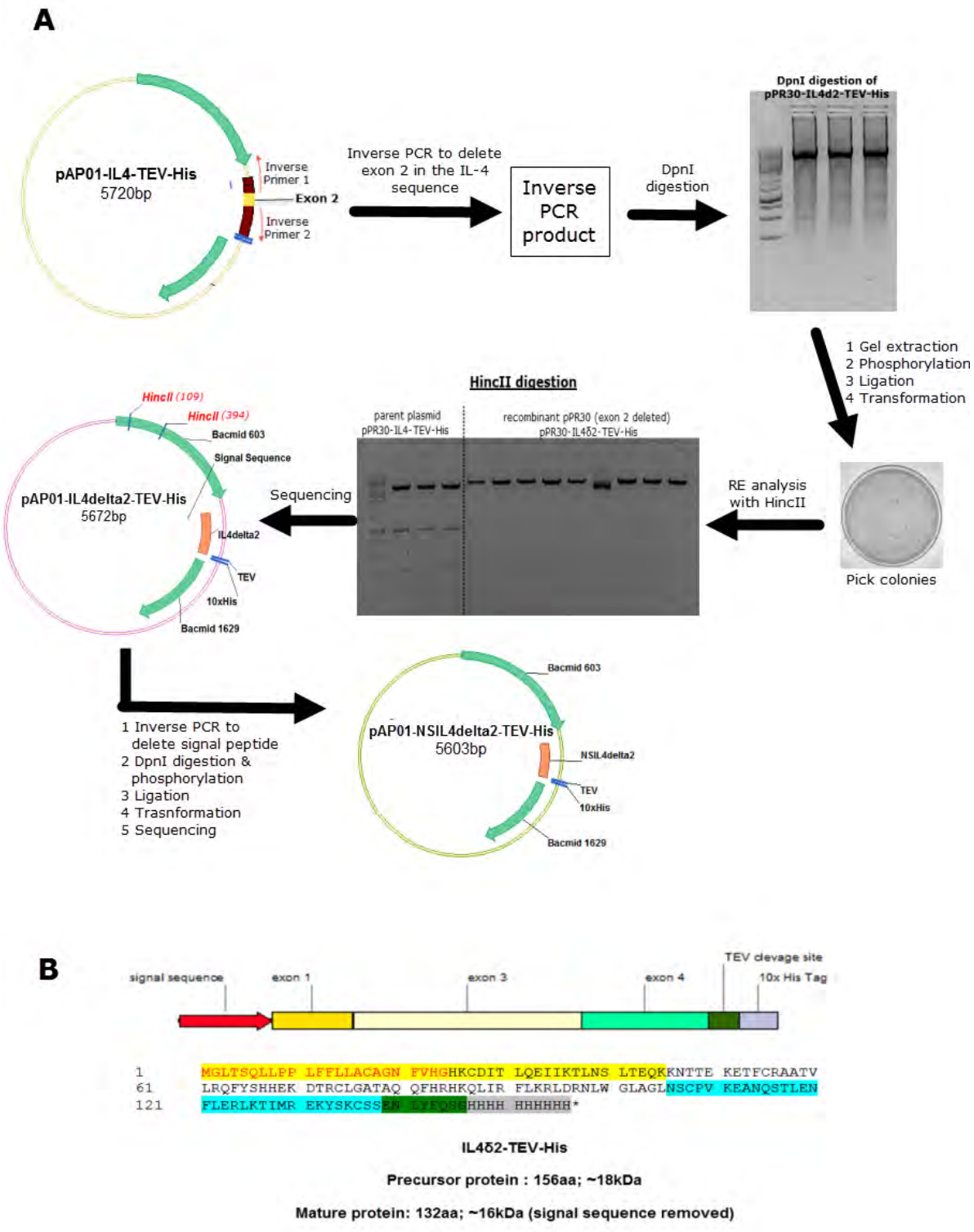

A
pAP01-IL4-TEV-His
5720bp
Inverse Primer 1
Exon 2
Inverse Primer 2
Inverse PCR to delete exon 2 in the IL-4 sequence
Inverse PCR product
DpnI digestion
DpnI digestion of pPR30-IL4d2-TEV-His
1 Gel extraction
2 Phosphorylation
3 Ligation
4 Transformation
Pick colonies
RE analysis with HincII
HincII digestion
parent plasmid pPR30-IL4-TEV-His
recombinant pPR30 (exon 2 deleted) pPR30-IL4δ2-TEV-His
Sequencing
HincII (109)
HincII (394)
Bacmid 603
Signal Sequence
pAP01-IL4delta2-TEV-His
5672bp
IL4delta2
TEV
10xHis
Bacmid 1629
1 Inverse PCR to delete signal peptide
2 DpnI digestion & phosphorylation
3 Ligation
4 Trasnformation
5 Sequencing
pAP01-NSIL4delta2-TEV-His
5603bp
NSIL4delta2
TEV
10xHis
Bacmid 603
Bacmid 1629
B
signal sequence
exon 1
exon 3
exon 4
TEV clevage site
10x His Tag
1 MGLTSQLLPP LFFLLACAGN FVHGHKCDIT LQEIIKTLNS LTEQKKNTTE KETFCRAATV
61 LRQFYSHHEK DTRCLGATAQ QFHRHKQLIR FLKRLDRNLW GLAGLNSCPV KEANQSTLEN
121 FLERLKTIMR EKYSKCSSEN LYFQSGHHHH HHHHHH*
IL4δ2-TEV-His
Precursor protein : 156aa; ~18kDa
Mature protein: 132aa; ~16kDa (signal sequence removed)

Figure 4.6 (A) the cloning steps to produce the pAP01-IL-4δ2-TEV-His and pAP01-NSIL-4δ2-TEV-His construct and (B) The structure and amino acid sequence of IL-4δ2-TEV-His sequence inserted into pAP01. The NSIL-4δ2-TEV-His sequence is identical to the IL-4δ2-TEV-His sequence except that the signal sequence, which comprises the first 24 amino acids (shown in red), are deleted.

4.3.3.2 Protein expression and nickel chelate affinity chromatography purification of His tagged recombinant IL-4δ2 protein

Transfection of pAP01-IL-4δ2-TEV-His and production of high titre viral stocks were performed as previously described (section 4.2.2.3). Initial expression experiments using conditions similar to rIL-4 (MOI of 10 and harvesting of cell supernatants at 3 days PI) resulted in no expression of protein. Small scale experiments revealed that His tagged-IL4δ2 was present in the cell lysates but not the supernatants suggesting that the expressed IL4δ2 was not secreted but remained intracellular (Figure 4.7). The signal peptide (the first 24 amino acids) in IL-4, and probably in IL4δ2 as well, is required for protein secretion and is cleaved to produce the mature functional protein. However, it was difficult to determine if the signal peptide was cleaved in the IL4δ2-TEV-His protein expressed here because the difference in size (~1-2 kDa) of rIL4δ2 before and after signal peptide cleavage was too small to resolve on an SDS PAGE gel. Given that proper protein function requires removal of the signal sequence, another construct was created where the signal peptide was removed (pAP01-NSIL-4δ2-TEV-His; section 4.3.3.1). This construct was used for subsequent His-tagged rIL4δ2 protein expressions. Optimized conditions (an MOI of 10 and 2 days PI) were used to infect a ~500ml insect cell culture at a concentration of $1.5x10^6$/ml. Given the intracellular location of the rIL4δ2 protein, cells were harvested, lysed and purified as described in section 4.2.2.8. SDS-PAGE gel electrophoresis and Western blot analysis using anti-His and anti-IL-4 antibodies confirmed the presence of His-tagged rIL4δ2 (~14kDa) in the concentrated eluted fraction (Figure 4.8). However, the purity of the rIL4δ2 protein was only ~30-40% based on band densitometry analysis (Lane 7 in figure 4.8A) A number of strategies [529] were employed in an attempt to improve purity including: increasing imidazole concentrations in the wash buffer up to 100mM or varying imidazole

concentrations in the elution buffer to differentially elute the nonspecific proteins; increasing NaCl concentration up to 500mM and Triton-X 100 up to 1% in the binding buffer; performing affinity purification utilizing Co^{2+} (Thermo Scientific) rather than Ni^{2+} as the metal-ion affinity matrix [530, 531]. However, none of these substantially improved the purity, and in some cases, even reduced the yield of rIL4δ2. Furthermore, the Western Blot analysis revealed that most of the protein was insoluble (Lane 3 of Figure 4.8B). Attempts to improve solubility by varying the constituents of the lysis buffer (varying buffers, salt concentrations and detergents), using harsher cell lysis methods (sonication) or commercial lysis buffers (IPER lysis reagent, Thermo Scientific) were unsuccessful (Figure 4.9A-D). rIL4δ2 protein yield (~5µg from a 500ml culture) was much lower compared to rIL-4. However, sufficient protein was obtained to functionally assess IL4δ2 (chapter 5).

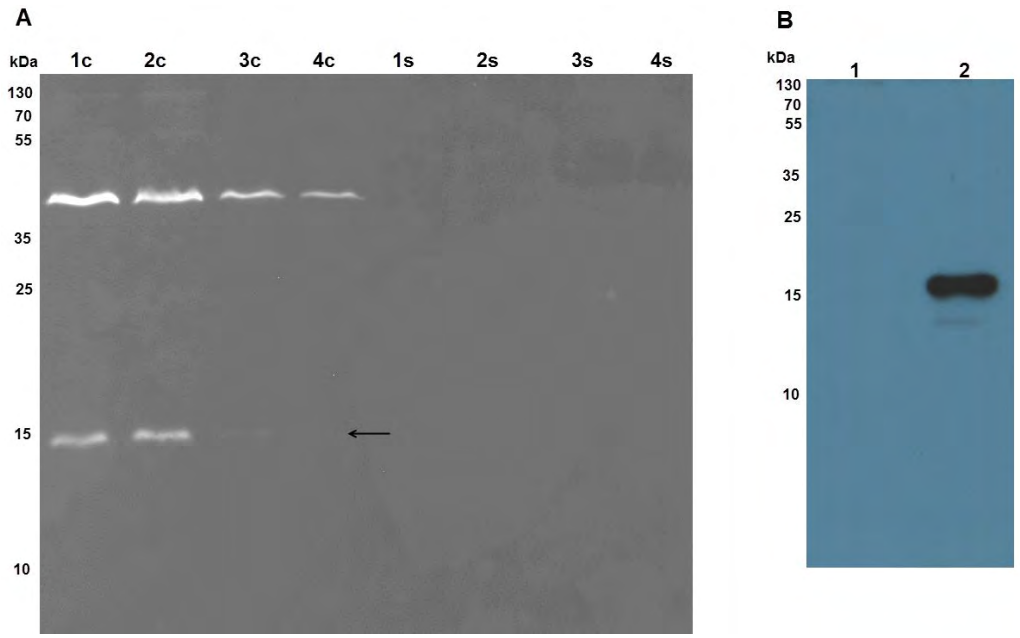


Figure 4.7. Cellular (intracellular vs extracellular) localization and optimization of conditions for the expression His tagged recombinant IL-4δ2 (rIL-4δ2). (A) Western blot analysis of crude cell lysates and cell supernatants at different days of harvest post infection (PI) and different multiplicity of infections (MOI) – Lanes 1c-4c show crude cell lysates and lanes 1s-4s shows cell supernatants at (1) Day 2 PI and MOI 5 (2) Day 2 PI and MOI 10 (3) Day 3 PI and MOI 5 (4) Day 3 PI and MOI 10. IL-4δ2 has a size of ~15kDa (arrow). (B) Western blot analysis of purified and concentrated IL4δ2 (Day 2 PI and MOI 10) in (1) cell supernatants and (2) crude lysates show that

protein is not secreted and remains intracellular. Molecular weight markers are shown on the left in kiloDaltons (kDa). Anti-His antibody was used at a 1:5000 dilution. 15µl of each fraction was loaded onto the gel.

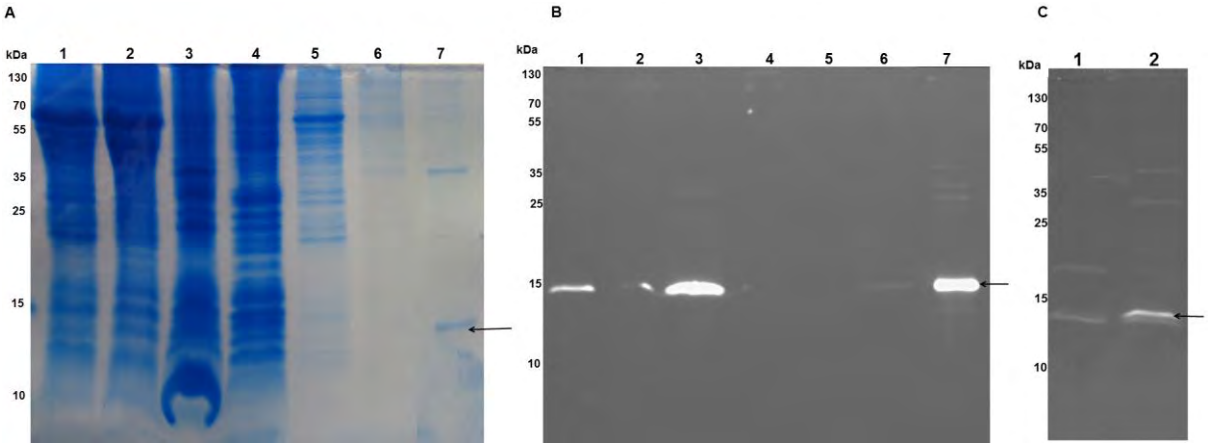


Figure 4.8. Purification of His tagged recombinant IL-4δ2 (rIL-4δ2). using Ni^{+} affinity column.(A) 15% SDS PAGE gel and (B) Western blot analysis (anti-His antibody at 1:5000 dilution) of rIL-4δ2 purification fractions showing (1) clarified rIL-4δ2 cell lysate (2) clarified cell lysate of negative control (uninfected *Sf21* cells) (3) Insoluble fraction (4) Insoluble fraction of negative control (5) rIL-4δ2 flow-through after column binding (6) rIL-4δ2 wash with 500mM imidazole (7) eluted and concentrated rIL-4δ2 fraction. In lane 7, a band migrating just below the 15kDa molecular weight marker (~14kDa) represented the partially purified rIL-4δ2 protein (arrow). Based on the Western blot, most of the rIL-4δ2 protein is located in the insoluble cellular fraction (lane 3 in B) (C) Western blot analysis of (1) clarified rIL-4δ2 cell lysate and (2) Insoluble fraction using anti-IL4 antibody (1:2000) confirms the identity of rIL-4δ2 at ~14kDa (arrow). Molecular weight markers are shown on the left in kiloDaltons (kDa). 15µl of each fraction was loaded onto the gel.

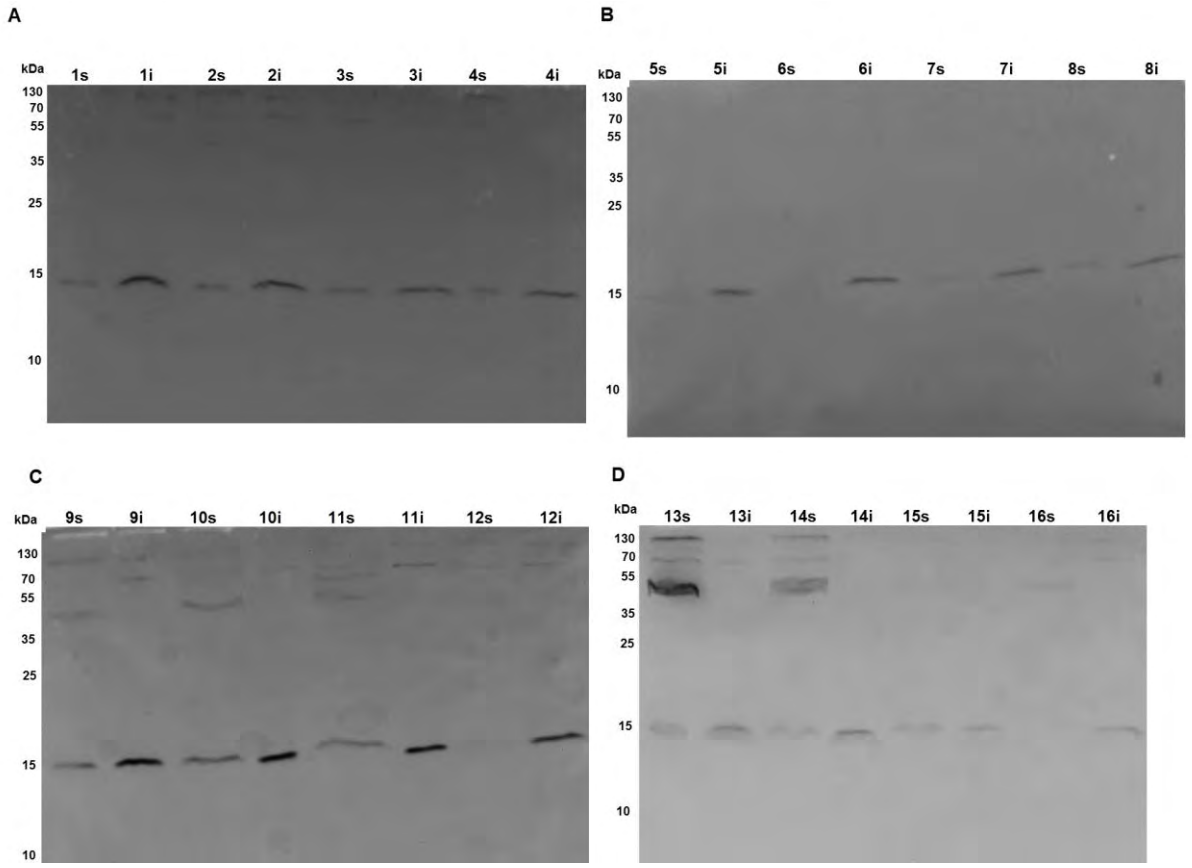


Figure 4.9. Optimization of cell lysis buffer parameters to increase the solubility of His-tagged recombinant IL-4δ2 (rIL-4δ2). Western blot analysis (A-D) of IL4δ2 soluble (s) and insoluble (i) cellular fractions using the following in the cell lysis buffer (1) $NaPO_4$ at pH6 (2) $NaPO_4$ at pH7 (3) $NaPO_4$ at pH8 (4) Tris at pH7.5 (5) HEPES at pH 7.5 (6) KCl 10mM (7) KCl 100mM (8) KCl 500mM (9) 0.1% Triton X-100 (10) 0.5% Triton X-100 (11) 1% Triton X-100 (12) IPER commercial lysis buffer (13) 0.1% sodium deoxycholate (14) 0.5% sodium deoxycholate (15) 1% sodium deoxycholate (16) 1% CHAPS. Anti-His antibody was used at 1:2000. Molecular weight markers are shown on the left in kiloDaltons (kDa). 15 µl of each fraction was loaded onto the gel.

4.3.3.3 Cloning of GST-tagged IL-4δ2 construct

A Glutathione-S-Transferase (GST) tag has been shown to increase the solubility of fusion proteins in a number of expression systems [532]. As such, a GST-tagged IL4δ2 construct was generated in an attempt to improve the solubility of IL4δ2. Initial cloning experiments to produce this construct were unsuccessful. Generation of this construct was therefore

outsourced to ExonBio (ExonBio, USA), where GST was introduced into the pAP01 vector followed by the IL4 cDNA sequence at the C-terminus of the GST tag and a TEV cleavage site between the GST tag and IL-4 cDNA. The subsequent construct, called pAP01-GST-TEV-IL-4, was further manipulated to produce the IL4δ2 construct. However, the position of the GST tag at the N-terminus of the IL-4 sequence prevents cleavage of the signal peptide by the insect cell machinery during protein expression. As a result, an inverse PCR was performed, using primers AP5_Inv1 and AP5_Inv2 (Appendix section B), to delete the IL-4 signal peptide and create the pAP01-GST-TEV-NSIL-4 construct, as previously described. Once DNA sequencing confirmed the removal of the signal sequence in pAP01-GST-TEV-NSIL-4, this construct was used in a second inverse PCR (using primers AP3_Inv1 and AP3_Inv2) to delete exon 2 and generate the pAP01-GST-TEV-NSIL-4δ2 construct. The cloning steps used to generate pAP01-GST-TEV-NSIL-4δ2 are shown in Figure 4.10A and the cDNA sequence of this construct is shown in section B of the Appendix. The structure and translated amino acid sequence of pAP01-GST-TEV-NSIL4δ2 are shown in Figure 4.10B.

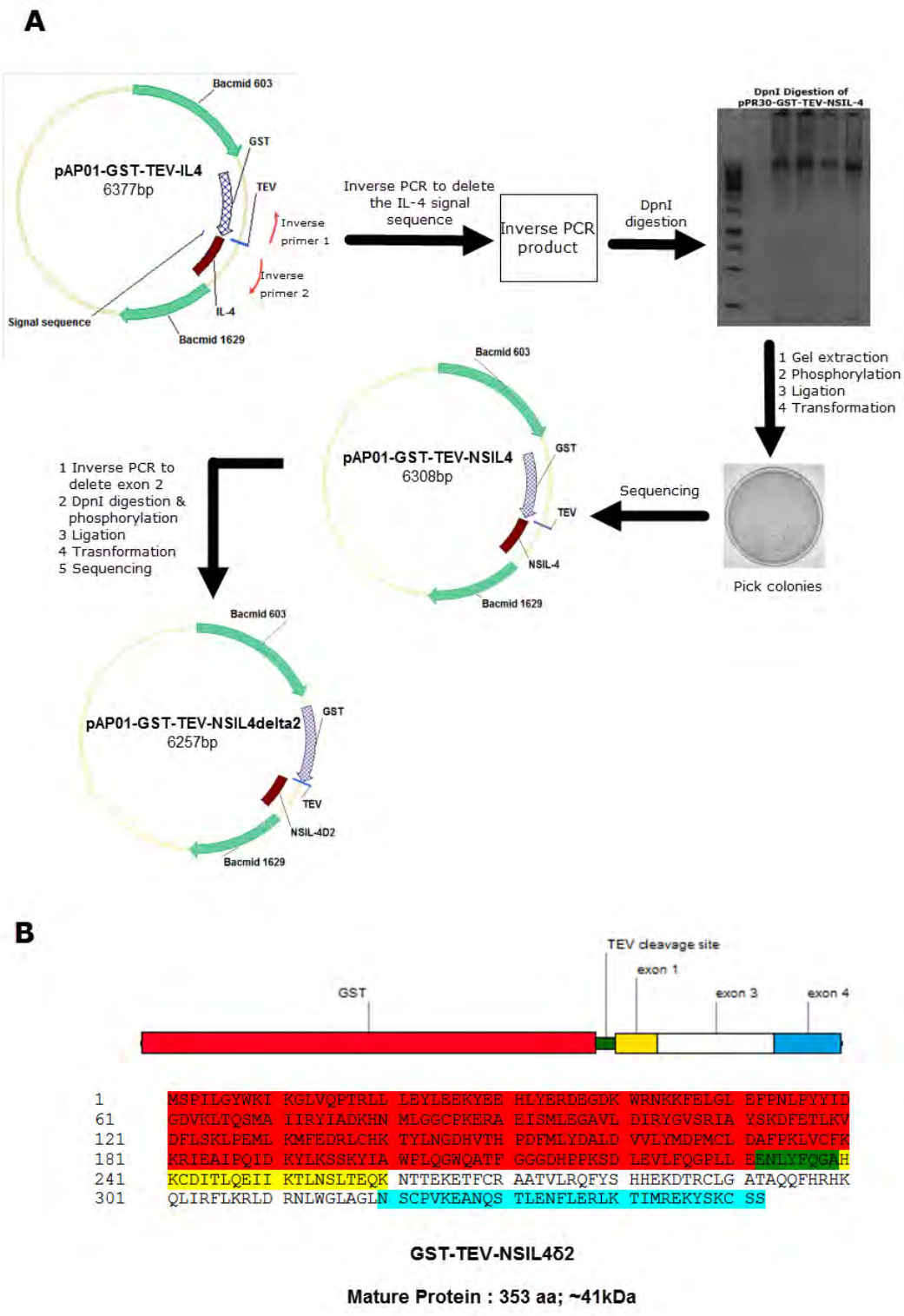


Figure 4.10 (A) the cloning steps to produce the pAP01-GST-TEV-IL4δ2 construct and (B) the structure and translated amino acid sequence of GST-TEV-IL4δ2.

4.3.3.4 Protein expression and purification of GST tagged recombinant IL-4δ2 protein using magnetic beads

Transfection of pAP01-GST-TEV-IL4δ2 and production of high titre viral stocks were performed as previously described (section 4.2.2.3). Small scale expression experiments revealed that an MOI of 20 and harvesting at 3 days PI was optimal for protein expression. Given the lack of a signal peptide, GST-tagged IL4δ2 was expressed in the intracellular compartment. These optimized conditions were used to infect a ~500ml insect cell culture at a cell concentration of $1.5x10^6$/ml. Cells were lysed, harvested and purified as described in section 4.2.2.8. SDS-PAGE and Western analyses using anti-GST antibody confirmed the presence of 2 proteins in the purified fractions; a band at ~41 kDa, which corresponded to the size of the GST-tagged IL4δ2, and a second band at ~25kDa (lane 4 of Figure 4.11A). Purification of uninfected insect cell lysates revealed that this 25kDa protein was also present in uninfected cells (lane 8 of figure 4.11A) suggesting that it is an intrinsic insect cell protein that is being co-purified along with the GST-tagged IL4δ2. A previous study [533] has also reported the co-purification of this glutathione binding protein (GluBP) during GST purification. The large amount of GluBP in the uninfected cells suggests that this protein is in much larger quantities than the GST- IL4δ2 protein and is likely competing for available binding sites leading to a reduced IL4δ2 yield. This competing effect is maintained even if increasing amounts of magnetic beads are added to the lysates during purification. Pichet *et al* [533] suggested that GluBP has a lower affinity for glutathione than GST and will elute at a lower concentration of glutathione. As a result, differential elution of the contaminating protein was attempted by gradually increasing the concentration of glutathione in the elution buffer from 5mM to 50mM. However, the GluBP eluted at all concentration of glutathione that were tested (figure 4.11B). Also, initial attempts to cleave the GST tag using ProTEV, in order to release the IL4δ2 protein from the beads while leaving the GluBP attached, were unsuccessful due to the almost nil recovery of cleaved IL4δ2. Use of size exclusion chromatography or further optimization of cleavage conditions may be necessary to isolate IL4δ2 protein but, at this point, protein expression using this construct was not pursued further.

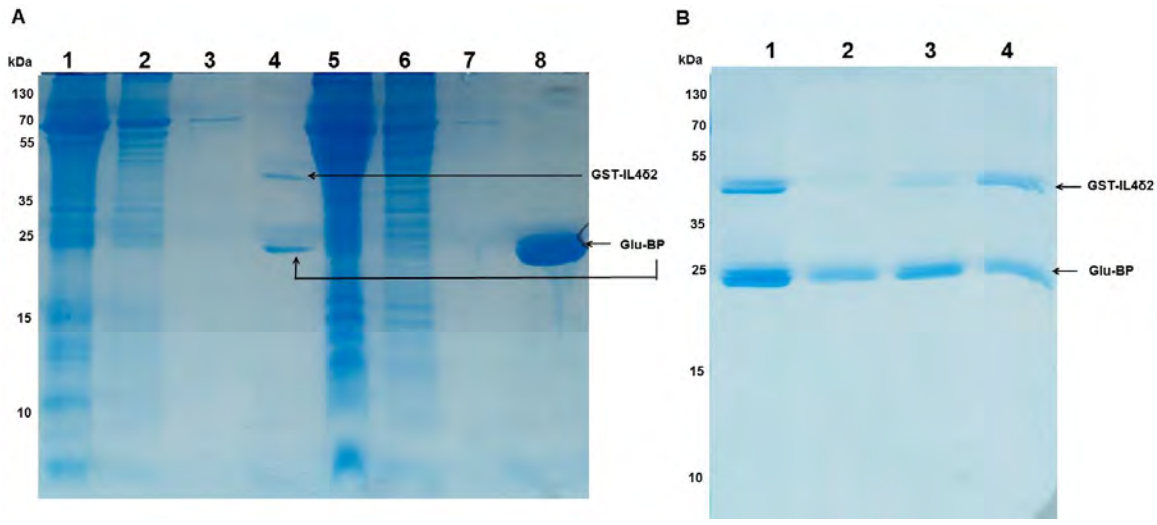


Figure 4.11. Purification of GST tagged recombinant IL-4δ2 (GST-IL-4δ2; ~41 kDa) using glutathione magnetic beads and attempts to remove a contaminating intrinsic Glutathione binding insect cell protein (Glu-BP; ~24 kDa) (A) 12% SDS PAGE gel of GST-IL-4δ2 and uninfected *Sf21* insect cells purification fractions showing the presence of the Glu-BP "outcompeting" GST-IL-4δ2 for available binding sites on the beads (1) GST-IL-4δ2 clarified cell lysate (2) GST-IL-4δ2 flow-through after bead incubation (3) GST-IL-4δ2 wash (4) eluted GST-IL-4δ2 fraction (5) uninfected *Sf21* clarified cell lysate (6) uninfected *Sf21* control flow-through after bead incubation (7) uninfected *Sf21* wash (8) eluted uninfected *Sf21* fraction. (B) A 12% SDS-PAGE gel showing purified GST-IL-4δ2 fractions in an attempt to remove GluBP by using differential glutathione elution conditions [533]. Elution at (1) 5mM glutathione (2) 10mM glutathione (3) 20mM glutathione (4) 50mM glutathione failed to reduce the amount of Glu-BP present in the pure fraction. Molecular weight markers are shown on the left in kiloDaltons (kDa). 15µl of each fraction was loaded onto the gel.

4.4 Discussion

This chapter described the attempt to express recombinant IL-4 and IL-4δ2 using a baculovirus expression system. His-tagged rIL-4 and rIL-4δ2 was successfully expressed and purified by affinity purification in sufficient amounts for functional assessment. However, His-tagged rIL-4δ2 protein recovery and purity were low post-purification and the majority of protein was insoluble. Attempts to increase solubility and purity were unsuccessful. A GST-rIL-4δ2 construct was used to successfully express N-terminus GST-tagged rIL-4δ2. However, competitive binding with an intrinsic glutathione binding insect cell protein (GluBP) during the purification process resulted in low rIL-4δ2 recovery.

4.4.1 Expression of rIL-4 in baculovirus infected insect cells

In the above experiments, rIL-4 was cloned with its native signal peptide and the expressed protein was secreted into the extracellular media suggesting that the insect cell machinery was able to effectively recognize and cleave the signal peptide and fold the protein in its native conformation. Similar secretory expression of mammalian rIL-4 has been demonstrated in a baculovirus system [518, 534] and is also commercially available for a range of species. A His-tagged fusion protein was constructed to obtain purified protein by affinity purification rather than the use of virus-containing supernatants [534] for functional and immunological assays. A His-tag was chosen because of its small size and non-immunogenicity and usually does not affect protein folding or require removal following purification [535, 536]. Nonetheless, cleavage of the His tag was attempted but, due to low protein recovery post-cleavage, was not pursued. Two distinct bands were observed on the SDS-PAGE gel which may represent alternatively glycosylated forms of the protein [518], but this is unlikely given that only a single band was observed in the Western blot. IL-4 undergoes N-linked glycosylation in the mature protein. However, N-linked glycans in insect cells are not equivalent to mammalian cells as they have high mannose content and lack sialic acid [537]. Inappropriate glycosylation can have significant effects on protein stability, conformation and bioactivity [508, 509]. Functional assays were performed to determine rIL-4 bioactivity and will be discussed in Chapter 5.

4.4.2 Expression of rIL-4δ2 in baculovirus infected insect cells

To my knowledge, this is the first study to attempt expression of the IL-4 splice variant, IL-4δ2, in a baculovirus system. Unlike IL-4, IL-4δ2 was not secreted into the culture supernatant but remained in the intracellular compartment, suggesting an incompatibility of IL-4δ2 secretion in the insect cell secretory pathway. In eukaryotes, protein secretion is mediated by the signal peptide which, through binding to a signal recognition protein, directs the precursor protein to the endoplasmic reticulum (ER). Here, it is cleaved by signal peptidases, properly folded and glycosylated before transport to the Golgi apparatus for additional modifications and subsequent extracellular secretion [538, 539]. The efficiency of this process is a function of the host expression system and the protein itself [539]. In IL-4 and IL-4δ2, the signal peptide comprises the first 24 amino acids of the precursor protein which is cleaved to produce the mature protein. The observation that IL4, but not IL-4δ2, is secreted into the media under the direction of the same signal peptide suggests that the sequence and/or structure of IL-4δ2, rather than an inappropriate signal peptide, may be interfering with the insect cell secretory mechanisms. The IL-4 structure consists of 4 α-helix bundles with a short two-stranded anti-parallel β-sheet and is stabilized by 3 disulfide bonds[385, 386]. The omission of exon 2 in IL-4δ2 resulted in a protein structure which lacks the β-sheet and a disulfide bond due to deletion of a cysteine residue [385, 386]. Despite these structural differences, IL-4δ2 retains the majority of the hydrophobic core and native structure of IL-4 [386]. Furthermore, IL-4δ2 seems to exists as a naturally occurring protein in mammals [31] suggesting that the protein is stable in its natural environment and these effects on secretion may be specific to expression in a baculovirus system. There is evidence indicating that protein structure can influence the secretory pathway. Choo *et al* demonstrated that recognition of signal sequences by signal peptidase can extend into the mature protein sequence and subsequently affect signal peptide cleavage in various expression hosts [540]. Furthermore, studies of deletion mutants in different expression systems have demonstrated that protein structure can reduce the ability of the signal peptide to direct translocation to the ER [541, 542]. This effect may be due to a reduced affinity of the signal recognition protein for the signal peptide, interference with signal peptidase activity or a combination of the two [541]. A similar effect may be occurring with IL-4δ2 expression in this system. It was difficult to determine if the signal peptide was cleaved

because the difference in size (~1-2 kDa) of rIL4δ2 with or without the signal peptide was too small to adequately resolve on an SDS PAGE gel. Given that proper protein function requires removal of the signal sequence, rIL4δ2 was subsequently expressed using a construct where the signal peptide sequence was deleted (pAP01-NSIL-4δ2-TEV-His; section 4.3.3.1). However, it cannot be ruled out that the use of the intrinsic human IL-4 signal peptide may be suboptimal for expression in the baculovirus-insect cell system. Atamas *et al* successfully expressed IL-4δ2 in *P. pastoris* using a yeast specific α-factor secretory signal that directs extracellular secretion of the protein within this system [47]. In future experiments, replacement of the human IL-4 signal sequence with one more appropriate for a baculovirus system, such as the honey bee mettilin sequence [543], will be attempted to improve secretion of the protein.

While IL-4δ2 was successfully purified from cell lysates, the amount of recovered protein was low as a significant quantity remained as insoluble aggregates. Protein aggregation in a baculovirus system has been previously reported [514, 515], and is likely a direct consequence of failed protein secretion. In insect cells, the secretory pathways can be hindered by overproduction of protein due to the strong polyhedron promotor, a lack of appropriate chaperone molecules or deterioration of infected cells, all resulting in accumulation of proteins and subsequent aggregation within the infected cells [544]. Protein overexpression and cellular localization has been shown to cause aggregation of GFP in a baculovirus system [539]. In the rIL-4δ2 expression described here, removal of the signal sequence inevitably resulted in protein accumulation within the cell and likely led to the observed formation of insoluble aggregates. A probable mechanism of protein aggregation involves the formation of specific intermolecular interactions between partially folded protein intermediates [545]. Mutations in native proteins may also form similar interactions due to alterations in protein folding. Studies have shown that point mutations in human proteins expressed in different systems have a dramatic effect on the production of protein aggregates [546, 547]. In the case of IL-4δ2, which contains a free cysteine residue, intermolecular disulfide bonds may form between adjacent protein molecules [548] leading to the observed protein aggregation.

The low yield of IL-4δ2 also probably explains the low purity after protein purification, which is particularly evident when proteins are expressed intracellularly. Secreted proteins tend to be easier to purify because the majority of protein in the supernatant consists of the target recombinant protein [549]. Indeed, rIL-4 protein obtained from cell supernatants had a purity of >85%. In contrast, the large number of histidine-rich proteins in eukaryotic cells (including insect cells) makes it difficult to obtain His-tagged recombinant proteins of sufficient purity. A decahistidine tag was used to increase the affinity of IL-4 and IL-4δ2 fusion protein to Ni^{2+} beads during binding. However, IL-4δ2 still only consisted about 30% of the purified fraction. Similar purity issues occurred with the GST-tagged protein due the presence of an intrinsic GluBP.

4.4.3 Limitations and future investigations

Sufficient levels of rIL-4 were obtained from expression in the baculovirus system, whereas rIL-4δ2 expression proved to be more problematic and resulted in low protein yield. Due to time and resource constraints, further optimization of IL-4δ2 expression was not possible and additional investigations, detailed below, will be required to obtain sufficient amounts of IL-4δ2.

As stated above, IL-4δ2 protein was not secreted into the media even under the direction of the intrinsic IL-4 signal peptide. This was unexpected given that IL-4 was successfully processed and secreted into the media using the same signal peptide. Future experiments will replace the intrinsic IL-4 signal peptide with a sequence that directs secretion of proteins in baculovirus infected insect cells, such as the honey bee metillin leader sequence, which has been shown to significantly increase protein secretion and yield [543].

The presence of the His-tag on the C-terminal end of the protein may have affected rIL-4δ2 protein expression. Optimal placement of the tag, either on the C-terminal or the N-terminal end, is usually protein specific [536]. However, this is unlikely given that this did not affect rIL-4 expression, which has a similar protein structure. Furthermore, the His tag is small and

uncharged under physiological conditions so it usually does not interfere with protein folding and compartmentalization [536]. Nonetheless, movement of the His tag to the N-terminal end of the IL-4δ2 sequence will be attempted to determine if tag placement has a significant effect on expression levels, solubility or protein localization.

Cleavage of the affinity tag from expressed proteins was performed to try to improve the purity of the eluted proteins, particularly in the case of His- and GST-tagged rIL-4δ2. However, these efforts were unsuccessful because IL-4δ2 expression levels were very low before cleavage and were further reduced post-cleavage. Attempts to increase the expression levels of IL-4δ2 or further optimize the TEV cleavage conditions will be performed to remove the affinity tag without compromising protein yield.

The use of another expression system may be required if sufficient quantities of IL-4δ2 cannot be produced in the baculovirus system. Functional IL-4δ2 has been previously produced in yeast [47] and mammalian cells [32, 393]. The mammalian expression system is most likely optimal for IL-4δ2 expression because it best mimics the native environment. However, expression levels here are usually lower than insect cells and the use of adenovirus constructs requires additional biosafety considerations. In the *P. pastoris* system, Atamas *et al* used a yeast-specific leader sequence to express IL-4δ2 [47]. This system is a viable option but was not feasible when this study was being conducted. Expression of IL-4δ2 in *E. coli* has been previously reported [525] but was not used for reasons stated in Table 4.1. Indeed, most of the protein was expressed as insoluble inclusion bodies in this study. However, optimization of reaction conditions, the use of an appropriate promoter and signal peptides sequences specific for a prokaryotic system and efficient endotoxin removal techniques may produce IL-4δ2 protein suitable for immunological assays. Further investigation will be required to determine which expression system would be most appropriate.

4.5 Conclusion

Human rIL-4 was successfully cloned and expressed in a baculovirus insect cell expression system in adequate amounts for further downstream analyses. rIL-4δ2 was also successfully expressed but the protein yield was much lower compared to IL-4. Further investigations need to be performed to optimize IL-4δ2 expression in this system. In the following chapter, rIL-4 and rIL-4δ2 will be used in immunological assays to determine the activity of these proteins *in vitro*.

5. CHAPTER 5: Characterization of recombinant IL-4 and IL-4δ2 amino acid sequence and protein function

5.1 Introduction

IL-4 is a pleiotropic cytokine with multiple effects on different cell types including T and B-lymphocytes, monocytes, macrophages, and fibroblasts [347]. The effects of IL-4 in *in vitro* cultures have been well described. These include inducing T-cell proliferation [47, 392], driving Th2 differentiation [550], inducing CD23 expression and IgE production in B cells [48, 392] and inhibiting LPS-induced cyclooxygenase-2 expression and subsequent prostaglandin E2 secretion in monocytes [47, 48]. IL-4δ2, the naturally occurring splice variant of IL-4, is an IL-4 antagonist and suppresses IL-4-driven responses. For example, IL-4δ2 downregulates IL-4-induced T cell proliferation and CD23 expression on B lymphocytes and activates the production of pro-inflammatory cytokines IFN-γ, IL-6 and TNFα in human T-cell cultures [32, 47, 48, 392, 393]. Recently IL-4δ2 has been shown to be a naturally occurring protein and is associated with pulmonary inflammation in mice [502]. Like IL-4, IL-4δ2 binds to the IL-4 receptor (IL-4R) but at a lower affinity. Furthermore, it is presumed the binding of IL-4δ2 to the IL-4R does not induce a signal cascade in the cell [392].

The aim of this chapter is to confirm the amino acid sequence, by mass spectrometry, and function of recombinant IL-4 and IL-4δ2 expressed in a baculovirus-insect cell system using specific *in vitro* assays.

5.2 Methods

5.2.1 Mass spectrometry

The rIL-4 protein was analyzed by mass spectrometry (MS), performed by Dr. Brandy Young-Gqamana at the Blackburn Lab in the Department of Medical Biochemistry, University of Cape Town. 20µg of purified rIL-4 was used for MS analysis. The lysis buffer (8% SDS, 200mM Tris-HCl, 0.2M TCEP) was added to purified protein (chapter 4) followed by sonication for 1 minute. The sample was heated at 90^{o}C for 3 minutes then loaded onto a

0.5ml 3kDa MWCO centrifugal filter (Millipore) pretreated with 50mM Ammonium Bicarbonate (ABC) and proteolysed on the MWCO centrifugal filter using a FASP protocol [551]. In short, the sample was reduced, alkalyated and digested using lysis buffer, iodoacetamide and trypsin respectively. The sample was incubated overnight at 37^{o}C and the peptides were collected and further analyzed by product ion scan using a TSQ Vantage mass spectrometer (ThermoFisher). The data was processed using Skyline software.

5.2.2 PBMC isolation

Whole blood was obtained by venipuncture from 10 healthy controls. PBMCs were isolated by density centrifugation as described in section 2.4.2.

5.2.3 ^{3}H thymidine assay to measure T-cell proliferation

A total of 5x10^{4} PBMCs suspended in RPMI 1640 (Lonza) (supplemented with 10% pooled human AB serum and 1:100 penicillin and streptomycin) were seeded in 96-well round bottom plates. Cells were stimulated with anti-CD3 antibody (eBiosciences) at 250ng/ml and incubated at 37^{o}C and 5% CO_2 for 3 days. On day 3, specified amounts of rIL-4 (5-100ng/ml), rIL-4δ2 (100-1000ng/ml) or a combination of both were added to appropriate wells. In order to test the effect of neutralizing IL-4, anti-IL4 antibody (Abcam) was added at concentrations of 0.5-20µg/ml to wells containing rIL-4. Appropriate controls were also included; phytohaemagglutinin (PHA; Sigma Aldrich) at 10µg/ml was used as a positive control and the negative control used His-purified fractions of the pAP01 expression control (section 4.2.2.6). All controls and interventions were performed in triplicate at a final volume of 200µl. Cells were incubated for a further 3 days at 37^{o}C and 5% CO_2. After 3 days, 1µCi of ^{3}H-Thymidine (AEC Amersham) was added to each well and incubated at 37^{o}C for 18 hours. The cells were then harvested using the Wesbart 2020 Maxi cell harvester (Wesbart) and counted by liquid scintillation spectrophotometry using a Tri-Carb 2100 TR liquid scintillation counter (Packard). The mean counts per minute (CPM) for each condition was used to calculate the % proliferation, in the case of rIL-4, and the % suppression in the case of IL-4δ2 and anti-IL-4 antibody, using the formulas below:

$$\left(\frac{\text{(Mean CPM of rIL-4 intervention) - (Mean CPM of neg control)}}{\text{Mean CPM of negative control}} \times 100 \right) = \text{\% Proliferation}$$

$$100 - \left(\frac{\text{Mean CPM of intervention with rIL-4+rIL4δ2 } \underline{\textbf{OR}} \text{ +anti-IL-4 Ab}}{\text{Mean CPM of rIL-4 intervention}} \times 100 \right) = \text{\% Suppression}$$

5.2.4 Flow cytometry to measure CD23 expression on B-cells

A total of $1.5x10^6$ PBMCs were seeded in 24 well plates. Cells were stimulated with rIL-4 at specified concentrations (0.5-50ng/ml) and incubated at 37°C and 5% CO_2 for 3 days. Cells were then harvested and stained for specific surface markers using fluorescently labeled antibodies (Table 2.2) and subsequently analyzed on a BD LSR II flow cytometer (BD Biosciences) as outlined in section 2.4.6.

5.2.5 Data analyses

Statistical differences between controls and the different interventions were determined using the Wilcoxon matched-pairs signed rank test. A p value of <0.05 was deemed significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad) software and Microsoft Excel (Microsoft).

5.3 Results

5.3.1 Confirmation of rIL-4 amino acid sequence by mass spectrometry

Trypsin specifically cleaves polypeptides at the carboxy terminus of lysine (K) and arginine (R) residues. Trypsin digest of the rIL-4 protein and subsequent analysis of the mass

spectrum identified 3 peptide fragments; a 9 amino acid peptide at lysine position 36 (TLNSLTEQK), a 6 amino acid peptide at arginine position 71 (AATVLR) and a 4 amino acid peptide at lysine position 101 (QLIR). An acceptable signal intensity was produced for each peak in the mass spectrum (signal:noise was >~10). The identified peptides were compared to a database of known protein sequences using the NCBI BLAST sequencing tool (http://blast.ncbi.nlm.nih.gov/) and all fragments were 100% homologous to the human IL-4 protein sequence. The observed fragment ions for each identified peptide are shown in figure 5.4. The masses of the fragment ions allow the sequence of each peptide to be determined. This data, as well as the expected size observed on the SDS-PAGE gel and the reactivity with anti-IL-4 antibody by Western blot (Chapter 4), provides sufficient evidence to confirm the identity of the purified rIL-4.

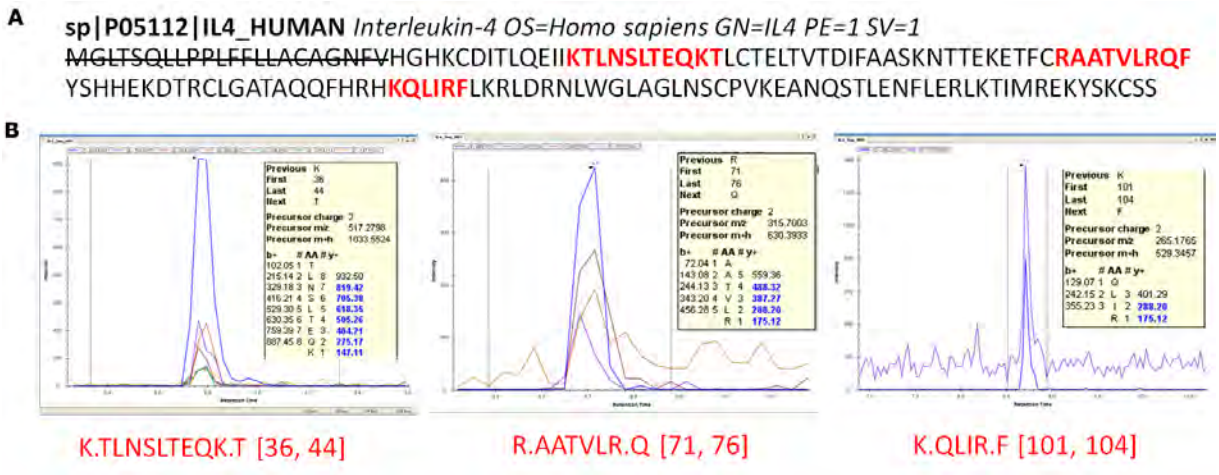


Figure 5.1. The confirmation of the recombinant IL-4 (rIL-4) protein using tandem mass spectrometry. Trypsin digestion of rIL-4 produced 3 peptide fragments. (A) The location of each fragment in the IL-4 protein sequence is shown above in red and the IL-4 signal peptide is crossed out as it is absent in the mature protein. (B) The fragment ion masses (m/z shown in blue) observed in the mass spectra provide the amino acid sequence for each peptide.

5.3.2 The effect of rIL-4 and rIL-4δ2 on T-cell proliferation

The addition of rIL-4 to anti-CD3 stimulated PBMCs (n=10) induced T-cell proliferation in a concentration dependent manner. Proliferation was 350% at 5ng/ml rIL-4 (p=0.002), 490% at

10ng/ml rIL-4 (p=0.002) and 670% at 20ng/ml rIL-4 (p=0.002) compared to the negative control (Figure 5 .2A). Proliferation in response to PHA (10μg/ml) stimulation was also observed. Neutralization of IL-4 using anti-IL-4 antibody (n=3) suppressed the observed T-cell proliferation. Addition of anti-IL-4 antibody (Ab) at 0.5, 5 and 20μg/ml suppressed T-cell proliferation induced by 20ng/ml rIL-4, by 65%, 88% and 94%, respectively. A similar pattern of suppression was observed when antibody was added to PBMCs stimulated with 100ng/ml rIL-4 (mean suppression was 26% at 0.5μg/ml anti-IL-4 Ab, 77% at 5μg/ml anti-IL-4 Ab and 82% at 20μg/ml anti-IL-4 Ab; Figure 5.2B). However the differences were not statistically significant.

Sufficient rIL-4δ2 was produced (Chapter 4) to assess its effect on IL-4-induced T-cell proliferation. The addition of rIL-4δ2 suppressed T-cell proliferation induced by 5ng/ml rIL-4 (mean suppression was 55% at 1000ng/ml rIL-4δ2, 44% at 500ng/ml rIL-4δ2 and 10% at 100ng/ml rIL-4δ2) and 10ng/ml rIL-4 (mean suppression was 30% at 500ng/ml rIL-4δ2 and 11% at 100ng/ml rIL-4δ2). The addition of rIL-4δ2 (100ng/ml) alone did not have any effect on proliferation as the CPM value was similar to the negative control (Figure 5.3). Results were not statistically significant as the assay was only performed on samples from 2 subjects.

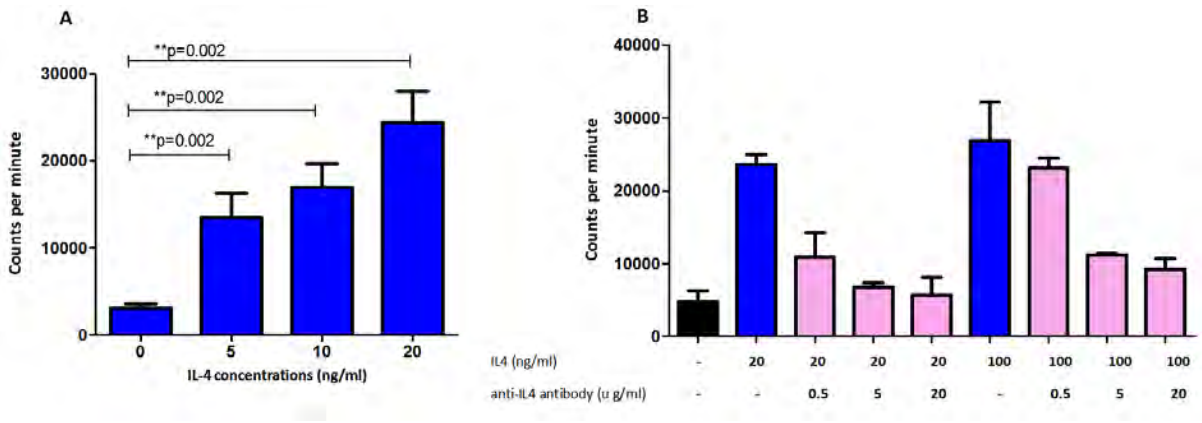


Figure 5.2. (A) Recombinant IL-4 induces proliferation of T-cells pre-activated with anti-CD3 in peripheral blood mononuclear cells of healthy participants (n=10) in a concentration dependent manner. (B) The addition of anti-IL-4 neutralization antibodies reduces the IL-4-mediated T-cell proliferation (n=3) in a concentration dependent manner. Proliferation was measured in a ^{3}H-Thymidine proliferation assay. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

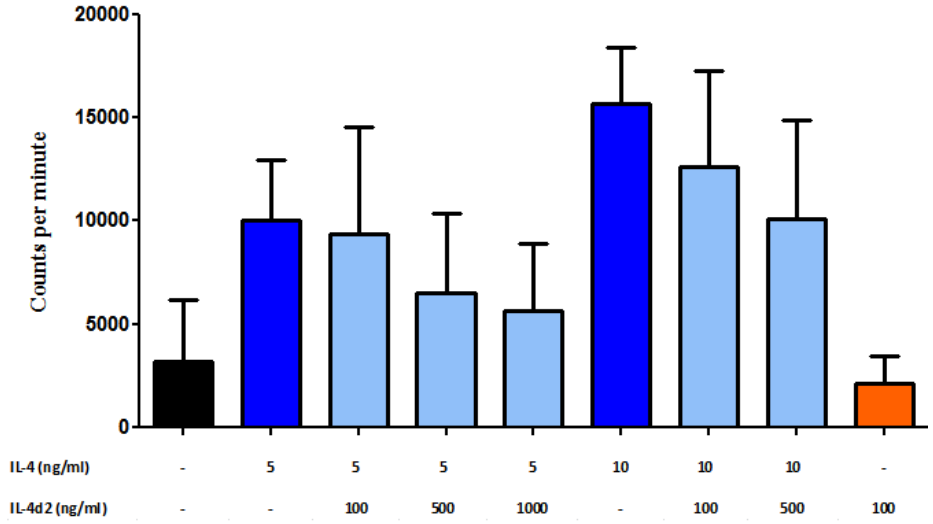


Figure 5.3. **IL4δ2 reduces the IL-4-mediated proliferation of T-cells pre-activated with anti-CD3 antibody in peripheral blood mononuclear cells of healthy participants (n=2) in a concentration dependent manner.** Proliferation was measured in a ^{3}H Thymidine proliferation assay. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

5.3.3 The effect of rIL-4 on CD23 expression in B-cells

The gating strategy used to identify CD23+ expressing B-cells in PBMCs is shown in Figure 5.4A. Lymphocytes were acquired based on cell size (FSC) and granularity (SSC). Cells were gated on CD3-CD19+ population to identify the B-lymphocyte population CD23+ cells were then identified within the B-lymphocyte (CD3-CD19+ population). CD23 expression was determined before and after the addition of rIL-4. The percentage (%) CD3-CD19+CD23+ was expressed relative to the B-lymphocyte population (CD3-CD19+).

rIL-4 induced CD23 expression on B-cells in healthy controls (n=6) in a concentration dependent manner. Median CD3-CD19+CD23+ levels increased when rIL-4 was added at 0.5ng/ml (29.9%, IQR 10.6-41.1; p=0.03), 1ng/ml (31.0%, IQR: 15.5-61.0; p=0.03), 5ng/ml (50.2%, IQR: 30.5-64.2; p=0.03), 10ng/ml (67.1%, IQR: 61.8-74.0; p=0.03) and 50ng/ml (69.7%, IQR: 64.6-75.2; p=0.03) compared to the negative control (3.1%, IQR: 0.9-8.0) (Figure 5.4B). PHA stimulation of PBMCs also resulted in increased levels of CD3-CD19+CD23+ cells. rIL-4δ2 was not assessed in this assay due to the limited amount of protein obtained from protein expression (chapter 4).

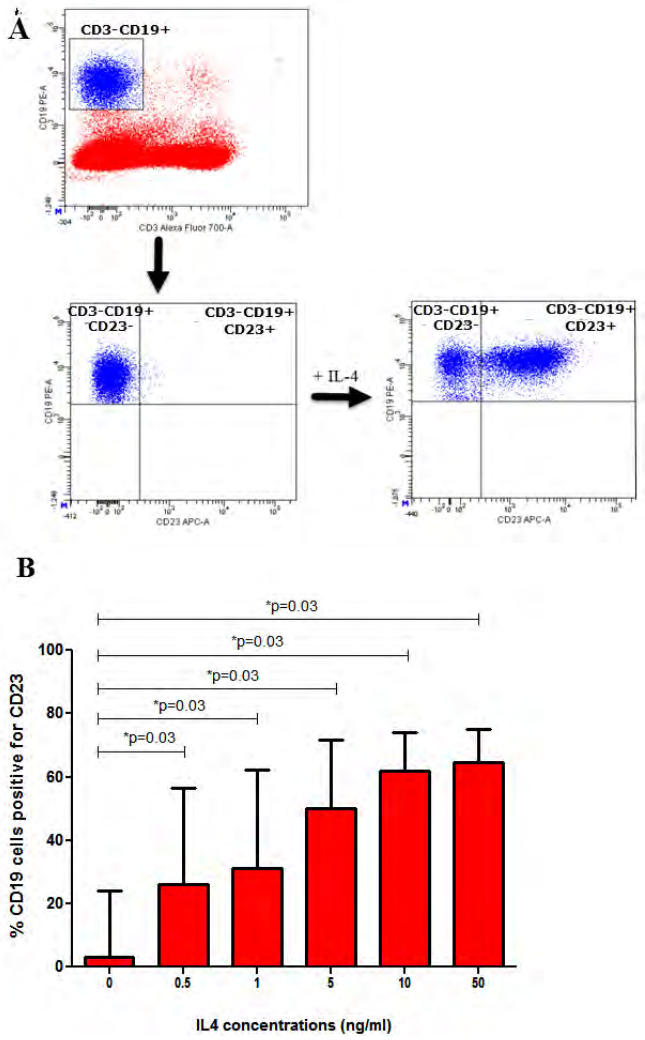


Figure 5.4. Recombinant IL4 induces expression of CD23 on B-cells (CD3+CD19+) in peripheral blood mononuclear cells of healthy participants (n=6) in a concentration dependent manner as measured by flow cytometry. The gating strategy for identification of CD3-CD19+CD23+ cells is shown in (A). The median frequency (%) and interquartile range of CD3-CD19+CD23+ cells at different concentrations of rIL-4 are shown in (B). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

5.4 Discussion

The identity of the purified rIL-4 protein was confirmed by mass spectrometry. rIL-4 was shown to induce T-cell proliferation in a concentration dependent manner and this proliferation was suppressed by the addition of anti-IL-4 neutralizing antibody. rIL-4 also

induced CD23 expression on B-cells in a concentration dependent manner. IL-4δ2 suppressed the observed IL-4-induced T-cell proliferation and had no effect on proliferation when added alone. The function of IL-4δ2 was not assessed in the B-cell CD23 assay or analyzed by mass spectrometry due to the limited amounts of recombinant protein available.

5.4.1 Assessment of IL-4 and IL-4δ2 function

The effects of IL-4 and IL-4δ2 on T-cell proliferation and B-cell CD23 expression confirms the bioactivity of rIL-4 and rIL-4δ2 proteins expressed in a baculovirus-insect cell system. Similar results were observed when these proteins were produced in a *P. pastoris* expression system [47, 48]. In the previous chapter (Chapter 4), rIL-4 and rIL-4δ2 were expressed and purified with the His affinity tag still attached to the mature protein because removal of the His tag resulted in insufficient protein recovery. As a result, His-tagged proteins were used and the data provided here shows that protein function remained intact, suggesting that the His-tag did not interfere with binding to IL-4 receptors. However, future investigations will determine if bioactivity is altered when the affinity tag is removed.

Very high concentrations of IL-4δ2 (~100-200 times the amount of IL-4) were found to be required to suppress IL-4-induced T-cell proliferation in PBMCs. Structural studies suggest that IL-4 contains 2 binding sites for its receptor (IL-4R) [552, 553]. Deletion of exon 2 in IL-4δ2 alters the protein structure and results in the loss of one of these binding sites [48]. Subsequently, the IL-4δ2 binding affinity for the IL-4R on T-cells is presumably reduced and much higher concentrations of IL-4δ2 are therefore required to „out-compete" IL-4 for the IL-4R binding sites.

IL-4 significantly increased CD23 expression, a low affinity IgE receptor, expression on B-cells at a lower concentration compared to what was needed for induction of T-cell proliferation. In a similar study, low concentrations of IL-4 effectively induced CD23 expression and required ~1000 times more IL-4δ2 than IL-4 to suppress this effect [48]. The apparent higher sensitivity of B-cells to IL-4 compared to T-cells, may be a reflection of the

different assay systems used or differences in the receptor systems on B-cells compared to T-cells.

Other studies have used similar concentrations of IL-4 and IL-4δ2 to test their bioactivity *in vitro* [47, 48, 392]. However, the effective concentrations of IL-4 and IL-4δ2 *in vivo* are significantly lower and may reflect differences in the stability and bioactivity of recombinantly expressed proteins compared to their native counterparts. Luzina *et al* [393] observed differences in the bioactivity of adenovirus-mediated gene delivery of IL-4δ2 in mice compared to *in vitro* assays using IL-4δ2 expressed in mammalian cells. One plausible explanation is the presence of chaperone proteins *in vivo,* which preserve protein stability and bioactivity, but are absent *in vitro* [393]. Co-expression of chaperone proteins in conjunction with the recombinant protein in an appropriate expression system can sometimes enhance protein folding and bioactivity [537]. The extent of recombinant protein bioactivity is also dependent on the expression system used. As discussed in chapter 4, N-linked glycosylation patterns in insect cell expressed proteins are different from those expressed in mammalian cells due to their high mannose content and lack of sialic acid [507, 537]. Inappropriate glycosylation can have a significant impact on protein stability and function [508, 509]. For example, human erythropoietin is 1000 times more active *in vivo* compared to its de-sialylated form [554]. Naturally occurring IL-4, and probably IL-4δ2 as well, undergoes N-glycosylation. Atamas *et al* [47] demonstrated that de-glycosylated IL-4 and IL-4δ2 still retain their function *in vitro*. However, other evidence suggests that inappropriate glycosylation can impact bioactivity to a greater extent than non-glycosylated forms. Two studies [555, 556] found that glycosylated forms of IL-4 expressed in *P. pastoris* and *COS* cells have lower bioactivity compared to their de-glycosylated forms. This may be explained by the presence of mannose on N-glycans which allows for rapid uptake of these isoforms by cultured cells and subsequent cellular degradation [555]. Also, the lack of sialic acid residues means these proteins are more prone to proteolytic attack [557, 558]. The ultimate consequence of reduced recombinant protein stability and bioactivity in *in vitro* experiments is that high concentrations of protein are required to observe a significant effect.

5.4.2 Limitations

The effect of glycosylation on the function of IL-4 or IL-4δ2 was not tested. As mentioned above, inappropriate glycosylation, which occurs in insect cells, can affect protein function [508, 509]. However, rIL-4 and rIL-4δ2 were found to be active at concentrations similar to those used in functional assays in other studies [48, 392]. Further investigations will be required, where the proteins are de-glycosylated using PNGase and their function compared to untreated protein, to determine if differential glycosylation has any effect on protein bioactivity.

High concentrations of IL-4δ2 were required to reduce IL-4-induced T-cell proliferation. Given low protein yield obtained for IL-4δ2 (Chapter 4), it was not possible to assess its effect on CD23 expression in B cells or for mass spectrometric analysis. Once IL-4δ2 expression has been optimized and sufficient amounts are produced, these assays will be performed to confirm the structure and function of the protein.

5.5 Conclusion

The data presented here demonstrates that rIL-4 and rIL-4δ2 expressed in a baculovirus system are functional based on their ability to modulate T-cell proliferation and CD23 expression on B-cells. The role of rIL-4 on mycobacterial survival was subsequently investigated in an *in vitro* infection model. However, due to the low yield of rIL-4δ2 and the high concentrations required to produce an effect on IL-4-induced T-cell proliferation, it was not possible to assess the effect of IL-4δ2 in subsequent assays. Further studies will have to be performed in order to address the above.

6. CHAPTER 6: Effect of IL-4 on mycobacterial containment

6.1 Introduction

Innate and adaptive immune responses are required for effective control of *M.tb*. Innate mechanisms, facilitated by infected macrophages and possibly neutrophils [183], represent the first line of defence against *M.tb* infection [235, 479, 559, 560]. In some exposed individuals, the innate system may presumably be able to completely eliminate infection without initiation of adaptive immune mechanisms [5]. In the majority of exposed individuals however, the adaptive immune response, mediated principally by CD4 IFN-γ producing T-cells, plays a key role in control of infection [559]. As discussed previously, IFN-γ is responsible for activation of macrophages [228], CD8 mediated cytotoxicity [561-563] and recruitment of additional T-cells to the site of infection [559]. Despite high levels of IFN-γ at the site of disease [9, 11, 13-15, 482, 483], some individuals still progress to active TB. Preliminary evidence suggests that a subversive Th2 response may be undermining protective Th1 mediated immunity [16]. However, the direct relationship between IL-4 and host mycobactericidal activity remains unclear. In chapter 3, I have demonstrated that TB patients have higher IL-4 mRNA levels compared to presumed LTBI controls in peripheral blood and the IFN-γ:IL-4 ratio, which has been proposed as a surrogate marker for disease progression, is lower in TB patients compared to controls. These results have been confirmed in other studies [9, 21, 29, 416-418, 487, 504]. However, it is unknown if these high IL-4 levels is a by-product of excessive inflammation (irrelevant epiphenomenon) or plays an active role in causing TB progression. A number of mechanisms, that are associated with protection against TB, are downregulated by IL-4 [16]. To my knowledge, there have been no published studies that directly assessed the impact of IL-4 on mycobacterial containment. In the previous chapters, I have expressed and functionally assessed recombinant IL-4 (rIL-4) protein in a baculovirus insect cell expression system. The aim in this chapter is to assess the effect of adding rIL-4, either to effector cells and/or macrophages on mycobacterial survival within infected monocyte derived macrophages.

6.2 Methods

6.2.1 Mycobacterial containment assay overview

A mycobacterial containment assay was used to determine the effect of rIL-4 on the ability of effector cells and macrophages to control the intracellular containment of *M.tb* within monocyte derived macrophages (MDMs). Approximately 70ml of peripheral blood was collected from patients with active pulmonary TB and presumed LTBI controls. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (section 2.4.2). Monocytes were cultured for 5 days to generate monocyte derived macrophages (section 2.4.4). Simultaneously, PBMCs were cultured and stimulated with PPD (Statens Serum Institute) for 6 days to generate pre-primed effector cells. On day 5, previously grown stocks of H37*Rv* (section 2.4.5), a virulent laboratory strain of *M.tb*, were used to infect MDMs for 18 hours at a multiplicity of infection (MOI) of 3:1. On day 6, non-ingested H37*Rv* was removed by washing with warm RPMI and the pre-primed effector cells were co-cultured with infected MDMs for 48 hours followed by lysis of the infected MDMs. Serial dilutions of cell lysates (containing H37*Rv*) were made and plated on 7H10 Middlebrook agar. Colonies were counted and expressed as colony forming units/ml (CFU/ml). Appropriate controls containing infected MDMs but no effector cells (both with and without removal of non-ingested H37*Rv* by washing) were included. In order to assess the effect of IL-4 on mycobacterial growth, rIL-4 (expressed and tested as described in chapter 4 and 5) was added at several concentrations at multiple time points during the assay. Infected MDMs were harvested at 48 hours and 120 hours post co-culture to determine the optimal time to harvest T-cells. These preliminary experiments were performed on presumed LTBI controls and are shown in section C of the Appendix. Based on these results, *M.tb* containment assays were then performed on samples from additional LTBI controls and TB patients. Descriptions of experimental controls and interventions are given below. An outline of the assay is shown in Figure 6.1 and details on stimulant concentrations and cell numbers are given in Table 6.1. Detailed descriptions of each step in the assay are provided in sections 6.2.1.1 to 6.2.5.

6.2.1.1 Experimental controls

6.2.1.1.1 MDMs infected with H37Rv

MDMs are generated (section 6.2.2) for 5 days and infected with H37*Rv* at an MOI of 3:1 for 18 hours. After 18 hours, the infected MDMs are either washed with warm RPMI to remove non-ingested bacteria (❷ in Figure 6.1 and Table 6.1) or not washed thus leaving the non-ingested bacteria in the well (❶ in figure 6.1 and Table 6.1). No effector cells were added to these wells. The % difference in CFU/ml between these two controls gave an indication of H37*Rv* uptake by MDMs (see Figure 6.2). The washed H37*Rv* infected MDM control was used as the baseline to determine the % *M.tb* containment.

6.2.1.1.2 PPD effectors

MDMs were generated, infected and washed as described in section 6.2.2. Concurrently, autologous PBMCs were stimulated with PPD (12µg/ml) for 6 days to generate PPD potential effector cells. On day 6, these PPD effectors were co-cultured with the infected MDMs (❸ in Figure 6.1 and Table 6.1).

6.2.1.2 Experimental interventions using rIL-4

6.2.1.2.1 PPD/IL-4 effectors

MDMs were generated, infected and washed as described in section 6.2.2. Concurrently, PBMCs were stimulated with PPD (12µg/ml) and IL-4 at concentration of 5, 20 and 100 ng/ml for 6 days to generate PPD effector cells. On day 6, these PPD effectors were co-cultured with the infected MDMs (❹ in Figure 6.1 and Table 6.1).

6.2.1.2.2 Exogenous IL-4

MDMs were generated, infected and washed as described in section 6.2.2. Concurrently, PBMCs were stimulated with PPD (12µg/ml) for 6 days to generate PPD effector cells. On day 6, these PPD effectors were co-cultured with the infected MDMs and rIL-4 was added at concentrations of 5, 20 and 100 ng/ml (❺ in Figure 6.1 and Table 6.1).

6.2.1.2.3 Neutralization of IL-4

„PPD/IL-4 effectors" wells were setup as stated in section 6.2.1.2.1 (4 in Figure 6.1 and Table 6.1). However, anti-IL-4 neutralizing antibody at 20µg/ml (Abcam) was added, together with PPD and rIL-4 (20 and 100ng/ml) to PBMCs for 6 days to generate effector cells. On day 6, these effectors were co-cultured with the infected MDMs.

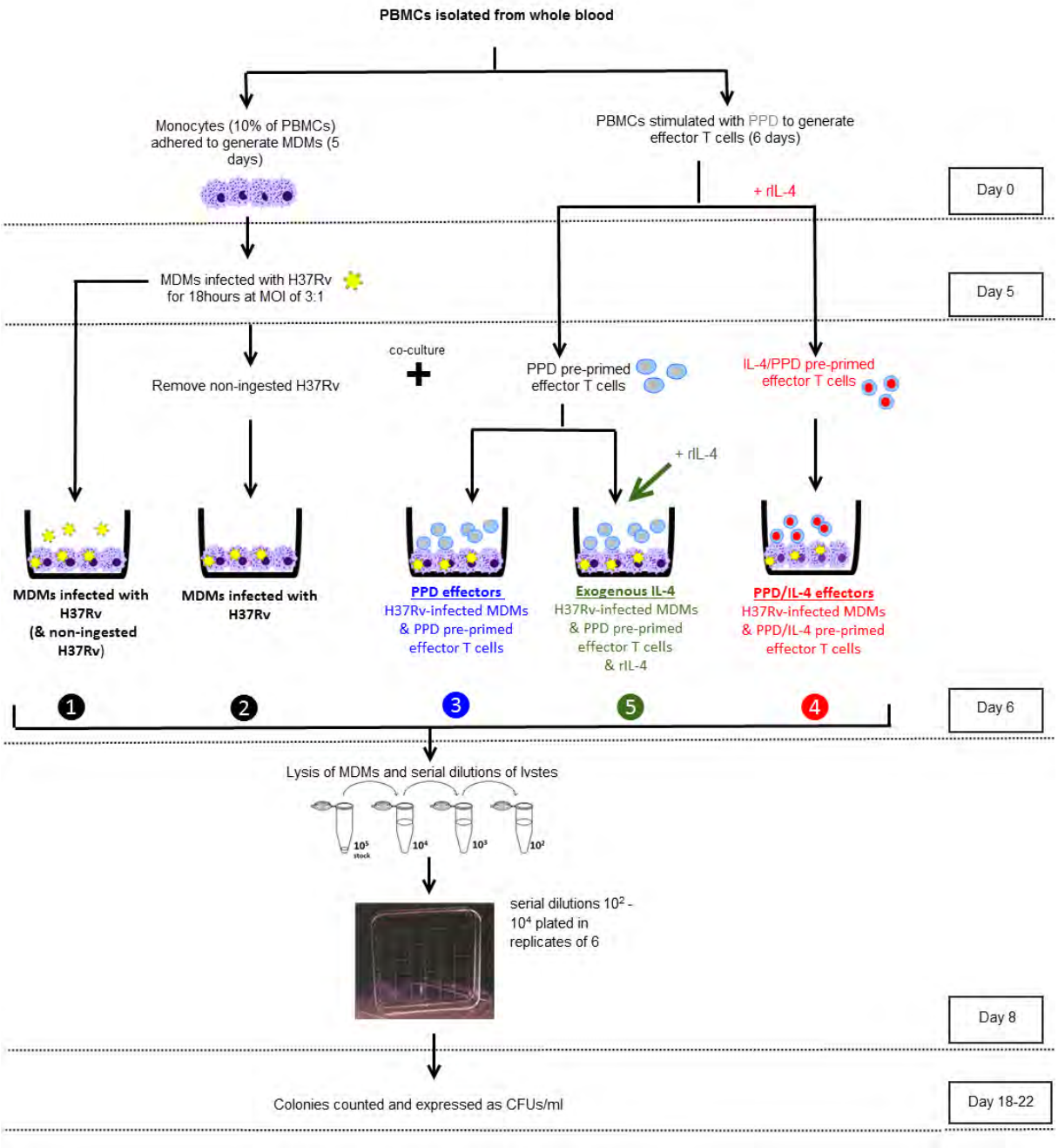


Figure 6.1. Overview of the mycobacterial containment assay to determine the effect of recombinant IL-4 (rIL-4) on *M.tb* containment within monocyte-derived macrophages. Culture conditions and stimulant concentrations of controls and interventions (labeled ❶ - ❺ in diagram) are further described in table 6.1

Table 6.1. Details of experimental setup including cell and stimulant concentrations in the mycobacterial containment assay (as described in Figure 6.1).

	No.*	Condition	DAY 0					DAY 6	
			MDMs		PBMCs				
			Cell number	stimulant	Cell number	stimulant		Cells co-cultured with infected MDMs	stimulant
				rIL-4 (ng/ml)		PPD (µg/ml)	rIL-4 (ng/ml)		rIL-4 (ng/ml)
Controls	1	MDMs infected with H37Rv (&non-ingested H37Rv)	$2x10^5$	-	-	-	-	-	-
	2	MDMs infected with H37Rv	$2x10^5$	-	-	-	-	-	-
	3	PPD effectors	$2x10^5$	-	$2x10^5$	12	-	PPD effectors	-
Interventions	4	PPD/IL4 effectors	$2x10^5$	-	$2x10^5$	12	5, 20, & 100	PPD/IL4 effectors	-
	5	Exogenous IL4	$2x10^5$	-	$2x10^5$	12	-	PPD effectors	5, 20, & 100

*corresponds to the controls/interventions shown in figure 6.1

6.2.2 Generation of MDMs

MDMs (~2x10^5) were generated in 96-well flat bottom plates as described in section 2.4.4.

6.2.3 Culture and stimulation of PBMCs

PBMCs were isolated (section 2.4.2) and 2x10^5 were seeded in 96-well round bottom plates (Nunc). Cells were stimulated with PPD (12µg/ml), PPD + rIL-4 (5, 20 and 100ng/ml) or PPD + rIL-4 (20, 100 ng/ml) + anti-IL-4 antibody (20µg/ml) in a final volume of 200 µl/well. Cells were incubated at 37°C in a 5% CO_2 humidified chamber for 6 days.

6.2.4 Infection of MDMs with H37Rv and co-culture of pre-primed effector cells and infected MDMs

Following generation of MDMs after 5 days, non-adherent T-cells removed by gentle washing with warm RPMI 1640 (Lonza). Adherent MDMs were infected with H37*Rv*, at an MOI of 3:1. This MOI was chosen based on preliminary optimization experiments, but additionally as a low MOI has been shown to reduce bacterial clumping during infection and maintains MDM viability *in vitro* [564]. Based on this MOI, a total of 6x10^5 CFUs were added to each well and incubated at 37°C and 5% CO_2 for 18 hours. Cells were then washed with warm RPMI to remove non-ingested H37*Rv* (except in the H37*Rv*-infected MDM control that was not washed; ❶ in figure 6.1 and Table 6.1). The pre-primed effector cells, generated by stimulation of PBMCs, were harvested and washed in RPMI. These effector cells were then added to the well containing H37*Rv* infected MDMs in a final volume of 250µl. The co-cultured cells were incubated at 37°C and 5% CO_2 humidified chamber for 48 hours. In the „Exogenous IL-4“ intervention (section 6.2.1.2.2 and ❺ in Figure 6.1 and Table 6.1), rIL-4 was added to the co-cultured cells at concentrations of 5, 20 and 100ng/ml.

6.2.5 Lysis of infected cells and plating of cell lysates

Following co-culture, cells were washed with warm RPMI and the supernatants discarded to remove cell debris and extracellular H37*Rv* (except in the H37*Rv*-infected MDM control that

was not washed; ❶ in figure 6.1 and Table 6.1). 200µl of H_2O was then added to each well to lyse infected cells and release intracellular bacteria. These lysates were then harvested and four 10-fold serial dilutions were made in 0.25% Tween-80 solution (0.25 % Tween 80 (Merck) in PBS). Six 10µl replicates of each dilution was pipetted onto each grid of a gridded 7H10 Middlebrooks agar plate. The plates were incubated at 37°C for 10-14 days or until visible colonies had formed. Colonies were counted using an inverted microscope.

6.2.6 Data analysis

Colonies were counted and expressed as colony forming units per ml (CFU/ml). Controls were performed in duplicate and the average CFU/ml was reported. In order to normalize the data to account for inter-patient variability, the percentage (%) mycobacterial containment relative to the MDM only control was also reported. An increase in % mycobacterial containment represents decreased *M.tb* survival whereas a reduction in % mycobacterial containment represents an increase in *M.tb* survival. The % mycobacterial containment was calculated as follows:

$$100 - \left(\frac{\text{Experimental intervention (CFU/ml)}}{\text{H37Rv-infected MDM control only (CFU/ml)}} \times 100 \right) = \text{\% Mycobacterial containment}$$

Differences between experimental controls and interventions, in terms of absolute CFU/ml and % mycobacterial survival, were determined using the Wilcoxon matched-pairs signed rank test and Mann-Whitney U test. A p value of <0.05 was deemed significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad) software and Microsoft Excel (Microsoft).

6.3 Results

6.3.1 Mycobacterial uptake by monocyte derived macrophages

As stated in section 6.2.1.1.1, after infection of MDMs with H37*Rv*, non-ingested bacteria were either removed by washing (❷ in Figure 6.1 and Table 6.1) or were not washed and left in the well (❶ in figure 6.1 and Table 6.1). The „unwashed" H37*Rv* infected MDM control represents the total amount of bacteria added to the well and the difference between these 2 controls represents the *M.tb* uptake by MDMs. When all participants (Figure 6.2A; n=10) were considered, the CFU/ml decreased from 268.4x10^3 in the „unwashed" control to 76.7x10^3 in the washed control, representing a 34% uptake of H37*Rv* by macrophages. Viable mycobacterium decreased from 268.4x10^3 to 51.5x10^3 CFU/ml in TB patients (n=6) and 316.5 x 10^3 to 109.5x10^3 CFU/ml in presumed LTBI controls (n=4) in „unwashed" compared to „washed" MDM controls, representing a median *M.tb* uptake of 19% and 34% respectively (p=0.11; Figure 6.2B).

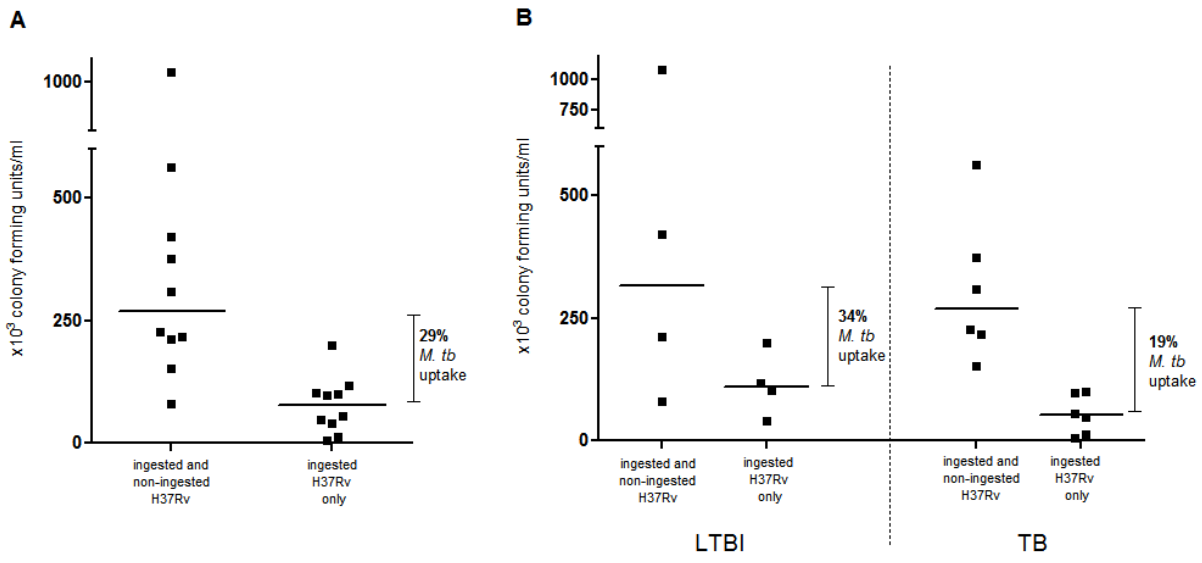


Figure 6.2 Median uptake of H37*Rv* by monocyte derived macrophages (MDMs) in (A) all study participants and (B) in patients with tuberculosis (n=6) and latently infected controls (n=4). In the „ingested and non-ingested H37*Rv*" column, MDMs were not washed after infection with H37*Rv* and thus represent the total amount of H37*Rv* added to each well for infection. In the "ingested H37*Rv* only" column, MDMs were washed after 18 hours of infection to remove any non-ingested bacteria and therefore represent only the H37*Rv* that was engulfed by the MDMs.

6.3.2 Effect of recombinant IL4 on mycobacterial containment in TB patients and LTBI controls

6.3.2.1 PPD effectors

The mycobacterial containment assay was performed in a total of 5 presumed LTBI controls and 8 TB patients. There was a significant decrease in median CFU/ml when the infected MDM‟s were co-cultured with PPD effectors in TB patients (n=8) compared to „H37*Rv* infected MDM only‟ control ($30.7x10^3$ *vs*. $15.7x10^3$ CFU/ml; p=0.008) (Figure 6.3A; blue circles). Additionally a significant increase in mycobacterial containment (decreased *M.tb* survival) of 48% (p=0.008) was demonstrated (Figure 6.4A). A similar CFU/ml reduction was observed in LTBI controls ($101.7x10^3$ *vs*. $28.0x10^3$ CFU/ml) which equated to a 72% increase in mycobacterial containment (decreased *M.tb* survival) (Figure 6.3B and 6.4B) although this did not reach statistical significance (p=0.06). However, this lack of significance could be a result of the small sample size in the LTBI group (n=5).

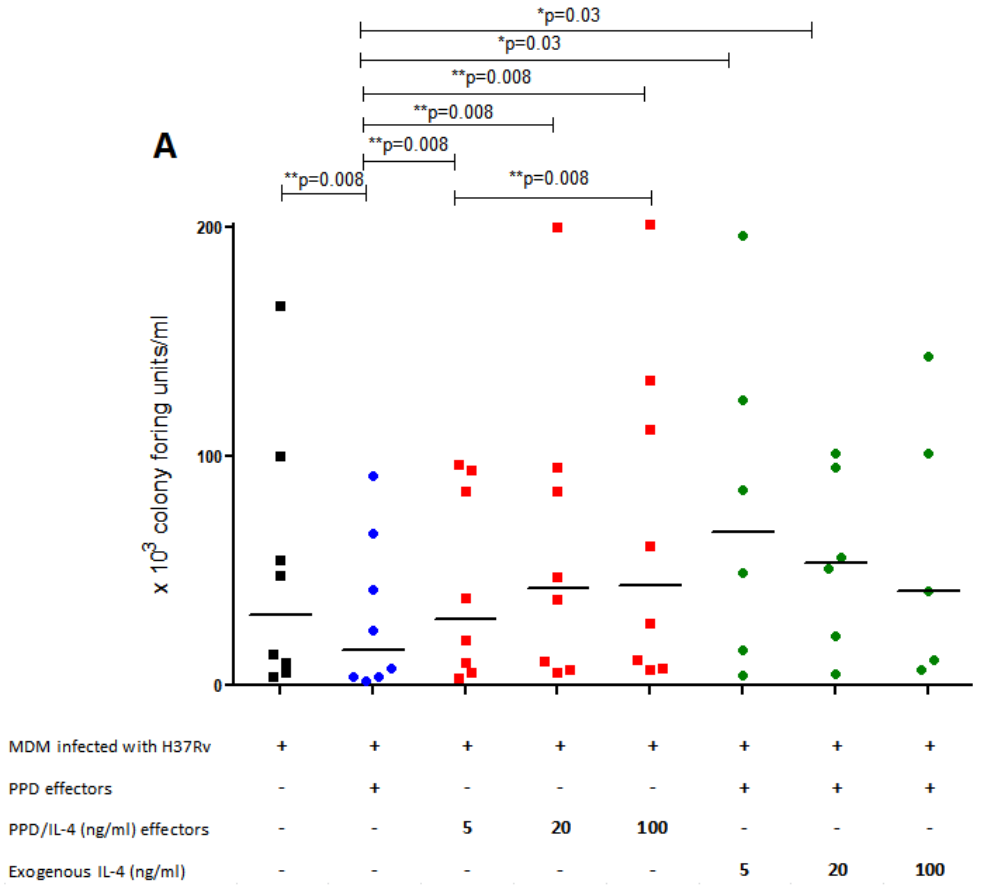
6.3.2.2 PPD/IL-4 effectors

In the „PPD/IL-4 effectors‟ interventions, rIL-4 was added together with PPD to PBMCs to generate PPD/IL-4 effectors, which were co-cultured with infected MDMs (section 6.2.1.2.1). In TB patients, compared to the „PPD effectors‟ control, there was a significant increase in the median CFU/ml when rIL-4 was added at 5ng/ml ($29.1x10^3$; p=0.008), at 20ng/ml ($42.5x10^3$; p=0.008) and at 100ng/ml ($43.8x10^3$; p=0.008) (Figure 6.3A; red squares). Subsequently there was a significant decrease in mycobacterial containment (increased *M.tb* survival), at each concentration (1%, -73% and -33%, respectively), compared to the „PPD effectors‟ control (48%) (Figure 6.4A; red squares). Absolute CFU/ml and % mycobacterial containment were significantly higher at 100ng/ml rIL-4 compared to 5ng/ml rIL-4 ($43.8x10^3$ *vs*. $29.1x10^3$ and -32% *vs.* 1%; p=0.008) indicating that the observed effect was dependent on the concentration of rIL-4 added. A similar concentration-dependent increase in absolute CFU/ml and decrease in % mycobacterial containment (increased *M.tb* survival) was observed in presumed LTBI controls when rIL-4 was added ($45.6x10^3$ CFU/ml and 64% at 5ng/ml; 70 x 10^3 CFU/ml and 18% at 20ng/ml; $108.3x10^6$ CFU/ml and -6% at 100ng/ml) (Figure 6.3B and 6.4B; red squares). However, there were no significant

differences compared to the „PPD effectors" control in this group. Furthermore, while the % mycobacterial containment was generally lower in TB patients compared to LTBI controls in equivalent wells (for example, -32.5% in TB *vs.* -6% in LTBI at 100ng/ml rIL-4), these differences were not statistically significant (p=0.06). As stated in the previous section, the lack of statistical significance may be attributable to the small sample size in the LTBI group (n=5).

6.3.2.3 Exogenous IL-4

In the „Exogenous IL-4" intervention, rIL-4 was added when PPD effectors were co-cultured with infected MDMs (section 6.2.1.2.2). In TB patients (n=6), compared to the „PPD effector" control, there was a significant increase in median CFU/ml when rIL-4 was added at 5ng/ml and 20ng/ml ($71.7x10^3$ and $60x10^3$ CFU/ml respectively; p=0.03) (Figure 6.3A; green circles). However, when data was normalized to the MDM only control, the % mycobacterial containment was significant decreased at rIL-4 concentrations of 5ng/ml and 100ng/ml (-8% and 13%, respectively; p=0.03) (Figure 6.4A; green circles). In contrast to the „PPD/IL-4 effectors" intervention, the CFU/ml and % mycobacterial containment was similar in all concentrations of rIL-4 suggesting that this effect was not concentration-dependent. Similarly, higher CFU/ml and lower % mycobacterial containment (increased *M.tb* survival) was observed in presumed LTBI controls (Figure 6.3B and 6.4B; green circles). However, there were no significant differences compared to the „PPD effector" control.


A
*p=0.03
*p=0.03
**p=0.008
**p=0.008
**p=0.008
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**p=0.008
x 10^3 colony foring units/ml
200
100
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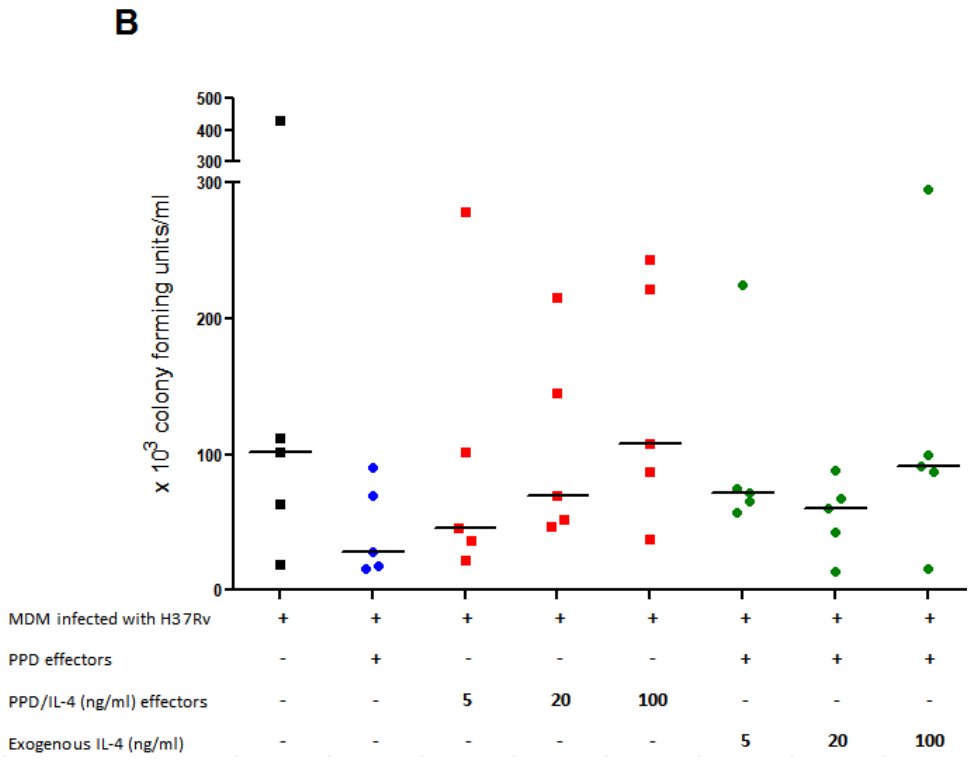
MDM infected with H37Rv + + + + + + + +
PPD effectors - + - - - + + +
PPD/IL-4 (ng/ml) effectors - - 5 20 100 - - -
Exogenous IL-4 (ng/ml) - - - - - 5 20 100


B
x 10^3 colony forming units/ml
500
400
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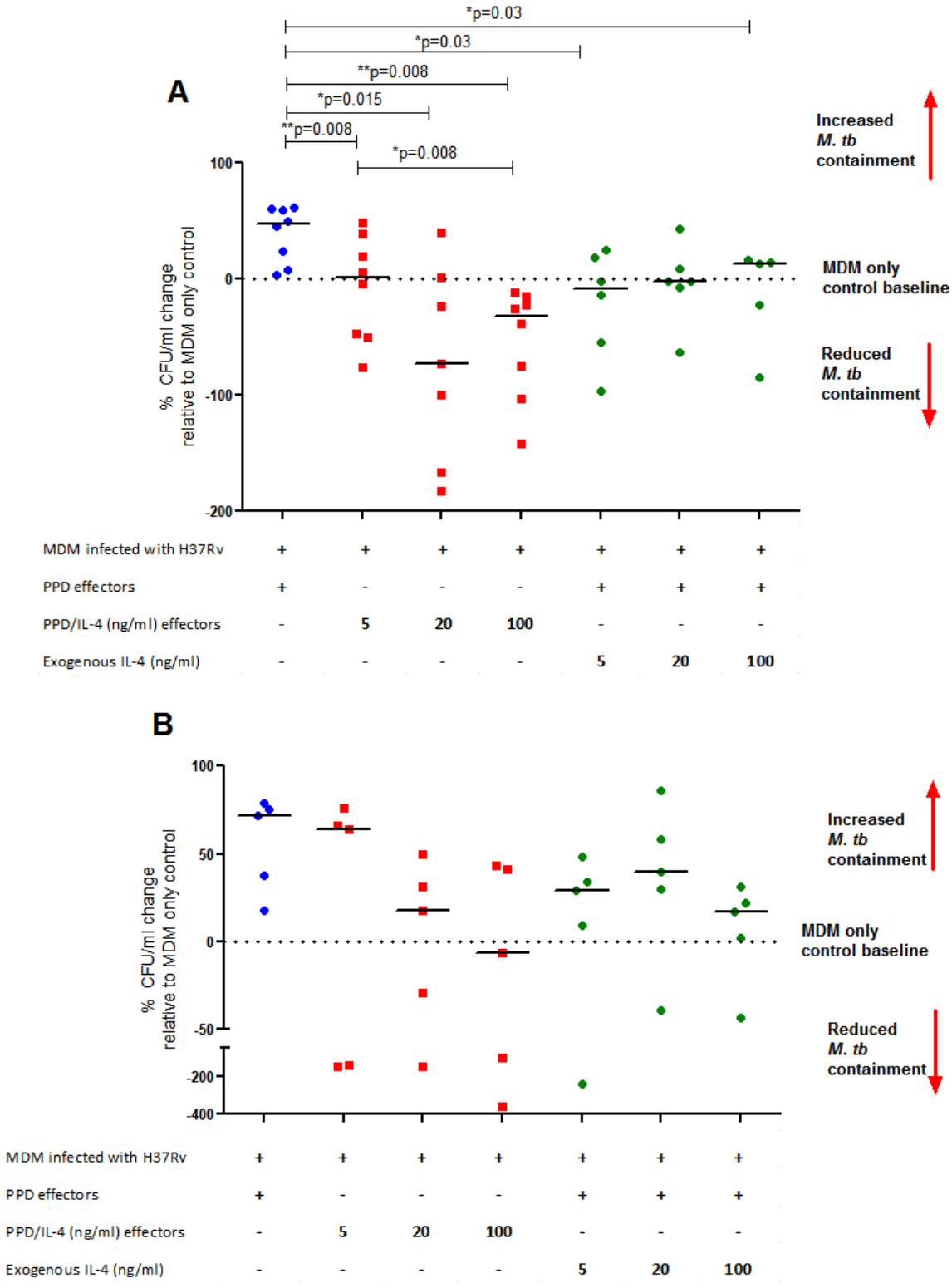
MDM infected with H37Rv + + + + + + + +
PPD effectors - + - - - + + +
PPD/IL-4 (ng/ml) effectors - - 5 20 100 - - -
Exogenous IL-4 (ng/ml) - - - - - 5 20 100

Figure 6.3. The effect on IL-4 on mycobacterial containment in monocyte derived macrophages (MDMs) of pulmonary tuberculosis patients (TB; n=8) and latently infected controls (LTBI; n=5) expressed as absolute CFU/ml. H37*Rv*-infected MDMs plated by themselves (black squares) or were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 5, 20 and 100ng/ml (red squares). PPD effectors were also added together with rIL-4 to infected MDMs (green circles) Median colony forming units (CFU/ml) are expressed for (A) TB patients and (B) LTBI controls. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.


A
*p=0.03
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**p=0.008
*p=0.015
**p=0.008
*p=0.008
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-100
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% CFU/ml change relative to MDM only control
Increased M. tb containment
MDM only control baseline
Reduced M. tb containment
MDM infected with H37Rv + + + + + + +
PPD effectors + - - - + + +
PPD/IL-4 (ng/ml) effectors - 5 20 100 - - -
Exogenous IL-4 (ng/ml) - - - - 5 20 100
B
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% CFU/ml change relative to MDM only control
Increased M. tb containment
MDM only control baseline
Reduced M. tb containment
MDM infected with H37Rv + + + + + + +
PPD effectors + - - - + + +
PPD/IL-4 (ng/ml) effectors - 5 20 100 - - -
Exogenous IL-4 (ng/ml) - - - - 5 20 100

Figure 6.4. The effect on IL-4 on mycobacterial containment in monocyte derived macrophages (MDMs) of (A) pulmonary tuberculosis patients (TB; n=8) and (B) latently infected controls (LTBI; n=5) expressed as % mycobacterial containment. H37*Rv*-infected MDMs plated by themselves (black squares) or were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 5, 20 and 100ng/ml (red squares). PPD effectors were also added together with rIL-4 to infected MDMs (green circles; TB, n=5). The % mycobacterial containment was expressed relative to the „H37*Rv* infected MDM only" control. Increased *M.tb* containment indicates reduction *M.tb* survival whereas decreased *M.tb* containment indicates an increase in *M.tb* survival. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

6.3.2.4 Neutralization of IL-4

Given the more pronounced and concentration-dependent increase in CFU/ml and decrease in % mycobacterial containment that was observed in the „PPD/IL-4 effector" intervention, I attempted to determine the effect of neutralizing rIL-4 in this intervention in TB patients (n=6) by adding anti-IL-4 antibody (20µg/ml) at the same time as rIL-4 (as described in section 6.2.1.2.3). As already shown, addition of rIL-4 at 20ng/ml and 100ng/ml resulted in an increase in CFU/ml compared to the „PPD effectors" control ($76.5x10^3$ and $62.4x10^3$ vs. $34.8x10^3$, respectively; Figure 6.5A) and a subsequent decrease in % mycobacterial containment (-20% and 5.5% vs. 48.5%; Figure 6.5B). Addition of anti-IL-4 antibody to the wells containing 20ng/ml rIL-4 significantly reduced the CFU/ml to levels similar to the „PPD effectors" control ($32.4x10^3$ CFU/ml; p=0.03). Addition of anti-IL-4 antibody also reduced CFU/ml in 100ng/ml rIL-4 wells but to a lesser extent ($49.6x10^3$ CFU/ml). Furthermore, the % mycobacterial containment increased in the anti-IL-4 antibody containing wells with rIL-4 at 20ng/ml (60%) and 100ng/ml (42%) to levels similar to the „PPD effectors" control (48.5%). However, the difference was only significant in the 20ng/ml rIL-4 well (p=0.03).

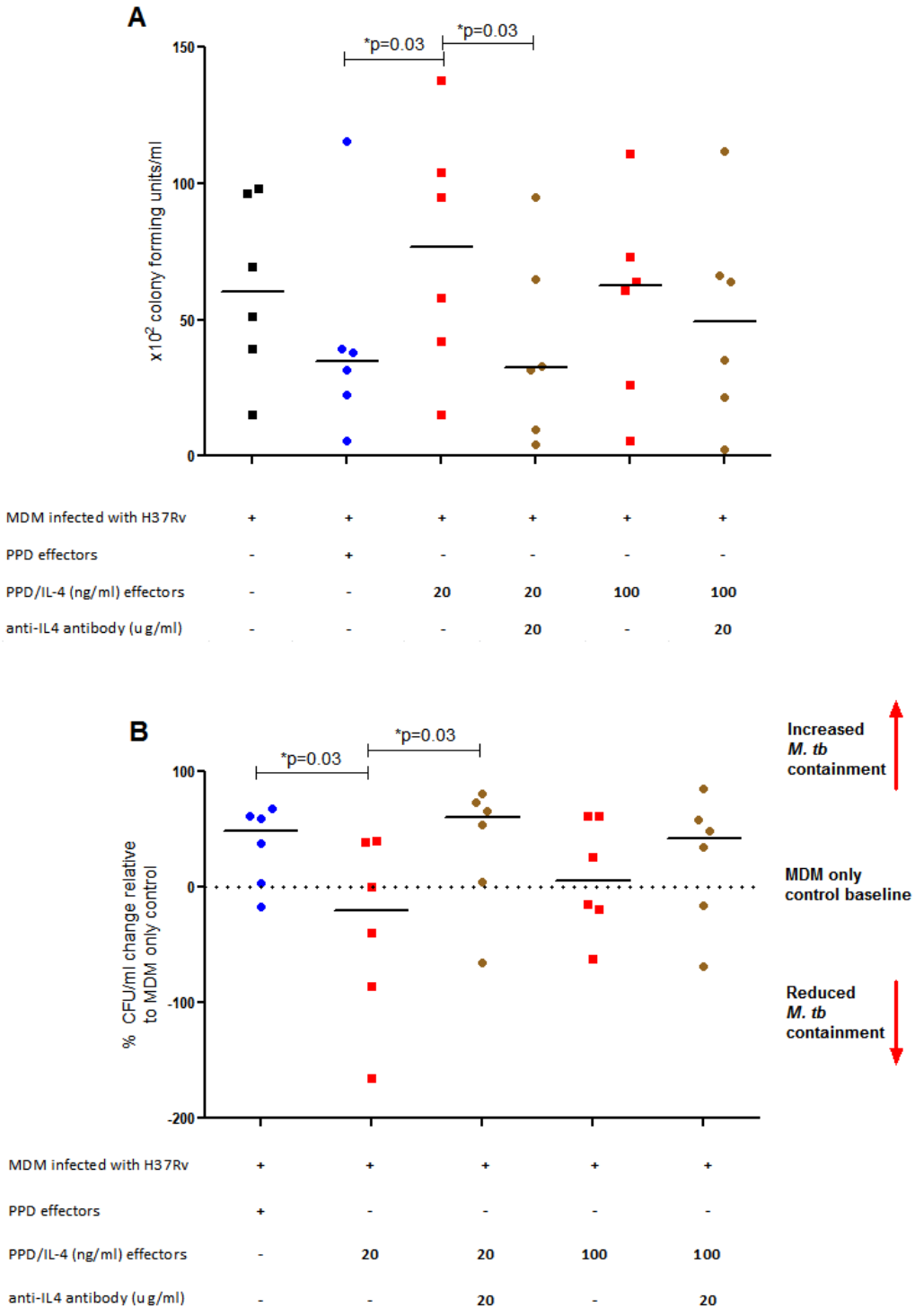


Figure 6.5 The effect of neutralizing IL-4, using anti-IL-4 antibodies, on mycobacterial containment in monocyte derived macrophages (MDMs) of pulmonary tuberculosis (TB) patients (n=6). H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells

pre-primed with purified protein derivation (PPD) alone (blue circles), PPD + rIL-4 (20 and 100ng/ml; red squares) or PPD+ rIL-4 + anti-IL4 antibody (20µg/ml; brown circles). Median colony forming units (CFU)/ml are expressed in (A) and the % mycobacterial containment i.e. change in CFUs relative to the „H37*Rv*-infected MDM only" reference control is expressed in (B). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

6.4 Discussion

There is substantial evidence that IL-4 is associated with progression to active TB but whether IL-4 can directly cause the observed immunopathology or is merely a by-product of inflammation remains unclear. In the preceding experiments, I have shown that IL-4 has the ability to subvert mycobacterial containment within monocyte derived macrophages. This effect is observed when rIL-4, together with PPD, is used to pre-prime effector T-cells („PPD/IL-4 effectors") or when rIL-4 is added to infected macrophages in the presence of PPD pre-primed effector T-cells („Exogenous IL-4"). The subversive effects in the former occur in an IL-4 dose-dependent manner. Furthermore, the IL-4 driven subversive effect was reversed with the addition of anti-IL-4 antibody in the „PPD/IL-4 effectors" intervention.

6.4.1 PPD pre-primed effector cells enhance mycobacterial containment

In the mycobacterial containment assay, macrophages were infected and co-cultured with effector T-cells, generated by pre-stimulating PBMCs with PPD. I observed a ~50% increase in mycobacterial containment with PPD effectors compared to no effectors (MDM only control) in TB patients. Similar levels of containment were observed in other *in vitro* co-culture infection models of TB [293, 564]. The *in vitro* infection model used here primarily assesses the role of adaptive immunity in restricting *M.tb* growth. This is largely mediated by IFN-γ-producing CD4 Th1 cells [559], which are essential for the initial [565-567] and long term control [568] of bacterial growth in mice, and are equally important in humans [569]. *In vitro* stimulation of PBMCs with PPD, which is a mixture of mycobacterial antigens, activates both resting and central memory CD4 T-cells [570, 571], usually toward a Th1 phenotype with the production of pro-inflammatory cytokines. These cytokines, including

IFN-γ, activate macrophages and cytotoxic CD8 T-cells [572] to restrict *M.tb* growth. In similar *in vitro* models of infection, Silver *et al.* demonstrated the importance of CD4 cells in restricting *M.tb* growth in peripheral monocytes [569] whereas Carranza *et al.* found that CD8 T-cells were more effective than CD4 cells in eliminating *M.tb* in alveolar macrophages [564]. These results highlight the important contribution of an antigen-specific Th1 response in TB immunity. Mycobacterial containment was somewhat higher in presumed LTBI controls (72%), which could indicate that their T-cell recall responses are more effective, compared to TB patients, in terms of their ability to control *M.tb* replication.

6.4.2 PPD and IL-4 pre-primed effectors subvert mycobacterial containment

The co-culture of PPD/IL-4 effectors and infected macrophages exhibited a reduction in *M.tb* containment by 50-120% compared to co-culture with PPD effectors alone, depending on the IL-4 concentration used. The addition of IL-4 at this point in the assay i.e. prior to pre-priming of T-cells, may be inhibiting differentiation of naïve T-cells to become Th1 effectors and driving them toward a Th2 phenotype as T-cell polarization is heavily influenced by the cytokine microenvironment [227]. Under Th2 polarizing conditions, IL-4 downregulates IFN-γ expression of naïve T-cells by inhibiting STAT6 activation and subsequently increasing the expression of GATA-3 [573, 574]. This downregulation of IFN-γ is possibly occurring in our model as well as leading to a reduction in mycobactericidal activity within macrophages. The Th1/Th2 paradigm not only applies to CD4 T-cells but extends to CD8, NK and γδ T-cells [575, 576] and IL-4 may be polarizing these cells in a similar manner to CD4 Th cells. The IL-4-concentration dependent effect on mycobacterial containment in this intervention suggests that there is some level of competitive inhibition between Th1 and Th2 activation. At increasing IL-4 concentrations, IL-4, which drives Th2 polarization, is outcompeting PPD, which drives Th1 polarization, to produce a predominant Th2 response. Furthermore, neutralization of IL-4, using anti-IL-4 antibodies, reverses this effect, most likely by restoring a predominantly Th1 driven phenotype among the T-cell population. However, IL-4 is highly pleiotropic and can have additional effects both on T-cells and macrophages (reviewed in [16]). A number of these mechanisms were investigated and will be discussed in chapter 7.

6.4.3 Exogenously added IL-4 to PPD pre-primed effectors subverts mycobacterial containment

In the „Exogenous IL-4" intervention, where IL-4 is added to the co-cultured PPD effectors and infected macrophages, mycobacterial containment was reduced by 35-56% compared to the „PPD effectors" control. This was less than the „PPD/IL4 effectors" intervention. In contrast to the „PPD/IL4 effectors", where addition of IL4 had a direct effect on development of a Th1 effector response, IL-4 added at this stage in the assay is likely to have a lesser impact on effector cells given that these cells would have fully differentiated in response to PPD to become predominantly Th1 effectors. Murine data suggests that, after 96 hours of stimulation under Th1 polarizing conditions, cells are fully committed to the Th1 lineage and, even if subjected to Th2 polarizing conditions, are unable revert to a Th2 phenotype [574]. Indeed, PBMCs are stimulated with PPD alone for 6 days (no IL-4 is added) in this assay suggesting that these are fully differentiated cells of a predominantly Th1 phenotype. However, these Th1 cells may still retain responsiveness to IL-4 [574] and are able to modulate IFN-γ expression, but to a lesser extent than in the „PPD/IL4 effector" intervention. This may explain the smaller decrease in mycobacterial containment in the „Exogenous IL-4" compared to „PPD/IL-4 effectors" intervention. Furthermore, rIL-4 may also directly affect macrophages in this model. IL-4 drives alternate activation of macrophages, which have anti-inflammatory functions and downregulate protective mycobactericidal mechanisms such as iNOS production [39, 40], TLR expression [434], autophagy [44] and apoptosis [41, 436]. The degree to which this occurs will be dependent on the plasticity of macrophages and to what extent they can switch between the classical and alternative phenotype. This has been shown in an *in vitro* model of *C. neoformans* infection [577] but may also occur in progressive TB *in vivo* [40].

6.4.4 Biological significance of IL-4 mediated subversion of mycobacterial containment

In our infection model, particularly in the „PPD/IL-4 effectors" intervention, addition of IL-4 artificially skews the Th1/Th2 balance to favour a Th2 response. This is most probably due to a dampening of mycobactericidal activities by both lymphocytes and macrophages leading to the observed reduction in mycobacterial containment. Whether this is occurring *in vivo* as

well will be dependent on the Th1/Th2 balance within the cellular environment, which is influenced by the pathogen, environmental factors and the genetic background of the host [227]. Evidence suggests that a pre-existing mixed Th1/Th2 response, induced by environmental mycobacteria, is present in individuals from developing countries [16]. Furthermore, *M.tb* infection is known to downregulate protective Th1 mechanisms [8]. Certain bacterial components, such as ManLAM, of virulent *M.tb* strains can also stimulate IL-4 production [427]. These factors may all be contributing to a disrupting Th2 response. This is further supported by the data presented in chapter 3 and previously published studies [9, 416-418, 503, 504] which show high IL-4 levels and a low Th1:Th2 ratio in TB patients compared to healthy controls. Impaired Th1 immunity is most apparent in the early stages of infection where an inadequate Th1 response fails to contain *M.tb* within the granuloma and leads to active disease.

6.4.5 Limitations

This *in vitro* model of mycobacterial infection assessed the capacity of lymphocytes and their soluble mediators to modulate the growth of *M.tb* within monocyte derived macrophages. However, the model is a „closed system" and lacks the milieu of cytokines, recruitment and involvement of other cell types and granuloma formation that occurs at the site of disease. Nonetheless, the aim of these experiments was to specifically assess the effect of IL-4 on the immune activities of lymphocytes and macrophages, as these cells are the most important in the immune response to TB and the pleiotropic effects of IL-4 are most evident on these cells.

It is unknown if mycobacterial containment is a result of direct killing of *M.tb* or an inhibition of its replicative state. Counting of CFUs was performed at ~14 days after plating for optimal assessment of colony numbers, as extensive overgrowth occurred after this period. Further growth may have occurred after this period if organisms were in a non-replicating state. However, previous optimization experiments in our lab did not find any additional growth even after 60 days of culture [293].

The biological relevance of the observed decrease in mycobacterial containment after co-culture for 48 hours is unclear. Similar studies have used different time periods ranging from 18 hours to 7 days [293, 564, 569]. However, given the 24 hour doubling time of *M.tb*, a 48-hour period allows for sufficient intracellular replication of *M.tb* to observe an effect on CFU counts while still maintaining MDM survival. Trypan blue exclusion staining of infected MDMs at this time point confirmed the viability of these cells. Furthermore, in my own optimization experiments, a 120-hour co-culture time period resulted in a significant drop in CFU counts, compared to 48 hours, which is most likely due to lysis of infected macrophages. As such, the choice of experimental conditions is justified.

The concentrations of IL-4 used in these assays may not be biologically relevant given that IL-4 is active at picomolar levels *in vivo*. The rIL-4 concentrations used here were derived from the concentrations used in the rIL-4 T-cell proliferation assay that produced a significant effect (chapter 5). Recombinant proteins can act differently to their naturally produced counterparts and is heavily dependent on the expression system used. For instance, certain proteins expressed in insect cells undergo inappropriate N-glycosylation resulting attachment of much simpler N-glycan sugars compared to mammalian expressed counterparts leading to reduced protein bioactivity [578] (discussed in chapter 4). Indeed, proteins expressed in a mammalian system do show improved stability compared to insect cell counterparts [506, 507]. Also, protein quantification methods, including Bradford and ELISA, fail to assess proper protein function and a portion of the measured IL-4 may be non-functional. Furthermore, the *in vivo* environment has mechanisms that increase bioactivity and maintain the effect of certain proteins. For example, human erythropoietin is 1000-fold more active *in vivo* compared to when it is used in *in vitro* experiments [554]. Similar mechanisms may also prolong the effect of IL-4. IL-4 has a very short half-life (<20 minutes) but soluble IL-4 receptors are able to sequester free IL-4 and prolong its activity. No IL-4 bioactivity could be determined in supernatants of T-cells activated in the presence of IL-4 for 6 days but bioactivity was markedly increased the presence of sIL-4R [362]. These findings justified the IL-4 concentrations used in my assays. Indeed, other *in vitro* stimulation experiments used similar IL-4 concentrations [47, 48, 392, 579], likely for the same reasons.

Considerable variation was observed in mycobacterial uptake by MDMs of TB patients and presumed LTBI controls. This variation may be explained by inter-participant biological variability, inherent variability in mycobacteria stocks or a result of the small sample size which, due to experimental and time constraints, was unavoidable.

Monocyte derived macrophages may have different phagocytic capacities and anti-mycobacterial properties compared to their tissue specific counterparts [580, 581]. It may have been more appropriate to use alveolar macrophages in the assay to better reproduce *M.tb* infection at the site of disease. However, blood, compared to BAL, is much more easily acquired and high PBMC numbers usually obtained. Furthermore, BAL can be prone to contamination with resident respiratory tract flora. Additionally, in a similar assay used to assess the effect of Tregs on *M.tb* containment, similar outcomes were obtained either using MDMs or alveolar macrophages [293].

The use of PPD to pre-activate PBMCs tends to favour CD4 activation over other T-cells, including CD8, γδ and NK T-cells. Perhaps the use of live bacteria or whole cell sonicates may better mimic the T-cell activation responses in vivo, as these antigen induce stronger activation of CD8, γδ and NK T-cells compared to soluble antigens such as PPD [570, 582].

Unfortunately, due to limited protein yields, the effect of IL4δ2 could not be assessed in this model. Given the limited data on its precise role in TB, it would have been interesting to perform these assays using both IL-4 and IL4δ2. However, IL4δ2 expression will be attempted in more appropriate systems to increase protein yield and we will attempt to determine the effect of IL4δ2 on mycobacterial containment as part of future work.

6.5 Conclusion

The ability of IL-4 to subvert mycobacterial containment within monocyte derived macrophages suggests that a subversive Th2 response can undermine protective immunity, likely by a downregulation of the Th1 response. However, it is unknown what other TB-

associated immune mechanisms, which can impact mycobacterial survival, may be affected by IL-4. In the next chapter, specific mechanisms of IL-4 mediated mycobacterial subversion will be investigated.

7. CHAPTER 7: Mechanisms by which IL-4 impacts mycobacterial containment

7.1 Introduction

The observation of high IL-4 levels in active TB has been documented [9, 15, 30, 288, 417, 418] and my results in chapter 3 support these findings. Furthermore, I have shown that, in an *in vitro* macrophage infection model, IL-4 is associated with a subversion of mycobacterial containment (chapter 6). However, the mechanisms involved in this subversion have not been fully elucidated. One possible mechanism is the IL-4-mediated downregulation of Th1 effector cells. In a murine model, this occurs through STAT-6 or STAT-6-induced regulation of T-bet and IFN-γ expression [574]. The anti-mycobacterial effects of IFN-γ on both T-cells and macrophages have been well described and include the induction of ROI and RNI species [235, 479], CD8 cytotoxicity [218], autophagy [158, 583] and apoptosis of infected cells [236].

IL-4 can also directly affect the ability of macrophages to kill *M.tb*. Harris *et al* showed that IL-4 inhibits starvation-induced autophagic control of *M.tb* in infected macrophages via Akt upregulation [44]. IL-4 polarizes macrophages to become alternatively activated and express high levels of pattern recognition receptor (PRRs), such as mannose receptor and DC-SIGN, which facilitate uptake of *M.tb* into these cells [432]. Alternatively activated macrophages can also downregulate *M.tb* killing mechanisms [39].

Regulatory T-cells (Tregs) have been implicated to be detrimental in *M.tb* infection as a high frequency of Tregs has been observed in TB patients compared to healthy controls [287, 288, 291-294] and Tregs have been shown to be associated with attenuated mycobacterial stasis [293]. However, the role of IL-4 in Treg development and maintenance is less clear, with studies, mostly performed in mice, suggesting that IL-4 can either stimulate [301, 303, 443, 444, 584] or inhibit [302, 445] the proliferation and activity of Tregs.

Thus, IL-4 has the potential to modulate multiple immune mechanisms involved in controlling *M.tb* infection. The aim of this chapter was to explore specific immune mechanisms (Treg and Th1 downregulation) that may be involved in the IL-4-associated subversion of mycobacterial containment observed in chapter 6. These data would provide a better understanding of how IL-4 may undermine a protective immune response leading to active disease.

7.2 Methods

7.2.1 Mycobacterial containment assay overview

A mycobacterial containment assay was performed, similar to that described in chapter 6, to determine the possible mechanisms involved in the observed IL-4 associated subversion of mycobacterial containment (Chapter 6). In contradistinction to chapter 6 however, cells were analyzed by flow cytometry to determine the expression levels of lymphocyte- and macrophage-specific surface and intracellular biomarkers, rather than lysing them to culture and grow intracellular H37*Rv*. Briefly, ~50ml of whole blood was obtained from TB patients. MDM generation and infection, PBMC stimulation and cell co-culture for 48 hours were performed as summarized in section 6.2.1. Infected MDMs and lymphocytes were then harvested, stained with fluorescently-labelled antibodies against specific cell surface and cytokine markers (outlined in table 7.2) and analyzed by flow cytometry. Appropriate controls were included in the assay, as outlined below. Based on the results in chapter 6, and due to cost and sample volume limitations, only the effect of IL-4 on biomarker expression in the „PPD/IL-4 effectors" intervention, was assessed as described in section 6.2.1.2.1 and below. Descriptions of experimental controls and interventions are given below. An outline of the assay is shown in Figure 7.1 and details on stimulants and cell numbers are given in Table 7.1.

7.2.1.1 Experimental controls

7.2.1.1.1 Uninfected MDMs and MDMs infected with H37Rv

The H37*Rv* infected MDM control was performed as described in section 6.2.1.1.1 (2 in Figure 7.1 and Table 7.1). A control well containing uninfected MDMs was also included. (1 in figure 7.1 and Table 7.1). No effector cells were added to these wells.

7.2.1.1.2 PPD effectors

PBMCs were stimulated with PPD for 6 days to generate PPD effectors, and subsequently co-cultured with infected MDMs as described in section 6.2.1.1.2 (3 in Figure 7.1 and Table 7.1).

7.2.1.2 Experimental interventions using rIL-4

7.2.1.2.1 PPD/IL-4 effectors

PBMCs were stimulated with PPD and rIL-4 at concentrations of 20 and 100ng/ml for 6 days to generate „PPD/IL-4 effectors". These „PPD/IL-4 effectors" were then co-cultured with infected MDMs, as described in section 6.2.1.2.1. (4 in Figure 7.1 and Table 7.1)

7.2.1.2.2 Neutralization of IL-4 with anti-IL4 or anti-IL-4R antibody

„PPD/IL-4 effectors" wells were setup as stated in section 7.2.1.2.1(4 in Figure 7.1 and Table 7.1). However, anti-IL-4 neutralizing antibody (20ug/ml; Abcam) or anti-IL-4 receptor neutralizing antibody (20µg/ml; Abcam) was added, together with PPD and rIL-4 (100ng/ml) to PBMCs for 6 days to generate effector cells. On day 6, these effectors were co-cultured with the infected MDMs.

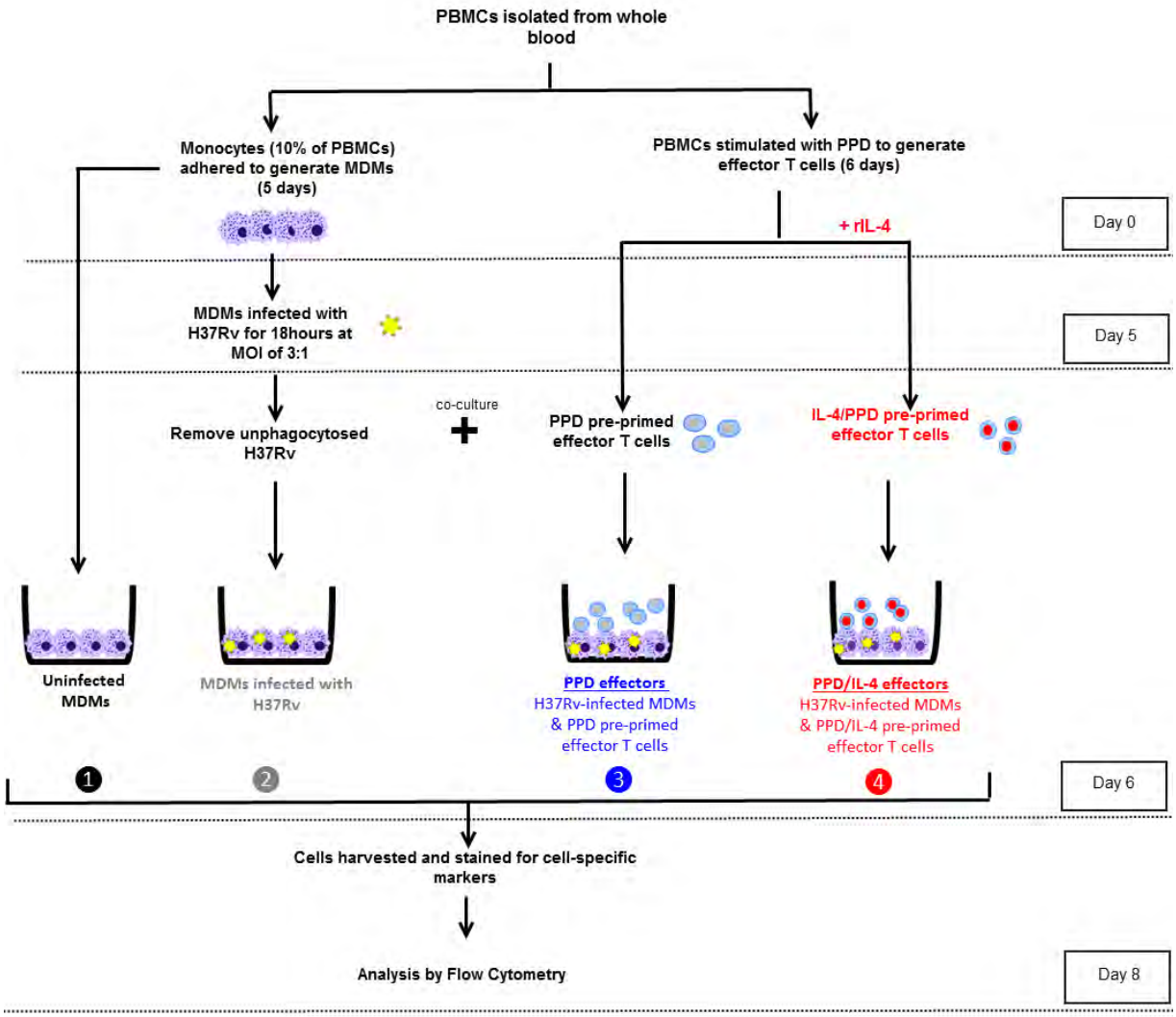


Figure 7.1. Overview of mycobacterial containment assay to determine the mechanisms involved in the IL-4-associated subversion mycobacterial containment within monocyte-derived macrophages in TB patients. Culture conditions and stimulant concentrations of controls and interventions (labeled ❶ - ❹ in diagram) are further described in table 7.1

Table 7.1. Details of experimental setup including cell and stimulant concentrations in the mycobacterial containment assay to determine mechanisms involved in the IL-4 associated subversion of mycobacterial containment in TB patients (as described in Figure 7.1).

	No.*	Condition	DAY 0					DAY 6	
			MDMs		PBMCs				
			Cell number	stimulant	Cell number	stimulant		Cells co-cultured with infected MDMs	stimulant
				rIL-4 (ng/ml)		PPD (µg/ml)	rIL-4 (ng/ml)		rIL-4 (ng/ml)
Controls	1	Uninfected MDMs	$6x10^5$	-	-	-	-	-	-
	2	MDMs infected with H37Rv	$6x10^5$	-	-	-	-	-	-
	3	PPD effectors	$6x10^5$	-	$6x10^5$	12	-	PPD effectors	-
Intervention	4	PPD/IL4 effectors	$6x10^5$	-	$6x10^5$	12	20, & 100	PPD/IL4 effectors	-

*corresponds to the controls/interventions shown in figure 7.1

7.2.2 Generation of MDMs

MDMs were generated as described in section 2.4.4. A total of $6x10^5$ MDMs were assumed to be plated in 24-well flat bottom plates as previous optimization experiments showed that approximately 10% of PBMCs were monocytes.

7.2.3 Culture and stimulation of PBMCs

PBMCs were isolated (section 2.4.2) and $2x10^5$ were cultured in triplicate in 96-well round bottom plates (Nunc). Cells were stimulated and incubated as described in section 6.2.3.

7.2.4 Infection of MDMs with H37Rv and co-culture of pre-primed effector cells and infected MDMs

MDMs were infected and co-cultured with pre-primed effector cells as described in section 6.2.4. An MOI of 3 equated to a total of $1.8x10^6$ CFUs being added to each well for infection. Triplicate wells of effector cells were harvested and pooled. These cells ($6x10^5$) were then added to the well containing H37Rv infected MDMs in a final volume of 1ml and incubated for 48 hours.

7.2.5 Cell harvesting, immunofluorescence staining and flow cytometry

All staining procedures were performed in a Biosafety level 3 laboratory. Golgi-stop, containing monensin (0.6µl/ml), was added to each well at least 5 hours before harvesting of cells in order to block the release of cytokines and proteins from the Golgi complex within the cell cytoplasm. Non-adherent cells were harvested and transferred to a 5ml FACS tube. Adherent cells were treated with 100ul of cold 0.5% EDTA (50ul EDTA (Sigma Aldrich) in 9.95ml of 1xPBS) for 30 minutes at room temperature followed by gentle scraping to lift MDMs from the plate. The wells were observed under an inverted microscope to establish whether all the cells had been removed. These cells were added to the same 5ml FACS tube. Cells were then stained for specific surface and extracellular markers using fluorescently-labelled antibodies (Table 7.2), as outlined in section 2.4.6. Cells were left in 4% para-formaldehyde overnight to kill any remaining H37*Rv* not removed during the staining procedure. Cells were analyzed on a BD LSR II flow cytometer.

Table 7.2. Fluorescently-labelled antibodies used to determine the mechanisms associated with IL-4 mediated subversion of mycobacterial containment by flow cytometry.

Monoclonal antibody	Fluorescent conjugate	Source	Staining Panel
CD3	Alexa fluor 700	Lymphocyte marker	1 & 2
CD4	APC H7	Th cell marker	
CD8	PeCy7	Cytotoxic T-cell marker	1
CD14	PeCy5	Macrophage markers	
CD16	PeCy5		
DC-SIGN	PerCP	Marker for alternative activation of macrophages	
IFNγ	PE	Th1 cytokines	
TNFα	APC		
LC3B	Alexa fluor 488	Autophagy marker	
CD25	PE	Lymphocyte activation/Treg marker	2
FOXP3	Alexa fluor 647	Treg transcription factor	
IL-10	Alexa fluor 488	Regulatory cytokine	
IFNγ	PECy7	Th1 cytokine	

7.2.6 Data analysis

The frequency of specific cell populations expressing biomarkers was determined using FACSDiva software (BD). Cells expressing the target biomarker were reported as a % of the total lymphocyte (CD3+CD4+ and CD3+CD8+) and macrophage (CD14+CD16+) populations. Differences in expression levels between experimental controls and interventions were determined using the Wilcoxon matched-pairs signed rank test and Mann-Whitney U test. A p-value of <0.05 was deemed significant. Statistical analyses were performed using GraphPad Prism 5.0 software and Microsoft Excel.

7.3 Results

7.3.1 Gating strategy for identification of lymphocytes and macrophages

The gating strategies for identification of biomarker expressing cells in the lymphocyte and macrophage populations are shown in figure 7.2. Lymphocytes and macrophages were acquired based on cell size (FSC) and cell granularity (SSC). Cells were gated on the CD14+/CD16+ populations for macrophages and CD3+ population for lymphocytes. CD4+ and CD8+ populations were identified within the CD3+lymphocyte populations. Cells expressing the target biomarkers were then identified in the gated macrophage (CD14+/16+), CD4+ and CD8+ lymphocyte (CD3+) populations. All subpopulation percentages displayed in the subsequent graphs were plotted relative to the total cell count in the gated population.

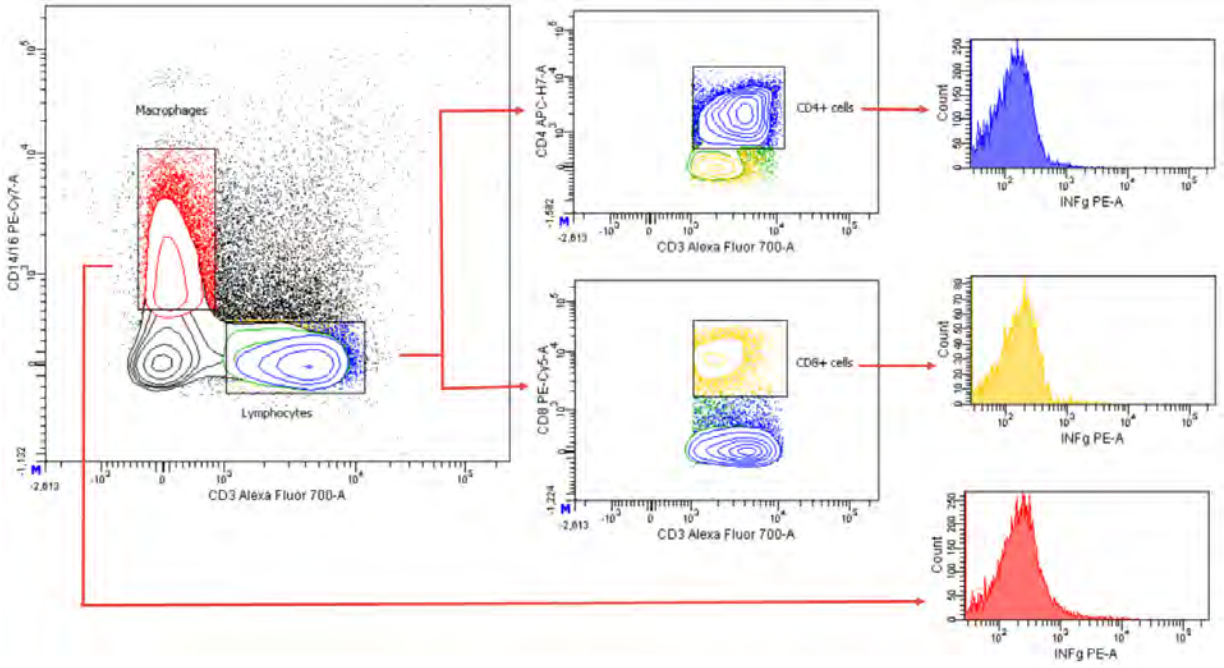


Figure 7.2 Gating strategies for identification of lymphocytes and macrophages expressing biomarkers

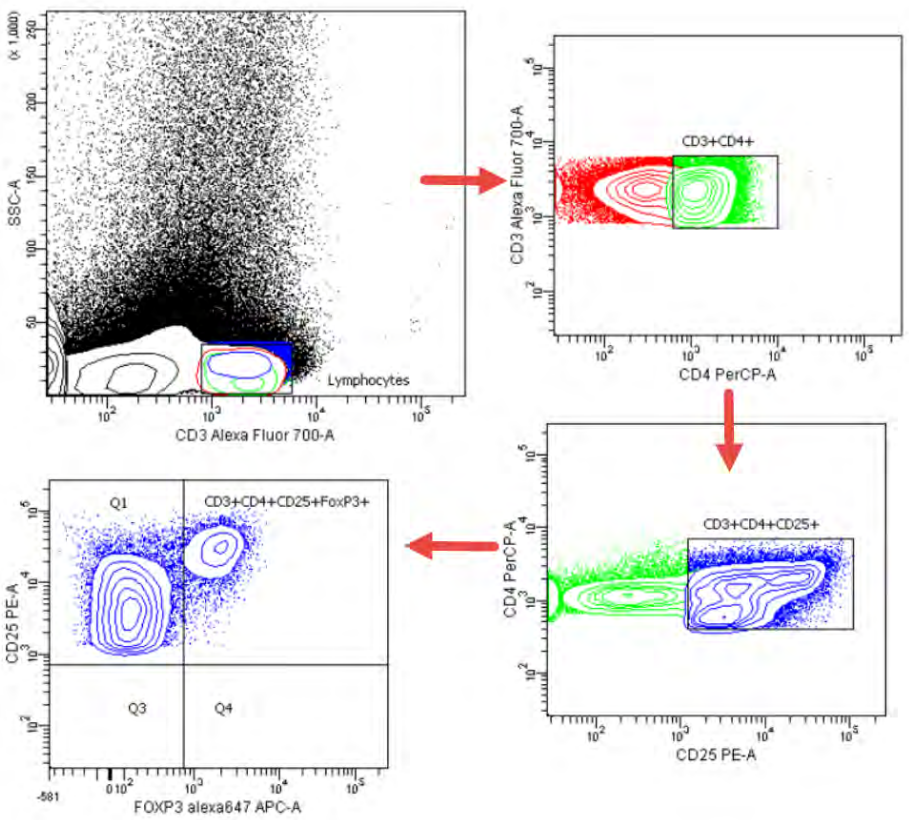
7.3.2 The effect of IL-4 on regulatory T-cell expression

The gating strategy for identification of Tregs (CD4+CD25+FoxP3+) in the lymphocyte population is shown in figure 7.3A. Lymphocytes were acquired based on cell size (FSC) and

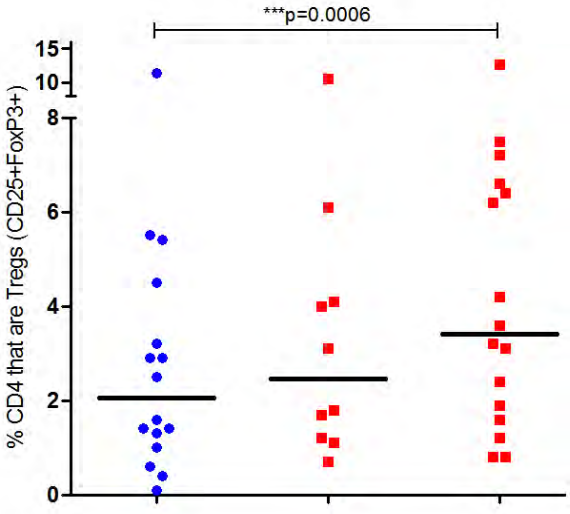
cell granularity (SSC). Cells were gated on CD3+CD4+ T-cells and then within that gate on CD4+CD25+ cells. FoxP3 + cells (Tregs) were identified within this population (figure 7.3). All subpopulation percentages displayed in the graphs were plotted relative to the total CD3+ cell count (lymphocytes).

There was a significant increase in median % Treg (CD4+CD25+FoxP3+) expression in „PPD/IL-4 effectors" (100ng/ml) compared to the „PPD effectors" control (p=0.0006) (Figure 7.3B). The expression of IL-10 by Tregs was also assessed. In the majority of patients, Tregs produced very little IL-10 either in the „PPD effectors" (median 3.3%) or the „PPD/IL-4 effectors" at 20ng/ml rIL4 (median 4.6%) and 100ng/ml (median 1.1%) (Table 7.3). There were no significant differences in Treg IL-10 expression between „PPD effectors" or „PPD/IL4 effectors".

A



B



MDM infected with H37Rv	+	+	+
PPD effectors	+	-	-
PPD/IL-4 (ng/ml) effectors	-	20	100

Figure 7.3. (A) The gating strategy for identification of regulatory T-cells (Tregs; CD4+CD25+FoxP3+) cells within the lymphocyte population and (B) the frequency (%) of CD4+CD25+Foxp3+ Tregs in the co-cultured lymphocyte population of the mycobacterial containment assay as measured by flow cytometry in pulmonary TB patients (n=16). H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 20ng/ml (n=10) and 100ng/ml (n=16) (red squares). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

Table 7.3 Median frequency of Regulatory T-cell (Tregs) expressing IL-10 in control (PPD effectors) and intervention (PPD/IL-4 effectors at 20 and 100ng/ml rIL-4) wells of the mycobacterial containment assay. Values are expressed as the median % of CD4+CD35+FoxP3+ cells and interquartile range in parentheses (IQR).

Analyte	Infected MDMs +		
	PPD effectors	PPD/IL-4 effectors (20ng/ml)	PPD/IL-4 effectors (100ng/ml)
% Tregs that express IL-10 Median % (IQR)	3.3 (0.5-24.5)	4.6 (1.6-13.6)	1.1 (0.6-11.4)

7.3.3 The effect of IL-4 on IFN-γ expression

There was significantly lower median CD4+IFN-γ expression in the „PPD/IL-4 effectors" well at 20 and 100ng/ml rIL-4 compared to the „PPD effectors" well ($p=0.01$ and $p=0.0005$, respectively). IFN-γ expression was significantly higher in the CD8+ lymphocyte population compared to the CD4+ population in the equivalent wells (PPD effectors, $p=0.04$; PPD/IL-4 effectors at 20ng/ml rIL-4, $p=0.015$; PPD/IL-4 effectors at 100ng/ml rIL-4, $p=0.001$).

However, there were no significant differences in CD8+IFN-γ expression between „PPD effectors" and „PPD/IL-4 effectors" (Figure 7.4A).

IFN-γ expression in the macrophage (CD14+CD16+) population was generally higher than in the lymphocyte (CD3+) population. Furthermore, IFN-γ expression was higher in uninfected MDMs compared to infected macrophages alone ($p=0.04$) and „PPD effectors" (containing infected MDMs and PPD effector cells; $p=0.02$). However, there were no significant differences in IFN-γ expression within the macrophage population between „PPD effectors" and „PPD/IL-4 effectors" (Figure 7.4B).

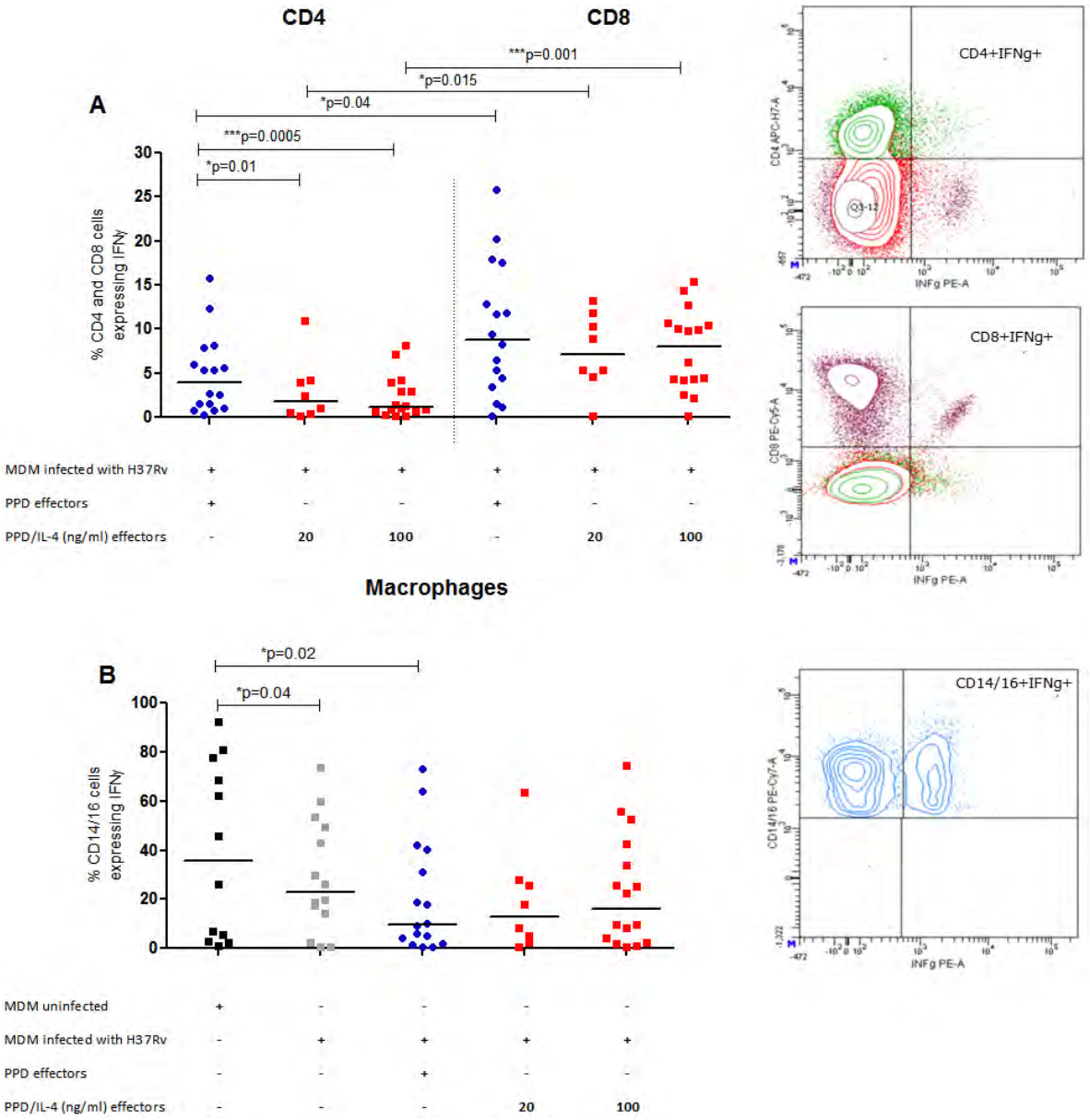


Figure 7.4. Frequency (%) of IFN-γ expressing cells in the co-cultured (A) CD4 and CD8 lymphocyte and (B) Macrophage (CD14/CD16) populations of the mycobacterial containment assay as measured by flow cytometry in TB patients. H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 20ng/ml (n=8) and 100ng/ml (n=16) (red squares). Uninfected and H37Rv-infected macrophages alone (n=12) were also cultured. Flow cytometry dot plots of the IFN-γ+ cells in the CD4+ (top), CD8+ (middle) and CD14+/16+ (bottom) populations are shown to the right of the corresponding graphs.

7.3.4 The effect of IL-4 on TNFα expression

There was significantly lower median CD4+TNFα+ expression in the „PPD/IL-4 effectors" well at 100ng/ml rIL-4 compared to the „PPD effectors" well (p=0.02). TNFα expression was similar in the CD4+ and CD8+ lymphocyte population. There were no significant differences in CD8+TNFα+ expression between „PPD effectors" and „PPD/IL-4 effectors" (p=0.8; Figure 7.5A).

TNFα expression in the macrophage (CD14+CD16+) population was similar to that in the lymphocyte (CD3+) population. There were no significant differences in TNFα expression within the macrophage population between „PPD effectors" and „PPD/IL-4 effectors" (Figure 7.5B)

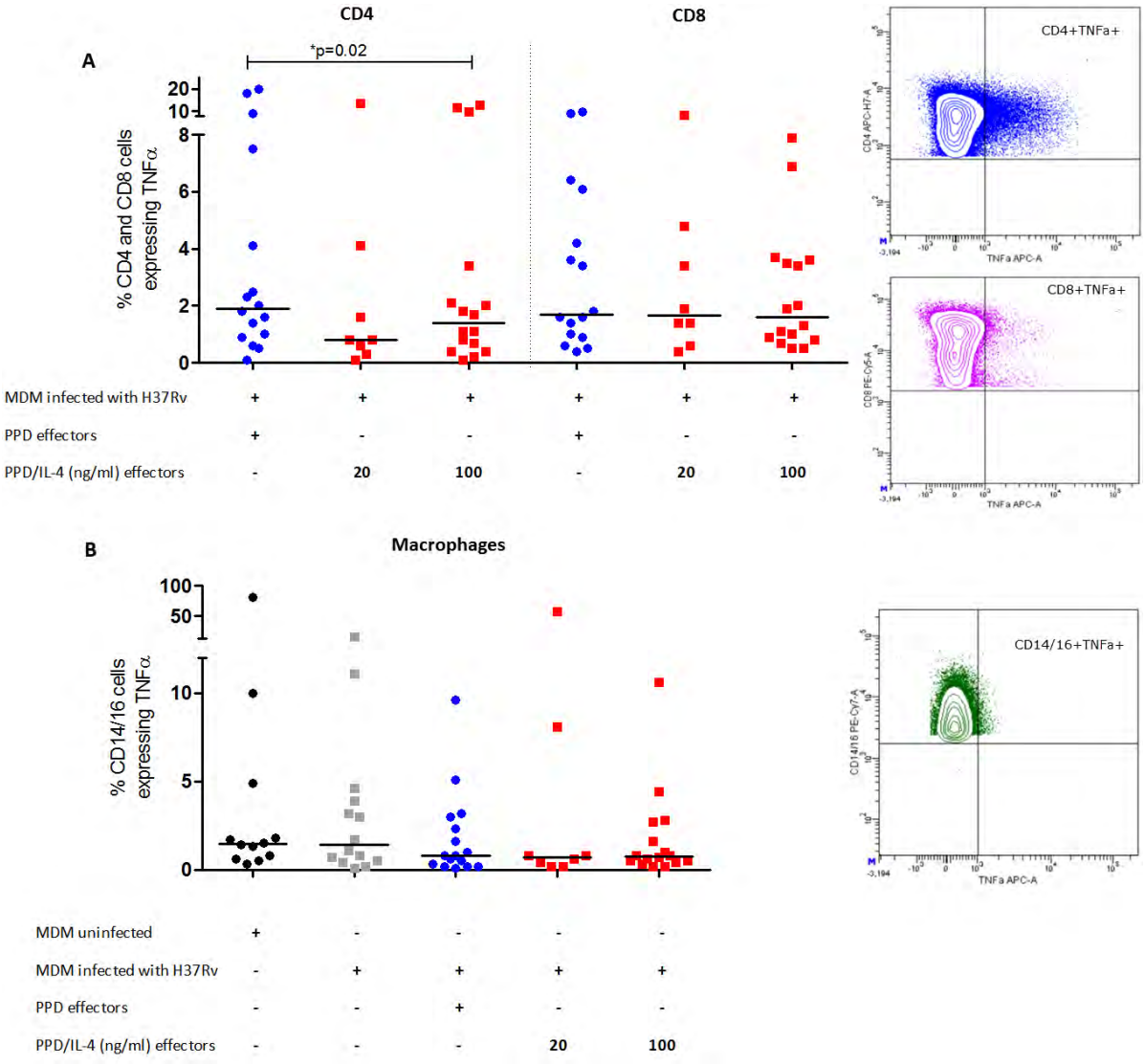


Figure 7.5. Frequency (%) of TNFα expressing cells in the co-cultured (A) CD4 and CD8 lymphocyte and (B) H37*Rv* infected macrophage (CD14/CD16) populations of the mycobacterial containment assay as measured by flow cytometry in TB patients. H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 20ng/ml (n=8) and 100ng/ml (n=16) (red squares). Uninfected and H37Rv-infected macrophages alone (n=12) were also cultured. Flow cytometry dot plot graphs of TNFα+ cells in the CD4+ (top), CD8+ (middle) and CD14+16+ (bottom) populations are shown to the right of the corresponding graphs.

7.3.5 The effect of IL-4 on DC-SIGN expression

Expression of CD4+DC-SIGN+ was generally very low and there were no significant differences between „PPD/IL-4 effectors" and „PPD effectors". DC-SIGN expression was up to 27 times higher in CD8+ compared to CD4+ lymphocytes in equivalent wells (PPD effectors, p=0.0006; PPD/IL-4 effectors at 20ng/ml rIL-4, p=0.015; PPD/IL-4 effectors at 100ng/ml rIL-4, p=0.0005). However, there were no significant differences in CD8+DC-SIGN+ expression between „PPD effectors" and „PPD/IL-4 effectors" (Figure 7.6A).

In macrophages (CD14+16+), DC-SIGN expression was significantly higher in „PPD/IL-4 effectors" at 100ng/ml rIL-4 compared to PPD effectors (p=0.02). DC-SIGN expression was also higher in uninfected MDMs compared to „PPD effectors" (containing infected macrophages and PPD effector cells; p=0.005) (Figure 7.6B).

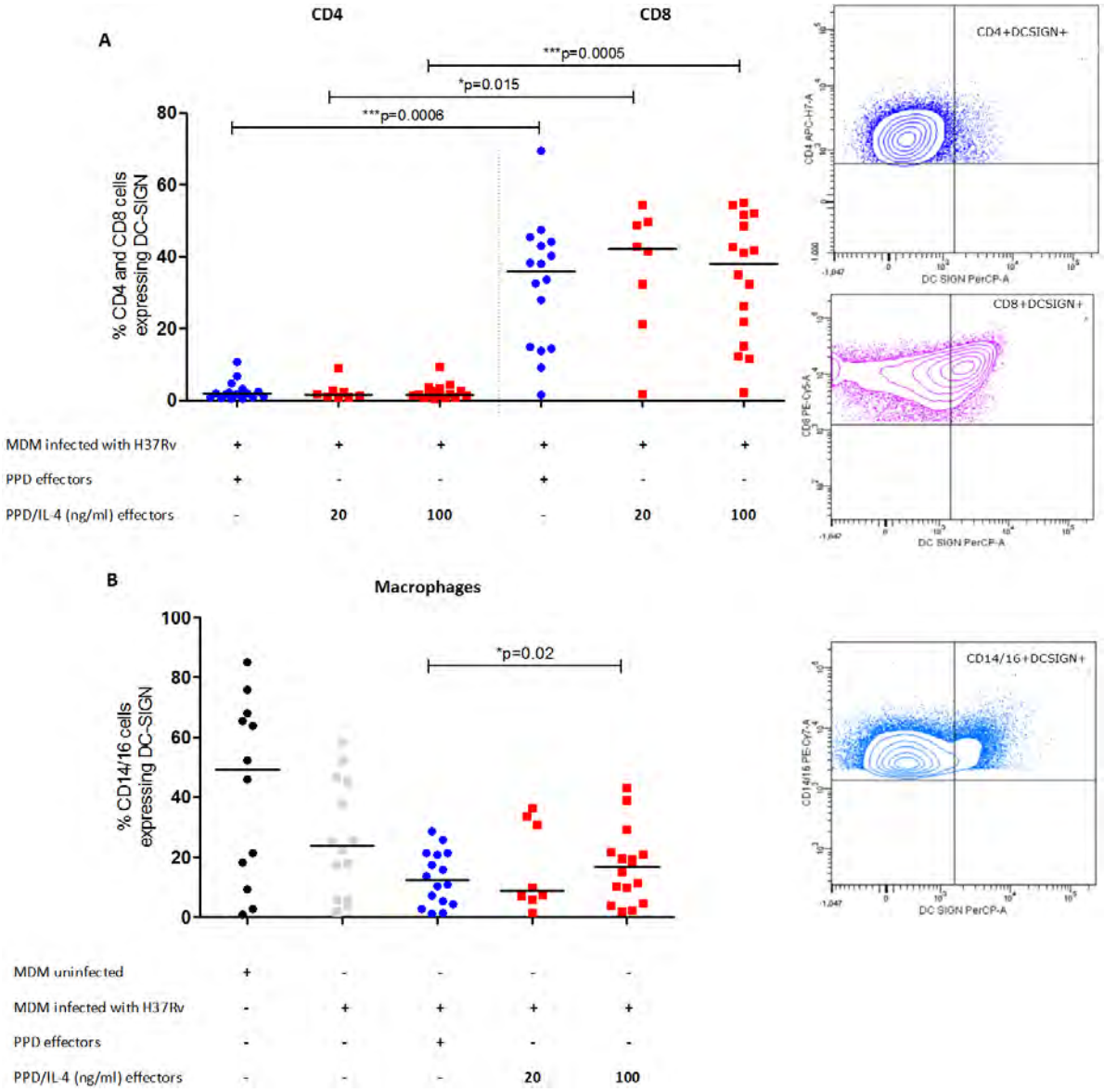


Figure 7.6. Frequency (%) of DC-SIGN expressing cells in the co-cultured (A) CD4 and CD8 lymphocyte and (B) H37*Rv* infected macrophage (CD14/CD16) populations of the mycobacterial containment assay as measured by flow cytometry in TB patients (n=16). H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 20ng/ml (n=8) and 100ng/ml (n=16) (red squares). Uninfected and H37Rv-infected macrophages alone (n=12) were also cultured. . Flow cytometry dot plots of the DC-SIGN+ cells in the CD4+ (top), CD8+ (middle) and CD14+/16+ (bottom) populations are shown to the right of the corresponding graphs.

7.3.6 The effect of IL-4 on LC3B expression (Autophagy)

The autophagy marker, LC3B was significantly higher in the CD8+ lymphocyte population compared to the CD4+ population in the equivalent wells (PPD effectors, p=0.01; PPD/IL-4 effectors at 20ng/ml rIL-4, p=0.008; PPD/IL-4 effectors at 100ng/ml rIL-4, p=0.009). There were no significant differences in CD4+LC3B+ or CD8+LC3B+ expression between „PPD effectors" and „PPD/IL-4 effectors" (Figure 7.7A).

LC3B expression in the macrophage (CD14+CD16+) population was generally higher than in the lymphocyte (CD3+) population. However, there were no significant differences in LC3B expression within the macrophage population between „PPD effectors" and „PPD/IL-4 effectors" (Figure 7.7B).

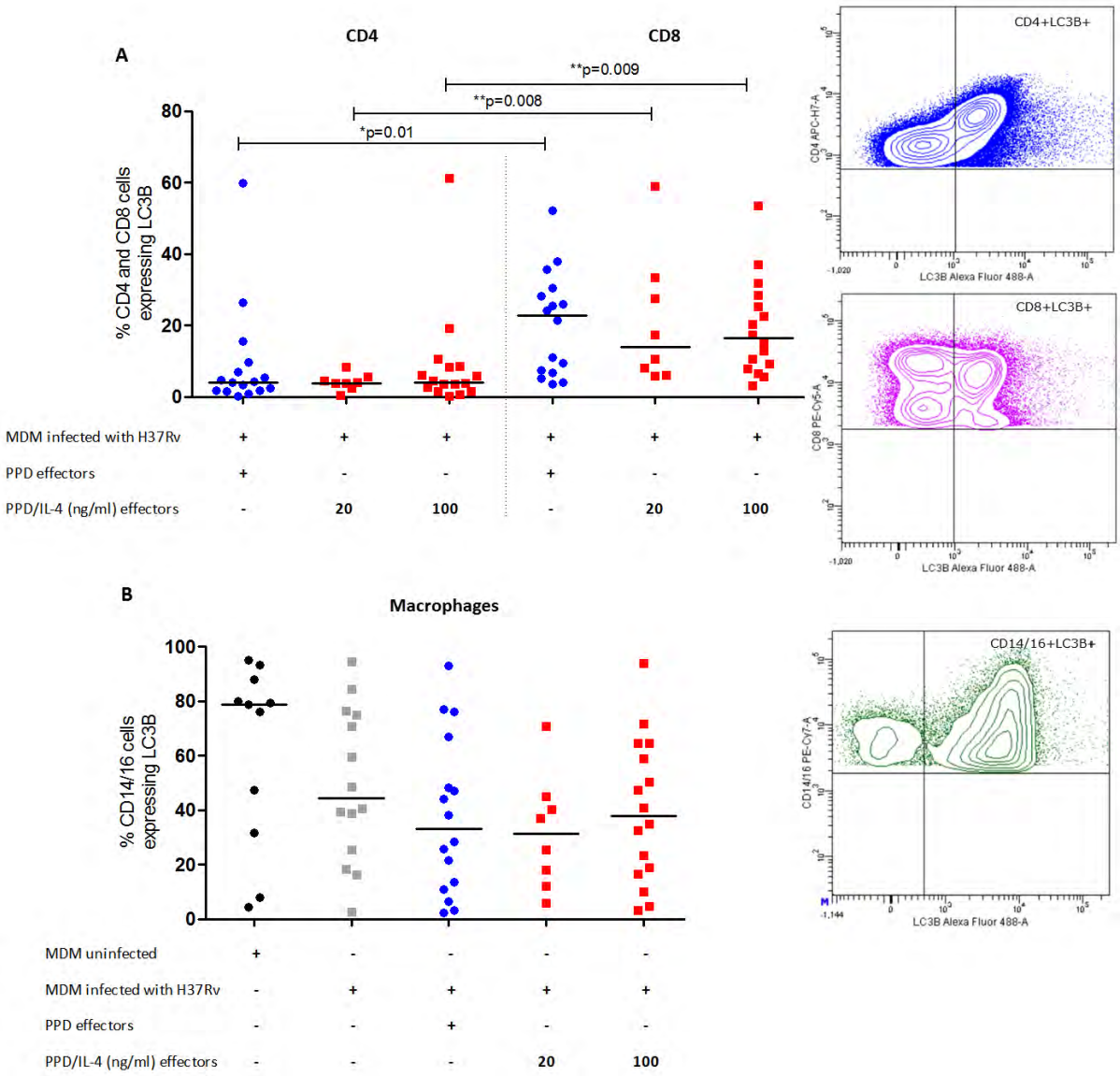


Figure 7.7. Frequency (%) of LC3B expressing cells in the co-cultured (A) CD4 and CD8 lymphocyte and (B) H37*Rv* infected macrophage (CD14/CD16) populations of the mycobacterial containment assay as measured by flow cytometry in TB patients (n=16). H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles) or PPD and rIL-4 at concentrations of 20ng/ml (n=8) and 100ng/ml (n=16) (red squares). Uninfected and H37Rv-infected macrophages alone (n=12) were also cultured. . Flow cytometry dot plots of the LC3B+ cells in the CD4+ (top), CD8+ (middle) and CD14+/16+ (bottom) populations are shown to the right of the corresponding graphs.

7.3.7 Neutralization of IL-4 and its effect on biomarker expression

In a subset of TB patients (n=6), the effect of blocking IL-4 was assessed by the addition of neutralizing anti-IL-4 antibody in the „PPD/IL-4 effectors". As expected, there was a significant increase in Treg expression (p=0.03) and decrease in IFN-γ expression (p=0.03) in „PPD/IL-4 effectors" at 100ng/ml rIL-4 compared to „PPD effectors". When anti-IL-4 antibody (20μg/ml) was added, there was a significant decrease in Treg expression (p=0.03; Figure 7.8A) and increase in IFN-γ expression (p=0.03; Figure 7.8B), compared to „PPD/IL4 effectors", to levels similar to the „PPD effectors" control. Addition of anti-IL-4R antibody (20μg/ml) produced similar results but there were no significant differences compared to the „PPD/IL-4 effector", most likely due to the low samples numbers in this intervention. There were no significant differences in the expression of TNFα, DC-SIGN or LC3B in the PPD/IL4 effectors with or without the addition of anti-IL-4 antibody (Table 7.4).

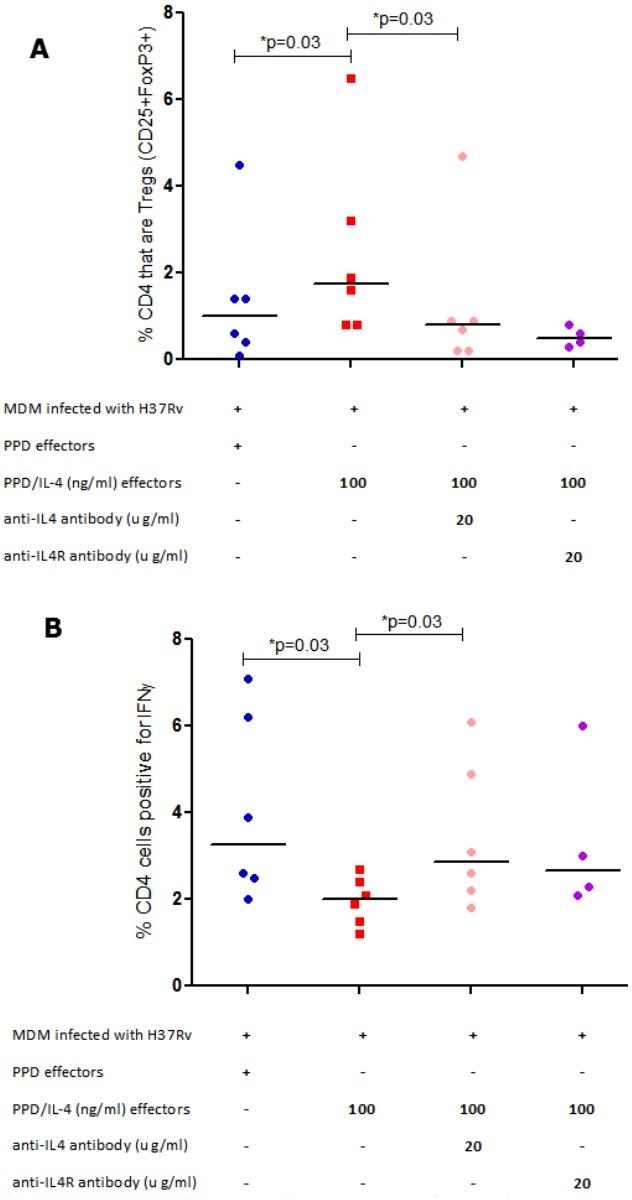


Figure 7.8. The effect of adding anti-IL-4 antibody on the frequency (%) of (A) regulatory T-cells (CD25+FoxP3+) and (B) IFN-γ expressing cells in the co-cultured CD4 lymphocyte population used in the mycobacterial containment assay as measured by flow cytometry in TB patients (n=6). H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (blue circles); PPD and rIL-4 at a concentration of 100ng/ml (red squares); PPD, rIL-4 and anti-IL4 antibody at a concentration of 20µg/ml (brown circles); PPD, rIL-4 and anti-IL4R antibody at a concentration of 20µg/ml (purple circles).

Table 7.4. Median frequency (%) of biomarker expressing lymphocytes (CD4, CD8) and macrophages (CD14/CD16+) in co-cultured cell populations of the mycobacterial killing assay with and without anti-IL-4 antibody as measured by flow cytometry. Peripheral blood mononuclear cells were obtained from pulmonary TB patients (n=6). H37*Rv*-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone **or** PPD and rIL-4 (100ng/ml) **or** PPD, rIL-4 (100ng/ml) and anti-IL4 antibody (20µg/ml). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

Analyte	CD4 T-Lymphocytes (CD3+CD4+)			
	PPD effectors	PPD/IL4 effectors (100ng/ml)	PPD/IL4 effectors & anti-IL4 Abs (20µg/ml)	p-value
IFN-γ	3.3 (2.4-6.5)*	2 (1.5-2.5)	2.9 (2.1-5.2)	[a]**p=0.03** [b]**p=0.03**
TNF-α	1.9 (0.7-2.9)	1.1 (0.4-1.9)	0.8 (0.4-1.9)	[a]p=0.06 [b]p=0.60
DC-SIGN	2.1 (1.6-7.7)	2.1 (1.4-5.7)	2.6 (1.1-4.7)	[a]p=0.53 [b]p=0.84
LC3B	8.3 (4.6-18.3)	8.4 (5.7-12.7)	6.9 (4.7-15)	[a]p=0.43 [b]p=0.69
Analyte	**CD8 T-Lymphocytes (CD3+CD8+)**			
	PPD effectors	PPD/IL4 effectors (100ng/ml)	PPD/IL4 effectors & anti-IL4 Abs (20µg/ml)	p-value
IFN-γ	14.8 (4.3-19.9)	10.6 (4-14.7)	11.6 (4.9-21.8)	[a]**p=0.03** [b]p=0.31
TNF-α	3.5 (1.5-6.9)	2.8 (1.1-4.5)	2.5 (1.1-5.8)	[a]p=0.06 [b]p=0.63
DC-SIGN	37.7 (24.6-46)	33.7 (23.4-49.2)	39.5 (30.4-50.6)	[a]p=1.0 [b]p=0.03
LC3B	17.6 (6.7-31.7)	15.2 (6.7-23.3)	15.3 (5-30)	[a]p=0.31 [b]p=1.0
Analyte	**Macrophages (CD14+CD16+)**			
	PPD effectors	PPD/IL4 effectors (100ng/ml)	PPD/IL4 effectors & anti-IL4 Abs (20µg/ml)	p-value
IFN-γ	7.2 (1.9-24.4)	9.2 (2.4-36.1)	9.4 (3.6-34.1)	[a]p=0.29 [b]p=0.84
TNF-α	0.3 (0.2-1.8)	0.7 (0.3-1.3)	0.6 (0.2-0.8)	[a]p=1.0 [b]p=0.14
DC-SIGN	5.7 (2.4-22.1)	7.1 (3.3-25.4)	6.6 (3.2-23.8)	[a]p=0.31 [b]p=0.84
LC3B	17.4 (3.1-47.4)	22.4 (4.4-43.3)	22.4 (4.6-41.4)	[a]p=0.84 [b]p=0.63

[a]p- p-value comparing median PPD effectors vs. PPD/IL4 effector;

[b]p – p-value comparing median anti-IL4 antibody vs. PPD/IL-4 effector

*values are expressed as the median % of cells expressing the specific analyte with the interquartile range in parentheses

7.4 Discussion

In chapter 6, I showed that pre-priming effector T-cells with PPD, in the presence of IL-4, results in a subversion of mycobacterial containment within infected monocyte derived macrophages. In the preceding experiments, the possible mechanisms that may drive this IL-4-mediated subversive effect on mycobacterial containment were investigated by flow cytometry. The addition of IL-4 resulted in a significant increase in Treg (CD4+CD25+FoxP3+) expression in the „PPD/IL-4 effectors" wells compared to „PPD effectors". Concurrently, there was a significant decrease in CD4+ T-cell expression of IFN-γ and TNFα and DC-SIGN expression on macrophages (CD14+CD16+). The addition of anti-IL-4 antibody reversed the expression patterns of CD4+IFN-γ and Tregs to levels similar to the „PPD effectors" control. Expression of the autophagy marker, LC3B, was not affected by the addition of IL-4, either in the macrophage or lymphocyte populations. Thus, IL-4-mediated subversion of *M.tb* containment is characterised by increased Treg expression, decreased CD4+ lymphocyte expression of Th1 cytokines (IFN-γ and TNFα) and increased DC-SIGN expression in macrophages.

7.4.1 IL-4 increase Treg expression

The pre-priming of PBMCs with PPD, in the presence of IL-4, resulted in a significant increase in the number of CD4+CD25+FoxP3+ regulatory T-cells (Tregs). IL-4 has been implicated in the development and maintenance of Tregs but the data are conflicting. Some studies have shown that IL-4 induces CD4+CD25- cells to become Tregs in both mice [301, 302, 443] and humans [303] whereas others have shown IL-4 inhibits Treg proliferation through the blocking of TGF-β [445]. Furthermore, IL-4 can either maintain Tregs [301, 444, 584] or suppress their function [302]. The data presented supports a proliferative role for IL-4 in Treg development and the observed differences, compared to other studies, are likely due to the *in vitro* model used (human vs murine), the timing of exposure to IL-4 and the type of antigen stimulation.

Tregs play a major role in the control of autoimmunity and immunopathological responses by downregulating inflammatory Th responses [267]. Traditionally, two major types of CD4+

Tregs have been described. Natural Tregs (nTregs), produced in the thymus, are anergic and are secreted into the periphery where they exert their suppressive effect. Inducible Tregs (iTregs), on the other hand, expand in response to antigenic stimulation and secrete immunomodulatory cytokines such as TGF-β and IL-10 [585]. Recent evidence suggests that nTregs can also expand in response to antigenic stimulation [586, 587]. Furthermore, a subset of nTregs, in response to IL-4 and antigen stimulation, undergoes clonal expansion to become antigen specific nTregs, termed Ts2. These are distinct from conventional nTregs, not only in their ability to respond to antigen but also in the expression of IL5Rα. These cells are able to suppress Th1, Th17 and macrophages [588, 589]. The presented data suggests that PPD stimulation, in the presence of IL-4, drives development of FoxP3+ nTregs as they produce very little IL-10 (Table 7.2).

The role of Tregs in TB has been extensively described. A number of studies have found that Tregs are high in TB patients [291-294, 590-593], tend to accumulate at the site of disease [291-294, 298] and decrease following anti-TB treatment [591, 593]. A Th1 response is the likely target of suppression by these Tregs, as Treg levels either correlate with reduced IFN-γ expression or functionally downregulate IFN-γ in these cells [287, 292-294, 592]. This is a likely scenario in my model as IFN-γ was also downregulated with the addition of IL-4. Additionally, Anti-TNF therapy is associated with increased Treg function and subsequent susceptibility to TB [296-298]. Two studies have directly demonstrated that Tregs favour mycobacterial survival by downregulation of anti-mycobacterial responses [293, 298]. In contrast, a murine model found that TB pathogen load was unaffected by Treg mediated depression of antigen specific cytokine responses [594]. However, none have previously shown a direct involvement of IL-4.

In my model, the addition of IL-4 results in increased Treg and decreased IFN-γ expression whereas neutralization of IL-4 simultaneously reverses these effects. Furthermore, blocking of the IL-4 receptor produces similar results to blocking of IL-4 protein suggesting that these effects are facilitated through signalling via the IL-4 receptor. IL-4-driven Treg effects have been shown to be mediated via the IL-4Rα in mice [443]. Taken together, these data suggest IL-4 may be promoting the expansion of nTregs which suppress the protective Th1 response

and result in a subversion of mycobacterial containment (chapter 6). However, the mechanism by which Tregs suppress the Th1 response, whether by a direct cell-to-cell contact mechanism [278] or through the production of regulatory cytokines [595, 596], is unknown. My data suggests the former, given that these Tregs produce very little IL-10. Nonetheless, further investigation is required to elucidate these mechanisms.

7.4.2 IL-4 downregulates the Th1 response

The addition of rIL-4 resulted in decreased expression of IFN-γ and TNFα in the CD4 T lymphocyte population but not in the CD8 or macrophage populations suggesting that the most significant effect of IL-4 in this model was a downregulation of the CD4 Th1 response. In a similar *in vitro* infection model, IFN-γ was produced primarily by CD4 T-cells but was less effective at restricting *M.tb* growth compared to CD8 T-cells, which mainly produced TNFα [564]. While IFN-γ production by other T-cells (including CD8, NK and γδ T-cells), is important, production of IFN-γ by these cells, cannot compensate for a deficiency in CD4+ T-cells [568, 597]. The vital role of IFN-γ in *M.tb* control has been discussed earlier, and includes activation of mycobactericidal activity in macrophages [228] and cytotoxic mechanisms in CD8 cells, through production of granzyme and perforin and Fas-FasL mediated apoptosis [217, 598], as well as further recruitment of T-cells [559].

TNFα is also an essential mediator in TB immunity as it recruits monocytes to the site of infection [124], is a strong inducer of apoptosis [140, 144, 145] in infected macrophages and is essential in granuloma formation as demonstrated in both murine and human TB [599, 600]. Indeed, anti-TNFα therapy can result in increased susceptibility to TB [249]. Previous studies have shown that TNFα is primarily produced by phagocytic cells, particularly at the site of disease [250], but also by T-lymphocytes [601]. In a murine model, a deficiency in myeloid derived TNFα caused early susceptibility to disease but was then controlled by recruitment of TNFα producing T-cells, which sustained protection during the later stages of infection [602]. In the model presented here, IL-4 reduced TNFα expression in CD4+ T-cells suggesting that IL-4 may undermine the effects of TNFα during the early stages of infection.

Given the importance of these cytokines, it is not surprising that the reduced Th1 response is consistent with the decrease in mycobacterial containment observed in chapter 6.

The downregulation of Th1 cytokines, in response to IL-4, may be occurring either by the increase in Treg activity (discussed in 7.4.1) and/or the skewing of the Th1/Th2 balance. T-cell priming with PPD alone primarily induces a Th1 response, most likely through MHC processing and activation of the IL-12/STAT4 signalling pathway [603]. However, the effect of adding IL-4, together with PPD, to un-primed T-cells before commitment to a specific T-cell lineage, may drive these cells toward a Th2 phenotype [574]. As a result, there is a concomitant reduction in the number of Th1 effectors and subsequently, reduced IFN-γ and TNFα expression. This result is most notable at higher rIL-4 concentrations suggesting that there is a competitive effect between Th1 and Th2 activation. Th2 activation, driven by IL-4, is perhaps outcompeting Th1 activation, via PPD stimulation, to produce a predominant Th2 response. Given that blocking of the IL-4 receptor, using anti-IL4R antibody, restored IFN-γ levels in a similar manner to blocking of IL-4, it is likely that Th2 polarization is occurring via an IL-4R signalling pathway.

There were no significant differences in IFN-γ or TNFα production in macrophages, with or without the addition of IL-4, suggesting that polarized Th2 effector cells did not affect the production of these cytokines in macrophages. However, IFN-γ expression was significantly decreased in infected compared to uninfected macrophages. This was similar to another H37Rv infection model of resting macrophages [604] and may indicate a decrease in macrophage numbers post-infection as a result of apoptosis or a downregulation of proinflammatory cytokine production by these macrophages. Indeed, *M.tb* inhibits classical Th1-mediated macrophage activation, because it blocks phagosome maturation [605], lysosome fusion [156], presentation via MHC Class I [606] and triggering via the IFN-γ receptor [607].

7.4.3 IL-4 increases DC-SIGN expression on macrophages

DC-SIGN is a C-type lectin expressed on the surface of phagocytes and facilitates entry of *M.tb* into phagocytic cells through binding of lipoarabinomanan (LAM). While *M.tb* entry into macrophages occurs predominantly through mannose receptors [608], it can also occur via DC-SIGN [609]. Increased expression of DC-SIGN was found in alveolar macrophages of TB patients and is thought to facilitate the spread of infection [205, 609]. Increased DC-SIGN expression is also a hallmark of alternative macrophage activation, and results in a downregulation of macrophage anti-mycobacterial mechanisms [39].

The addition of PPD/IL-4 driven effector T-cells results in increased DC-SIGN expression on macrophages. This is not surprising given that IL-4 is a potent inducer of alternate macrophage activation [351]. In this model, IL-4 polarizes T-cells to a predominantly Th2 response. The secretion of IL-4 by these polarized Th2 cells during co-culture with infected macrophages is likely to drive alternative activation of macrophages, characterized by, in this case, upregulation of DC-SIGN. In addition to increased surface expression of these pattern recognition receptors (PRRs), such as DC-SIGN and mannose receptor, alternatively activated macrophages exhibit increased production of arginase and subsequent downregulation of iNOS [39, 40]. The cytokine expression profile of these macrophages also changes with decreases in IFN-γ and TNFα production and increases in IL-10 and soluble TNF receptor secretion, leading to the formation of inactive TNFα-TNF receptor complexes [147, 435].

FoxP3+ Tregs can also induce alternative activation of macrophages *in vitro* [610]. If this is the case, then the increased Treg expression observed here may also be contributing to the increase in DC-SIGN levels. However, there was no decrease in IFN-γ or TNFα expression in macrophages with the addition of PPD/IL-4 driven effectors. This suggests that perhaps these macrophages are in a transition state where they express some markers of alternative activation but still retain their ability to express M1 associated pro inflammatory cytokines. This will depend on the plasticity of macrophages and their ability to switch between classical and alternate activation. Indeed, plasticity of macrophages has been demonstrated *in vitro* in *C. neoformans* infection [577].

An interesting finding is the high expression of DC-SIGN on CD8+ T-cells. DC-SIGN expression is usually restricted to DCs and macrophages. However, DC-SIGN-expressing B-cells have also been described, which facilitate HIV-1 infection of T-cells and increase in response to IL-4 [611]. However, there was no increased expression of DC-SIGN on CD8+ cells when stimulated in the presence of IL-4. This is the first study to provide evidence of DC-SIGN expression on CD8+ T-cells. *M.tb* binds to DC-SIGN and facilitates entry into macrophages and DCs but it is highly unlikely that it has the same function on CD8+ T-cells given that these cells have accessory cytotoxic functions and do not internalize *M.tb*. It is possible that DC-SIGN facilitates *M.tb* infection of macrophages or DCs in a way similar to B-cell-mediated HIV-1 infection [611] or its cytotoxic activity is activated by directly binding to mycobacterial PAMPs, such as ManLAM, independent of MHC I presentation. However, DC-SIGN expression was only assessed in TB patients and it is unknown if this receptor is highly expressed on CD8+ T-cells in other disease conditions or in healthy individuals. Further investigation is required to determine the significance of DC-SIGN expression on CD8+ T-cells in TB and whether it extends to other diseases as well.

7.4.4 LC3B production

Autophagy is a lysosomal degradation pathway for cytoplasmic materials and damaged organelles and involves the formation of autophagosomes and subsequent fusion with lysosomes. There is extensive evidence on the role of autophagy as a protective mechanism against *M.tb* (reviewed in [155]). Autophagy directly kills *M.tb* within macrophages in response to pro-inflammatory cytokines such as IFN-γ and TNFα [158, 159], vitamin D via cathelicidin [162] or through TLRs [160, 161]. In contrast, IL-4 and IL-13 can inhibit autophagy via two different mechanisms; starvation induced autophagy is blocked by upregulation of Akt whereas IFN-γ induced autophagy is inhibited via the STAT-6 pathway [44].

The most common measure of autophagy is the microtubule-associated protein light chain 3 (LC3), which localizes to autophagosomal membranes. Upon synthesis, unprocessed LC3

undergoes a C-terminal cleavage event to yield the cytosolic form, LC3-I. When autophagy is induced, LC3-I undergoes lipidation to form LC3-II, which then becomes associated with autophagosomes. Lysosomal fusion with the autophagosome results in degradation of the vesicle contents along with LC3-II. Thus, the presence of LC3 in autophagosomes, as well as the conversion of LC3-I to LC3-II, indicates autophagic induction. A fluorescently conjugated antibody against LC3B, the most commonly measured human LC3 isoform, was used in this assay to measure endogenous levels of LC3 protein. No difference in the level of LC3B with or without the addition of IL-4 in macrophages or in lymphocytes was found. This is in contrast to other studies which found that IL-4 caused a reduction in starvation-induced autophagy [44] and Vitamin D-mediated autophagy [612] within macrophages. These differences may be due to the method of LC3B quantification: confocal microscopy to determine the level of LC3 punctate cells [612] or immunoblotting to determine the LC3-II to LC3-I ratio [44]. Indeed, LC3B level is a good indicator of autophagy, as it correlates with the number of autophagosomes [613], but it does have its limitations. Variations in expression levels of LC3-I and LC3-II in different cell types, different antibody affinities of LC3-II compared to LC3-I as well as the degradation of LC3-II during autophagy can result in false conclusions concerning the extent of autophagy [614, 615]. This may be the reason why no differences in the IL-4 interventions were observed. It has been suggested that measurement of static levels of autophagy may not be sufficient and rather determining the level of „autophagic flux", which measures the rate of removal by autophagy, is a more sensitive technique [614, 615].

7.4.5 The biological significance of these findings

Using an *in vitro M.tb* infection model, I have shown the multiple subversive effects of IL-4 on cellular anti-mycobacterial mechanisms. A similar situation may also be occurring *in vivo*. At the site of disease, IFN-γ and TNFα are important for restricting *M.tb* growth and granuloma formation but excessive production has immunopathological consequences and can lead to tissue necrosis and cavity formation [8, 601]. Tregs function to prevent such immunopathology but also counteract these protective Th1 responses. High CD4+CD25+FoxP3+Tregs levels have been found in granulomas of both humans and

experimental animal TB models [291, 298, 616]. Furthermore, an early induction of Tregs results in delayed T-cell responses in the lung [300], likely by a direct suppression of effector T-cells. If IL-4 is indeed a driver of Treg proliferation, then a Th2 response in TB will likely disrupt the effector T-cell/Treg balance leading to a reduced Th1 response. The effect of alternate macrophage activation in the downregulation of protective responses in TB has been shown in mice *in vivo* [39, 40]. Evidence also suggests that a switch in macrophage polarization occurs from iNOS-producing classically activated macrophages to arginase-producing alternatively activated macrophages in progressive TB [40]. A shift in this macrophage ratio within the granuloma, likely driven by IL-4, reduces Th1 responses and promotes Treg expansion resulting in enhanced *M.tb* survival within these macrophages [597].

In contrast, a zebrafish model of *M. marinum* infection demonstrated that a complete lack of a Th2 response can lead to progressive infection [617]. This finding is interesting given the extensive evidence supporting the detrimental role of a Th2 response in TB. However, it does suggest that, to some extent, a Th2 response, including induction of Tregs, is probably necessary to prevent an uncontrolled inflammatory response and subsequent immunopathology.

Taking these data together, a delicate balance between the proinflammatory responses of macrophages and Th1 effector T-cells and immunoregulatory responses of Tregs at the site of disease is necessary to contain *M.tb* within the granuloma and simultaneously prevent excessive inflammation. A Th2-skewed response, which myself and others [9, 416-418] have shown to be present in TB, is likely disrupting this balance leading to a compromised immune response that cannot control the *M.tb* infection [38, 618].

7.4.6 Limitations

A number of assay limitations have already been presented in chapter 6 in addition to those discussed here.

These mechanistic studies were performed in „PPD/IL-4 effectors" interventions but not the „Exogenous IL-4" interventions. The number of PBMCs required in the above assay (to study mechanisms of IL-4 mediated subversion of *M.tb* containment) was much greater than what is needed for the mycobacterial containment assay to assess CFUs (chapter 6), mainly due to the high cell number requirements for flow cytometric analysis. As such, it was not possible to perform flow cytometry on both interventions and the „PPD/IL-4 effector intervention was chosen as it produced the most significant effect on mycobacterial containment, in terms of CFU/ml. However, „Exogenous IL-4" interventions will be investigated in future work to determine if these same mechanisms are at work when IL-4 is added at such a late stage to the model.

In addition to Tregs, activated T-cells produce low levels of FoxP3 [619, 620] and this may account for a rise in the number of Tregs (CD4+CD25+FoxP3+) in response to PPD and/or IL-4 stimulation. This is unlikely to have skewed the results given that expression of these cells was downregulated with the addition of anti-IL4 antibody even in the presence of PPD, suggesting the effect is truly an IL-4-driven expansion of Tregs. Future work will be performed to determine the identity, function and mode of suppression of these IL-4 induced Tregs.

Measurement of other markers of alternate macrophage activation, including arginase and mannose receptor was limited by cell numbers and the number of cytokines that could be effectively measured in each flow cytometry panel. It is likely that these markers would also have been increased and provided more supporting evidence. However, given that DC-SIGN is a well described marker of alternate macrophage activation, the data remain valid.

As discussed above, measurement of autophagy may have been more precise by measuring autophagic flux or immunoblotting techniques, rather than flow cytometric analysis of LC3B [614, 615]. However, PBMC isolation did not yield sufficient cells to perform separate experiment for measurement of autophagy. Furthermore, given the lack of antibodies to effectively distinguish between LC3-I and LC3-II, it was not possible to determine this ratio

by flow cytometry. Future experiments, which are beyond the scope of this study, will incorporate autophagic flux to more accurately determine the effect of IL-4 on autophagy.

7.5 Conclusion

The observed IL-4-associated subversion of mycobacterial containment within monocyte derived macrophages is mediated by a downregulation of a protective Th1 response (decreased IFN-γ and TNFα), likely through increased Treg activity, and alternatively activated macrophage polarization. In addition to IL-4, other Th2 and Th2-like cytokines may also be involved in undermining TB immunity. These data provide insights into immune mechanisms that can disrupt protective immunity and aid in identifying potential targets, such as a Th2 response, for design of future vaccines and immunotherapeutic agents. This prompted the work presented in the next chapter, where various Th1, Th2 and Th2-like cytokines and chemokines will be measured in TB and presumed LTBI samples from the periphery and site of disease.

8. CHAPTER 8: Levels of soluble Th1 and Th2-like cytokines and chemokines in supernatants from RD-1 antigen-driven peripheral blood and lung cells

8.1 Introduction

The data presented in the previous chapters provides evidence that a Th2 response, typified by IL-4, is present during TB infection and that it undermines protective Th1 mechanisms. However, it is unlikely that IL-4 alone drives this immune dysregulation given the complex cellular interactions and cytokine milieu found in the immune response to TB. Other cytokines and chemokines, which are thought to be detrimental in TB, are associated with a Th2 response. For example, IL-9, which has similar functions to IL-4, was recently shown to be produced by a distinct Th9 subset [49, 50] and is upregulated in active TB [344, 345]. Monocyte chemoattractant protein-1 (MCP-1) is considered a Th2 chemokine because of its role in the development and recruitment of Th2 cells [621-623] and high levels have been reported in TB patients compared to controls [624-627]. IL-10, an immunosuppressive cytokine produced by various Th subsets and alternatively activated macrophages, is often found to be upregulated in TB [33, 628, 629]. In contrast to the above mentioned cytokines, IL-17, similar to IFN-γ and TNFα, may have a protective role in TB as it is responsible for recruitment of neutrophils and early development of the granuloma [125, 630].

Studies have looked at these and other biomarkers in an attempt to identify a cytokine expression profile that distinguishes TB infected individuals from healthy controls. However, the data have been disparate. Studies have shown that IFN-γ and TNFα may either be increased [238, 240, 244, 631] or decreased [33, 243, 245-247, 632] in active TB. Studies measuring IL-10 [11, 15, 33, 246, 628, 629, 633] and MCP-1 [36, 624, 625, 627] levels have shown similar contrasting results. Furthermore, very few studies have investigated IL-9 in TB disease. Most reports examining the levels of soluble cytokines in response to antigenic stimulation focused primarily in the peripheral blood compartment. However, few have investigated both the site of disease and the periphery particularly in the context of a Th2 response. It is important to determine the local immune response at the site of disease in TB

as *M.tb* specific T-cells tend to accumulate here and, along with the highly activated resident tissue macrophages and other infiltrating cell types, produce a cytokine profile that can be different from the peripheral circulation [38]. Such investigations would provide useful information in identifying specific biomarker profiles which can aid in TB diagnosis or be used as targets in vaccine design and immunotherapeutic agents. Moreover, it is important to measure the soluble cytokines because, in contrast to mRNA, these proteins directly exert their effects on cells to generate immune responses.

The aim of this chapter was to identify specific cytokines and chemokines in *M.tb*-specific antigen stimulated cell cultures from the site of disease, specifically the lungs, and peripheral blood in TB patients and presumed LTBI controls.

8.2 Methods

8.2.1 PBMC and BAL cell isolation

Whole blood was obtained by venipuncture from 18 TB patients and 11 presumed LTBI controls. Broncho-alveolar lavage (BAL) fluid was collected from 10 TB patients and 8 presumed LTBI controls, as described in section 2.3.3. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation and BAL cells were isolated from BAL fluid as described in sections 2.4.2 and 2.4.3 respectively.

8.2.2 Cell culture

A total of $2.5x10^5$ BAL cells and PBMCs were plated in 96-well round bottom plates in triplicate. Cells were stimulated with the RD1 antigens ESAT-6 and CFP-10 (Oxford Immunotec) using manufacturer recommended concentrations (which are proprietary and cannot be disclosed) for 5 days in a final well volume of 200µl. Triplicate wells were pooled and harvested by centrifugation at 1200 rpm for 5 minutes. Supernatants were collected and stored in 1.5ml tubes at -80^oC.

8.2.3 Luminex MAP assay

A 7-plex Milliplex Human Cytokine MAP assay (Millipore) was performed for the following cytokines– IFN-γ, TNFα, IL-4, IL-9, MCP-1, IL-10 and IL-17 according to the manufacturer‟s instructions. Cell culture supernatants were assayed undiluted or diluted 10-fold in RPMI 1640 (Lonza). All samples were run in duplicate. Antibody-immobilized beads, quality controls and cytokine standards were prepared as per manufacturer‟s instructions. The standard curve of each analyte ranged from 1.6 – 10,000 pg/ml for each kit. 25µl of standards, quality controls and samples was added to appropriate wells of a 96-well filter plate. A mixture of fluorescent beads, conjugated to antibodies specific for the cytokines of interest, was added to each well and incubated with shaking at room temperature. The well contents were then washed with wash buffer using a vacuum manifold. Biotinylated detection antibody was added to the beads in the wells and incubated with shaking followed by another wash step. Phycoerythrin-conjugated streptavidin was then added to the well followed by a third incubation period and wash step. Beads were then resuspended in sheath fluid within the wells and immediately analyzed on a Bioplex Luminex 100 reader (Bio-Rad). Standard curve generation and quantification of analytes was performed using Bioplex Manager 6.0 (Bio-Rad).

8.2.4 Data analysis

Differences in median soluble cytokine and chemokine levels between the different groups (TB patients and LTBI controls) and different compartments (BAL vs peripheral blood) were determined using the Mann-Whitney U test. A p-value of <0.05 were deemed significant. Statistical analyses were performed using GraphPad Prism 5.0 software and Microsoft Excel.

8.3 Results

8.3.1 IFN-γ

IFN-γ levels were significantly lower in PBMC supernatants of TB patients compared to LTBI controls (433.0 pg/ml *vs*. 3757.0 pg/ml, respectively; p=0.02). In BAL supernatants, median IFN-γ levels were generally low and not significantly different in TB vs. LTBI (47.7

pg/ml *vs*. 1.0 pg/ml, respectively). IFN-γ was significantly higher in supernatants of PBMCs compared to BAL cells in LTBI controls (3757.0 pg/ml *vs*. 1.0 pg/ml, respectively; p=0.0006). (Figure 8.1A and Table 8.1).

8.3.2 TNFα

No significant differences in TNFα levels were observed in PBMC supernatants between TB patients and LTBI controls. Median TNFα levels were lower in BAL cell supernatants of TB vs. LTBI controls but these differences were not statistically significant (562.0 pg/ml *vs.* 2102.0 pg/ml, respectively; p=0.07). TNFα levels were significantly higher in supernatants of BAL cells compared to PBMCs in LTBI controls (2102.0 pg/ml *vs.* 847.0 pg/ml respectively; p=0.02) (Figure 8.1B and Table 8.1).

8.3.3 IL-9

IL-9 levels were not significantly different in the PBMC supernatants of TB patients and LTBI controls. IL-9 levels were significantly higher in BAL cell supernatants of TB vs. LTBI controls (3.5 pg/ml *vs.* 1.4 pg/ml, respectively; p=0.02). In TB patients, IL-9 levels were higher in the supernatants of BAL cells compared to PBMCs (3.5 pg/ml *vs.* 1.0 pg/ml, respectively; p=0.003) (Figure 8.1C and Table 8.1).

8.3.4 IL-4

IL-4 was mostly undetectable in TB patients vs. LTBI controls in PBMC (0.0pg/ml *vs.* 0.0pg/ml, respectively) and BAL cell supernatants (0.5pg/ml *vs.* 2.5pg/ml, respectively) (Figure 8.1D and Table 8.1).

8.3.5 MCP-1

Median MCP-1 levels were not significantly different in the PBMC supernatants of TB patients compared to LTBI controls. In BAL supernatants, MCP-1 was generally higher in TB vs. LTBI controls but these differences were not significant (15448 pg/ml *vs.* 7308 pg/ml,

respectively). MCP-1 was also higher, but not significant, in BAL compared to PBMC supernatants of TB patients (15448 pg/ml *vs.* 4025 pg/ml, respectively) (Figure 8.1E and Table 8.1).

8.3.6 IL-10

No significant differences in IL-10 levels were observed in TB vs. LTBI controls either in PBMC or BAL cell supernatants. However, median IL-10 levels were significantly higher in PBMC compared to BAL supernatants of LTBI controls (35.5 pg/ml *vs.* 2.7 pg/ml; p=0.01) (Figure 8.1F and Table 8.1).

8.3.7 IL-17

IL-17 expression was generally low in both groups. There were no significant differences in median IL-17 levels between TB patients and LTBI controls either in PBMC (2.8 pg/ml *vs.* 2.5 pg/ml, respectively) or BAL cell supernatants (4.8 pg/ml *vs.* 4.9 pg/ml, respectively) (Figure 8.1G and Table 8.1).

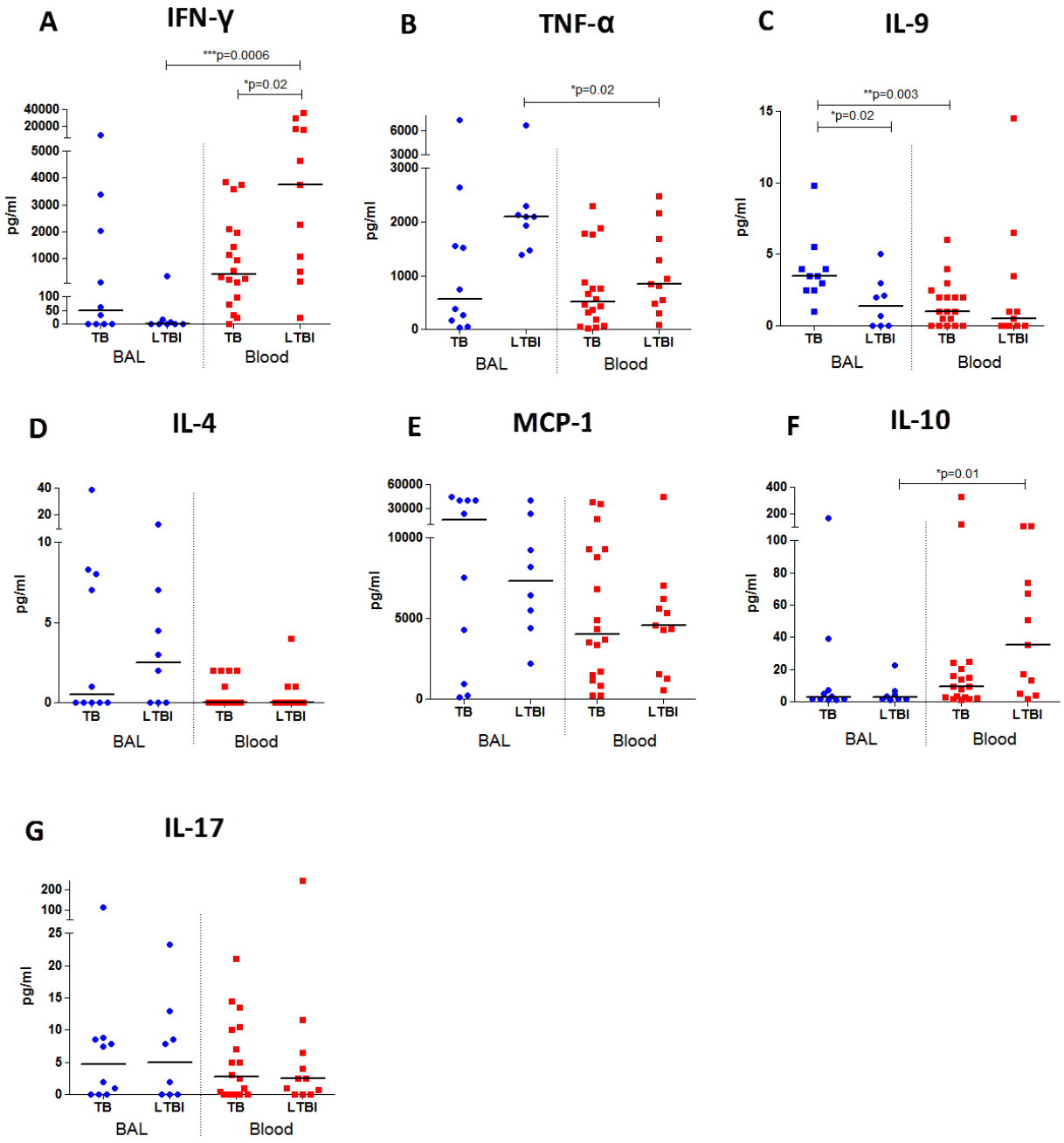


Figure 8.1. Soluble cytokine levels of (A) IFN-γ (B) TNFα (C) IL-17 (D) IL-4 (E) IL-9 (F) IL-10 (G) MCP-1 in ESAT-6 and CFP-10 stimulated broncho-alveolar lavage (BAL; blue circles) and peripheral blood mononuclear cell (Blood; red squares) culture supernatants from patients with pulmonary tuberculosis (TB; BAL= 10, Blood=18) and latently infected controls (LTBI; BAL=8, Blood=11) as measured by Luminex multiplex assay. Statistical analyses between group medians were performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

8.3.8 Ratio of Th2 or Th2 related cytokines/chemokines to Th1 cytokines

The ratio of IL-9:TNFα (Figure 8.2A) and MCP-1:TNFα (Figure 8.2B) was significantly higher in RD1-stimulated BAL cell supernatants of TB patients compared to LTBI controls (p=0.001 and p=0.03, respectively). In PBMC supernatants, the IL-9:IFN-γ ratio (Figure 8.2C) and MCP-1:IFN-γ (Figure 8.2D) ratio was significantly higher in TB patients compared to LTBI controls (p=0.049) but no significant differences were observed in the IL-10:IFN-γ (p=0.07), IL-9:TNFα (p=0.58) and MCP-1:TNFα (p=0.43) ratios (Figure 8.2E-G). IL-4 was not assessed in these ratios as it was undetectable in most samples. Additionally, the IL-9:IFNγ, MCP-1:IFN-γ and IL-10:IFN-γ ratios were not assessed in the BAL compartment because IFN-γ was undetectable in a large number of BAL samples.

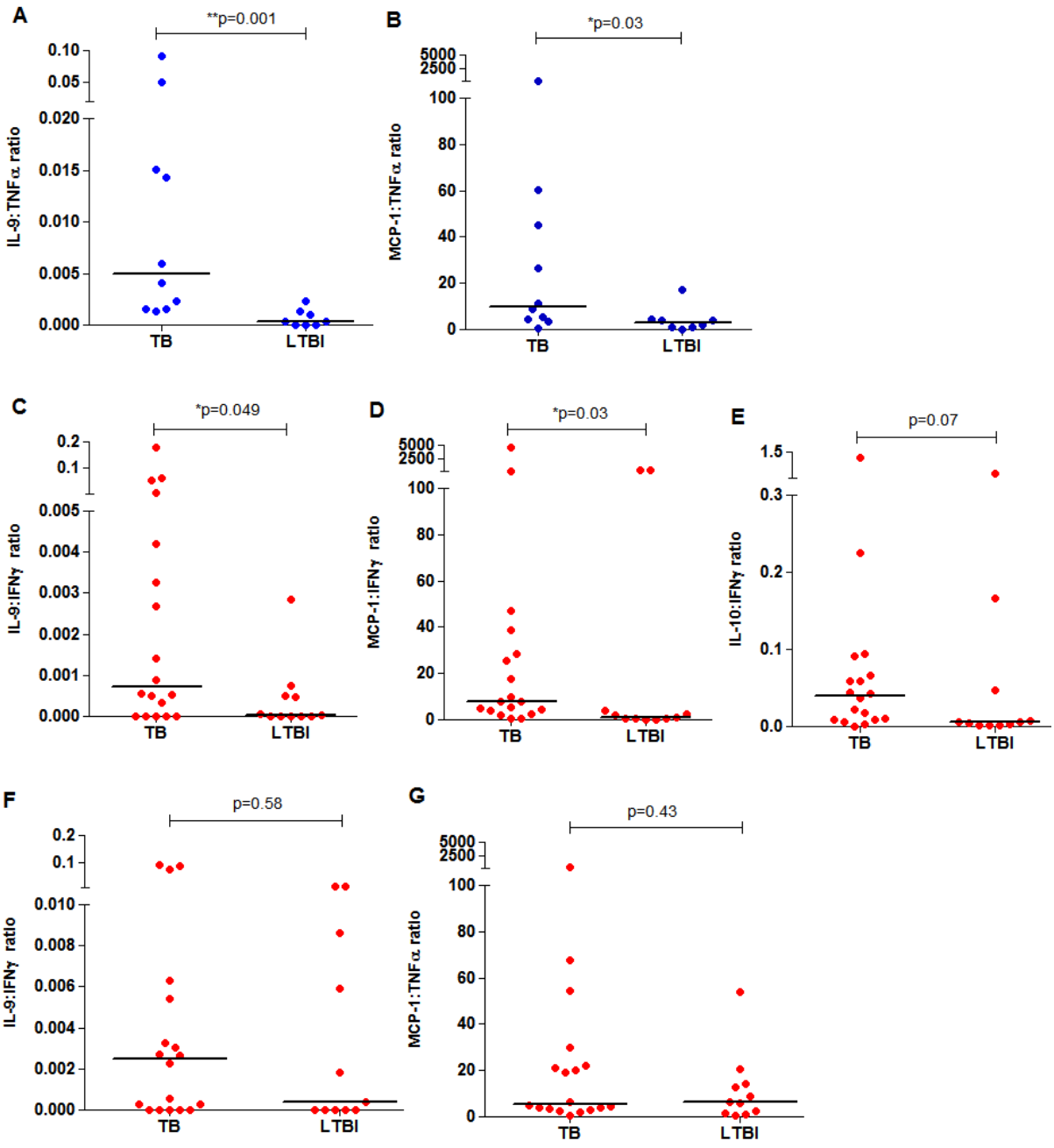


Figure 8.2. Cytokine ratios of (A)IL-9:TNFα and (B)MCP-1:TNFα in RD1-stimulated broncho-alveolar lavage (BAL; blue circles) cell culture supernatants; (C) IL-9:IFN-γ, (D) MCP-1:IFN-γ, (E) IL-10:IFN-γ, (F) IL-9:TNFα and (G) MCP-1:TNFα in RD1-stimulated peripheral blood mononuclear cell (Blood; red circles) culture supernatants from patients with pulmonary tuberculosis (TB; BAL= 10, Blood=18) and latently infected controls (LTBI; BAL=8, Blood=11). Statistical analyses between group medians were performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

Table 8.1. Median cytokine levels (pg/ml) of IFN-γ, TNFα, IL-9, IL-4, MCP-1, IL-10 and IL-17 in RD1 (ESAT-6 and CFP-10) stimulated broncho-alveolar lavage and peripheral blood cell culture supernatants from patients with pulmonary tuberculosis (TB) and latently infected controls (LTBI) as measured by Luminex multiplex assay. Cytokines are expressed in pg/ml and the interquartile range (IQR) is shown in parentheses. Statistical analyses between group medians were performed using the Mann-Whitney U test and $p<0.05$ was deemed significant.

Analyte pg/ml (IQR)	BAL		Blood		p-value
	TB (n=10)	LTBI (n=8)	TB (n=18)	LTBI (n=11)	
IFN-γ	47.7 (0-2384)	1.0 (0-14.8)	433.3 (89.4-1995)	3757.0 (511-16675)	[a]p= 0.16 **[b]p= 0.02** [c]p= 0.16 **[d]p= 0.0006**
TNF-α	562.3 (137.5-1825)	2102.0 (1586-2254)	507.0 (154.6-1107)	847.0 (478.5-1691)	[a]p= 0.07 [b]p= 0.17 [c]p= 0.83 **[d]p= 0.02**
IL-9	3.5 (2.5-4.4)	1.4 (0-2.8)	1.0 (0-2.2)	0.5 (0-3.5)	**[a]p= 0.02** [b]p= 0.61 **[c]p= 0.003** [d]p= 0.86
IL-4	0.5 (0-8.1)	2.5 (0-6.4)	0.0 (0-1.3)	0.0 (0-1.0)	[a]p= 0.96 [b]p= 0.88 [c]p= 0.13 [d]p= 0.06
MCP-1	15448.0 (761.1-40000)	7308.0 (4664-20251)	4025.0 (1404-9274)	4551.0 (1524-6201)	[a]p= 0.93 [b]p= 0.91 [c]p= 0.28 [d]p= 0.11
IL-10	2.7 (1.4-15.4)	2.7 (2-6.2)	9.5 (2.9-21.4)	35.5 (5-73.5)	[a]p= 1.0 [b]p= 0.11 [c]p= 0.14 **[d]p=0.01**
IL-17	4.8 (0-8.6)	4.9 (0-11.9)	2.8 (0-10.2)	2.5 (0-6.5)	[a]p= 0.96 [b]p= 0.89 [c]p= 0.84 [d]p= 0.80

p-values compare the median pg/ml in [a]TB *vs.* LTBI BAL, [b]TB *vs*. LTBI blood, [c]BAL *vs*. Blood TB, [d]BAL *vs.* Blood LTBI.

8.4 Discussion

A Luminex multiplex assay was performed to determine the levels of various cytokines and chemokines in RD1-stimulated PBMC and BAL cell culture supernatants of TB patients and presumed LTBI controls.

When cytokine levels between the different groups (TB *vs.* LTBI) within the same compartment were compared, increased IL-9 and decreased TNFα were observed in the BAL cell supernatants of TB patients compared to LTBI controls. However, although a definite trend was observed, the differences in TNFα levels were not significant. In PBMC supernatants, IFN-γ levels were lower in TB patients compared to LTBI controls. There were no significant differences in levels of IL-17, IL-4, IL-10 or MCP-1 between TB and presumed LTBI controls.

Comparison of cytokine levels between the different compartments (lung *vs.* peripheral blood) revealed significantly higher IL-9 and lower IFN-γ in the RD-1 stimulated cell supernatants of BAL compared to PBMCs in TB patients. In LTBI controls, higher TNFα and lower IL-10 was observed in the supernatants of BAL cells compared to PBMCs.

Analysis of various cytokine ratios revealed that the IL-9:TNFα and MCP-1:TNFα ratios were significantly higher in the BAL culture supernatants of TB patients compared to LTBI controls. Similarly, the IL-9:IFN-γ and MCP-1:IFN-γ ratios were significantly higher in PBMC supernatants of TB patients vs. LTBI controls. These results suggest that TB patients produce a distinct cytokine expression profile compared to LTBI controls and these differences are compartment-specific.

8.4.1 IFN-γ and TNFα

As discussed previously, Th1 cytokines, including IFN-γ and TNFα, activate mycobactericidal mechanisms to mount a protective immune response in *M.tb* infection [68]. Previous studies that measured IFN-γ in response to *in vitro* antigenic stimulation in PBMCs have been conflicting; some studies have reported significantly decreased IFN-γ levels in

active TB disease [33, 243-247], whereas others have demonstrated an increase in IFN-γ responses [238, 240-242] compared to healthy controls. The data presented here supports the former suggesting that there is a suboptimal Th1 response in TB patients. The differences in other studies may be due to variations in the experimental design including the specific antigen used for stimulation (*M.tb* whole sonicates vs. purified antigen) [240-242] or the length of the stimulation period [240, 242] which can have significant effects on cytokine expression [26, 634]. The extent of disease severity may also play a role as more severe disease is associated with lower IFN-γ levels in TB patients [240, 245, 635]. Possible explanations for the observed decrease in IFN-γ responses are the increased susceptibility of antigen specific T-cells to apoptosis in TB patients [636] or that IFN-γ secreting T-cells are recruited to the lungs during disease resulting in fewer circulating cells in the periphery [8].

IFN-γ levels in RD1-stimulated BAL cell culture supernatants were much lower compared to PBMCs. Studies assessing soluble cytokine levels from antigen-stimulated cells at the site of disease reported either lower [15] or higher production of IFN-γ [11, 482] compared to the periphery. These observed differences may be a reflection of the hyper-activated state of T-cells from the site of disease. Isolated T-cells from TB pleural effusions demonstrate accelerated proliferation and enhanced cytotoxicity [637, 638] and exhibit increased apoptosis [636] compared to peripheral blood T-cells. This effect is also observed in other chronic lung inflammatory diseases [639]. As such, further stimulation of hyper-responsive cells may result in enhanced cell death and decreased cytokine production. Another possibility is the relative abundance of T-cells present in the lung compared to the periphery. Despite lymphocytic infiltration into the lungs during pulmonary TB disease, peripheral blood usually has a higher proportion of T-cells compared to lung lavage, which consists mostly of alveolar macrophages [15]. Given that T-cells are the primary source of IFN-γ [11, 15], this disparity may also explain the low levels of IFN-γ secreted by RD1 stimulated cells from BAL.

As observed with IFN-γ, data on TNFα responses in TB has been disparate with studies reporting either increased levels [240, 244, 631] or decreased levels [411, 632] in antigen-driven PBMC and BAL cell supernatants of TB patients compared to healthy controls. I

found significantly higher levels in RD-1 stimulated supernatants of BAL cells compared to PBMCs of presumed LTBI controls. Furthermore, median TNFα levels were higher in BAL supernatants of presumed LTBI controls compared to TB patients, although these differences were not significant. The inconsistencies between these results and others studies may be due to technical variations in experimental design (discussed earlier) or the clinical status of the patient [632]. TNFα is thought to have a dual role in TB and the relative abundance of TNFα at various stages of disease may influence the disease outcome: low levels of TNFα result in suboptimal mycobactericidal activities and granuloma formation whereas high levels can cause enhanced inflammation and subsequent immunopathology [601]. The reduced TNFα levels in TB patients compared to presumed LTBI controls observed here, support the former. Low TNFα expression can also be a result of infection with hyper-virulent strains of *M.tb* [640] or increased expression of TNF receptors which sequester and inactivate TNFα [641].

TNFα was considerably higher than IFN-γ in RD1-stimulated BAL cell culture supernatants. As discussed above, BAL T-cells were most likely hyper-activated and died as a result of overstimulation, leading to low detectable levels of IFN-γ. However, this was not observed with TNFα and could be a reflection of the different cellular sources of these cytokines in lung lavage. In the lung, the dominant source of TNFα is phagocytic cells [250], whereas IFN-γ is mainly produced by CD4+ T cells [11, 15]. As such, the majority of TNFα being produced is probably from alveolar macrophages in the BAL.

8.4.2 IL-9

IL-9 was traditionally considered a Th2 cytokine but recent studies suggest that it can also be produced by Th17 [339, 642], Tregs [342] and the recently described Th9 cells [49, 50]. IL-9 functions in a similar manner to IL-4 as it promotes allergic airway inflammation in asthma and atopic allergy, eosinophil recruitment, IgE production and modulation of Treg activity (reviewed in [643]). There are few studies investigating IL-9 in TB [11, 334, 344, 345], most of which reported increased levels in response to *M.tb* stimulation either in PBMCs [344, 345] or pleural mesothelial cells [334]. My data is in agreement with these

studies as significantly increased IL-9 was found in RD1-stimulated BAL supernatants of TB patients compared to presumed LTBI controls. IL-9 was also significantly higher in the BAL supernatants compared to PBMC supernatants of TB patients. This finding of increased IL-9 production at the site of disease is similar to Ye *et al*, who reported a higher frequency of IL-9 producing cells in pleural effusion compared to blood in TB patients [334]. This may be the result of IL-9 producing cells being recruited from the periphery and accumulating at the site of disease [334].

A possible consequence of increased IL-9 in TB patients is the downregulation of the protective Th1 response and subsequent dampening of protective anti-mycobacterial mechanisms. Wu *et al* observed reduced IFN-γ mRNA expression upon the addition of exogenous IL-9 to PBMCs *in vitro* [344]. IL-9 also inhibited apoptosis in pleural mesothelial cells [334] and production of ROIs and TNFα in LPS stimulated monocytes [343]. The higher IL-9:TNFα cytokine ratio in stimulated BAL supernatants and IL-9:IFN-γ ratio in stimulated PBMC supernatants that was observed in TB patients compared to presumed LTBI controls support this notion (Figure 8.2A and C). Therefore, it is possible that IL-9 acts synergistically with IL-4 to bring about a Th2-associated disruption of the immune response. However, it is not known which T-cell subsets (Th2 or Th9) contribute to the observed IL-9 increase in TB patients. Further investigations on the cellular source of IL-9 were performed and will be discussed in chapter 9.

8.4.3 IL-4

IL-4 levels were mostly undetectable in both the PBMC and BAL supernatants of TB patients and presumed LTBI controls. This result is likely attributable to the difficulty in measuring IL-4, particularly at the protein level. Other studies have reported similar findings in serum, BAL fluid and *M.tb* antigen-stimulated supernatants [9, 35, 36, 629, 631]. As discussed in chapter 4, IL-4 is difficult to detect as it is physiologically active at low concentrations, it is rapidly internalized and can be sequestered by sIL4R. Even IL-4 mRNA levels detected by qPCR, which has superior sensitivity to Luminex, were low (chapter 3 and [9]). In contrast, other studies have reported higher levels of soluble IL-4 in TB patients

compared to healthy controls [11, 20, 241]. This may be due to the length of stimulation, method of detection and the use of pre-stimulation protocols. It is possible that IL-4 levels peaked earlier during stimulation and decreased to undetectable levels by day 5. ELISA, which has a similar range of detection to Luminex, is not as sensitive for measuring low expressing cytokines such as IL-4 [26], compared to ELISPOT and qPCR. Furthermore, RD1 antigens tend to be Th1 biased and may downregulate production of IL-4 [644].

8.4.4 MCP-1

MCP-1, also known as CCL2, is a chemokine responsible for recruiting monocytes and T-cells to the site of infection. It is required for effective granuloma formation and macrophage activation in the early stages of disease [645, 646]. However, high levels of MCP-1 can be detrimental to TB. Studies in Mexican, Korean and Zambian TB patients revealed that polymorphisms in the MCP-1 gene promotor region resulted in excessive production of MCP-1 and was associated with increased susceptibility to TB [647, 648]. Furthermore, MCP-1 is associated with the development of Th2 responses [621, 622, 649] and enhanced secretion of IL-4 [623].

In the data presented here, high levels of MCP-1 were mainly found in the BAL, which is expected given that the majority of MCP-1 is produced by macrophages [650]. MCP-1 levels were variable but median levels were higher in RD-1 stimulated BAL cell supernatants of TB patients compared to controls, although these differences were not significant. High levels of MCP-1 expression have been found in both the periphery and site of disease in TB patients compared to controls [624-627, 651]. Increased MCP-1 can downregulate the Th1 response, by inhibiting production of IL-12 [648] and IFN-γ [652]. A higher MCP-1:TNFα ratio was noted in stimulated BAL supernatants and MCP-1:IFN-γ in stimulated PBMC supernatants in TB patients compared to presumed LTBI controls, which supports this notion (Figure 8.2B and D).

8.4.5 IL-10

IL-10 is an anti-inflammatory cytokine produced by numerous cell types including T cells, B cells, neutrophils, macrophages and DCs [653]. Multiple CD4+ T-cell phenotypes also produce IL-10, including Th1, Th2, Th17 and certain types of Tregs [310]. In TB, IL-10 dampens protective immune responses by inhibiting production of pro-inflammatory cytokines, such as TNFα and IL-12 [305, 306], and enhancing iTreg differentiation [309]. IL-10 can also directly affect macrophages by inhibiting phagocytosis and production of ROIs and RNIs [307, 308]. However, IL-10 is also thought to limit excessive inflammation by downregulating these mechanisms [654].

In the data presented here, no significant differences were observed in IL-10 levels between TB patients and presumed LTBI controls either in RD1-stimulated BAL or PBMC culture supernatants. This is in contrast with other studies that found TB patients expressed higher levels of IL-10, compared to healthy controls, in response to antigenic stimulation of cells from the site of disease or peripheral blood [11, 33, 246, 628, 629]. In these TB patients, increased IL-10 production was often associated with lower levels of IFN-γ [33, 246]. However, the IL-10:IFN-γ ratio was not significantly different between TB patients and presumed LTBI controls (Figure 8.2E). The lack of statistical significance may be due to differences in experimental design (type of antigen, length of stimulation), or that the observed downregulation of Th1 responses was not dependent on production of IL-10 alone.

8.4.6 IL-17

IL-17 is a proinflammatory cytokine that is produced by various T-cells (CD4+, CD8+,γδ) including the recently described Th17 subset. In TB, IL-17 recruits and activates neutrophils, promotes secretion of inflammatory mediators including TNFα, IL-6 and MIP-2 [630] and contributes to early granuloma formation [125]. However, excessive IL-17 can lead to accumulation of large numbers of neutrophils in the lungs, alter their phenotype and cause immunopathology [265].

Low expression of soluble IL-17 levels was noted, which was not different in TB patients compared to presumed LTBI controls, either in the RD1 stimulated cell supernatants of BAL or PBMCs. This is in contrast to other studies which found that TB patients expressed increased levels of IL-17-producing cells, compared to healthy controls, in stimulated BAL cells [655] and PBMCs [293, 656]. However, these studies, which measured intracellular production of IL-17 by flow cytometry, still detected very few IL-17-producing CD4+ or CD8+ T-cells. Measurement of soluble IL-17 protein tends to be challenging as it is present at even lower concentrations and, similarly to IL-4, may be the result of rapid degradation of IL-17 protein or the inactivation of IL-17 by binding to IL-17 receptors [657].

8.4.7 Summary of findings in relation to a Th2 response

M.tb antigen-specific cells from TB patients exhibit a distinct cytokine expression profile compared to presumed LTBI controls and these cytokine secretion patterns are compartment-specific. TB patients showed increased IL-9 production and a higher IL-9:TNFα ratio at the site of disease compared to presumed LTBI controls. A similar effect occurred in the blood, where lower IFN-γ expression and a higher IL-9:IFN-γ ratio was observed. These data suggests a mixed Th1:Th2 response in TB patients and confirm the results obtained by qPCR (chapter 3). IL-4 is the principal cytokine produced by Th2 cells but, due to limitations in Luminex assay sensitivity, IL-4 was undetectable. As a result, no conclusions could be drawn on the effect of IL-4 in relation to expression of other cytokines in this assay. However, given that IL-4 and IL-9 have similar functions [643], it is possible that IL-9 acts in conjunction with IL-4 to subvert the protective Th1 response.

The ratio of MCP-1 to Th1 cytokines in the BAL (MCP-1:TNFα) and blood (MCP-1:IFN-γ) was higher in TB patients compared to presumed LTBI controls and suggests MCP-1 may also be involved in driving the Th2 response. MCP-1 is considered a Th2 chemokine in mice and humans because it is involved in Th2 polarization and recruitment [367]. In lepromatous leprosy, increased MCP-1 and lower TNFα resulted in unrestricted growth and dissemination of *M. leprae* [658]. A plausible scenario involves the recruitment of IL-9 producing T-cells to the site of disease, driven by MCP-1, and subsequent downregulation of protective Th1

mechanisms. These results provide further evidence that TB is characterized by a disruptive Th2 response.

8.4.8 Limitations

The choice of antigen used to stimulate cells in vitro and the length of stimulation can have a significant impact on cytokine production [26, 634]. Soluble antigens such as PPD and ESAT-6 are presented via the exogenous antigen processing pathway and primarily stimulate CD4+ T-cells, but not other T-cells [471]. PPD contains a mixture of mycobacterial antigens and responses to these proteins are not specific to *M.tb*, particularly in a setting such as South Africa where exposure to environmental mycobacteria and BCG vaccination is common. The RD1 antigens were chosen because they are *M.tb* specific and a combination of ESAT-6 and CFP-10 was used as it was shown to maximize the *M.tb* specific responses [659]. ESAT-6 and CFP-10 drive IL-4 production in PBMCs from TB patients [503]. In contrast, RD1 antigens have also been shown to be Th1 biased and downregulate Th2 responses [644].

A 5 day stimulation period was chosen as it was optimal for detection of soluble cytokines by ELISA [634] and previous studies have used similar incubation periods [238, 240, 241, 244, 247, 480]. However, a shorter stimulation time may have been more suitable for alveolar T-cells as these cells tend to be hyper-responsive and overstimulation may have caused enhanced cell death. Given that T-cells are the primary source of IFN-γ [11, 15], this could account for low IFN-γ levels in stimulated BAL cell supernatants. Furthermore, the optimal culture times for antigen induced responses can vary depending on the cytokine [634]. Given the complexity and limits of obtaining BAL cells, it was not feasible to do variable –cytokine specific time points optimized for each cytokine.

The small BAL sample size may have contributed to the lack of significant differences in cytokine levels between the TB and LTBI groups and subsequent interpretation of the data. Recruitment of participants to undergo an invasive bronchoscopy procedure was difficult and limited cell recovery often meant that immune assays needed to be prioritized based on the amount of alveolar cells that were available. Nonetheless, additional participants will be

recruited from other ongoing studies to increase the number of BAL samples in both the TB and LTBI groups.

8.5 Conclusion

M.tb antigen-stimulated cells from TB patients exhibit a distinct cytokine expression profile compared to presumed LTBI controls, characterized by increased IL-9 and reduced Th1 cytokine levels, and these secretion patterns are compartment-specific. These data suggest that cytokines and chemokines, apart from IL-4, may contribute to a Th2-associated disruption of the Th1 response. The IL-9 secretion pattern observed here prompted further investigation into the cellular source of IL-9 in TB.

9. CHAPTER 9: Analysis of IL-9 producing subpopulations of cells in lungs and peripheral blood

9.1 Introduction

IL-9 was traditionally considered a Th2 cytokine as it is associated with various Th2 responses and has a prominent role in Th2-associated diseases [660]. However, recent studies have demonstrated the multifunctional role of IL-9 and its production by different T-cell subsets. Of particular significance is the discovery of an IL-9 producing CD4 T-cell subset, known as Th9, which is distinct from other Th subsets including Th1, Th2 and Th17 cells [49, 50]. Th9 cells produce IL-9 as its signature cytokine and develop from naïve T-cells in response to IL-4 and TGFβ [49, 50, 322, 323]. Th9 cells can also develop from Th2 cells in the presence of TGFβ [50]. A number of transcription factors, such as PU.1, IRF4, STAT6 and GATA3, have been identified as necessary for Th9 development [49, 50, 326, 327] but these transcription factors are also required for the development of other T-cell subsets. Thus, the discovery of a single lineage defining Th9 transcription factor remains elusive. Th9 cells appear to contribute towards the immune response in allergic airway disease [326, 327, 332], asthma [661-663], autoimmune disease [49, 339, 642, 664] and parasitic infections [665-668]. Most of these data come from *in vitro* studies or from murine models and the significance of these cells in the immune response to human infection *in vivo*, particularly in the context of TB, have been under-explored.

A handful of studies have investigated IL-9 in TB [11, 334, 344, 345] and only one has attempted to define the phenotypic and functional characteristics of Th9 cells [334]. In the previous chapter, increased IL-9 measured by Luminex, was found at the site of disease in TB patients compared to LTBI controls. The aim of this chapter was to define the phenotypic characteristics of T-cells producing IL-9 in TB patients by flow cytometry.

9.2 Methods

9.2.1 PBMC and BAL cell isolation

Whole blood was obtained by venipuncture from 15 TB patients and 10 presumed LTBI controls. PBMCs were isolated by density centrifugation as described in section 2.4.2. Broncho-alveolar lavage (BAL) fluid was collected from participants (6 TB patients and 3 presumed LTBI controls) as previously described in section 2.3.3. BAL cells were isolated from BAL fluid as described in section 2.4.3.

9.2.2 Cell culture and immunofluorescence staining

A total of $1.5x10^6$ BAL cells and PBMCs were plated in 24 well plates. PBMCs and BAL cells were stimulated with PPD (12ug/ml) for 18 hours in a final well volume of 500µl. In a subset of TB patients and LTBI controls, PBMCs were stimulated either with PPD (12µg/ml) or RD1 antigens, ESAT-6 and CFP-10 (Oxford Immunotec), at manufacturer recommended concentrations (which are proprietary and cannot be disclosed) for 18 hours. Golgi-stop, containing monensin (0.6µl/ml), was added to each well 5 hours before harvesting. Cells were harvested and stained for specific surface and intracellular markers using fluorescently labeled antibodies (Table 2.2), and subsequently analyzed on a BD LSR II flow cytometer, as outlined in section 2.4.6.

9.2.3 Data analysis

The frequency of specific lymphocyte populations expressing IL-9 and other biomarkers was determined using FACSDiva software (BD) and were reported as a % of the total CD4+ and CD8+ lymphocyte populations. Differences in expression levels between TB patients and presumed LTBI controls in the different compartments were determined using the Mann-Whitney U test. A p-value of <0.05 were deemed significant. Statistical analyses were performed using GraphPad Prism 5.0 software and Microsoft Excel.

9.3 Results

9.3.1 Gating strategy for CD4+ and CD8+ lymphocytes expressing IL9

The gating strategy used to identify IL-9 expressing cells in the lymphocyte population is shown in Figure 9.1. Lymphocytes were acquired based on cell size (FSC) and granularity (SSC). Cells were gated on the CD3+CD4+ population for CD4+ lymphocytes and on CD3+CD8+ for CD8+ lymphocytes. Cells expressing IL-9 (IL9+) were identified within each of these populations. The frequency of CD4+IL9+ and CD8+IL9+ expressing cells were reported as a % of the total CD4+ or CD8+ lymphocyte population, respectively.

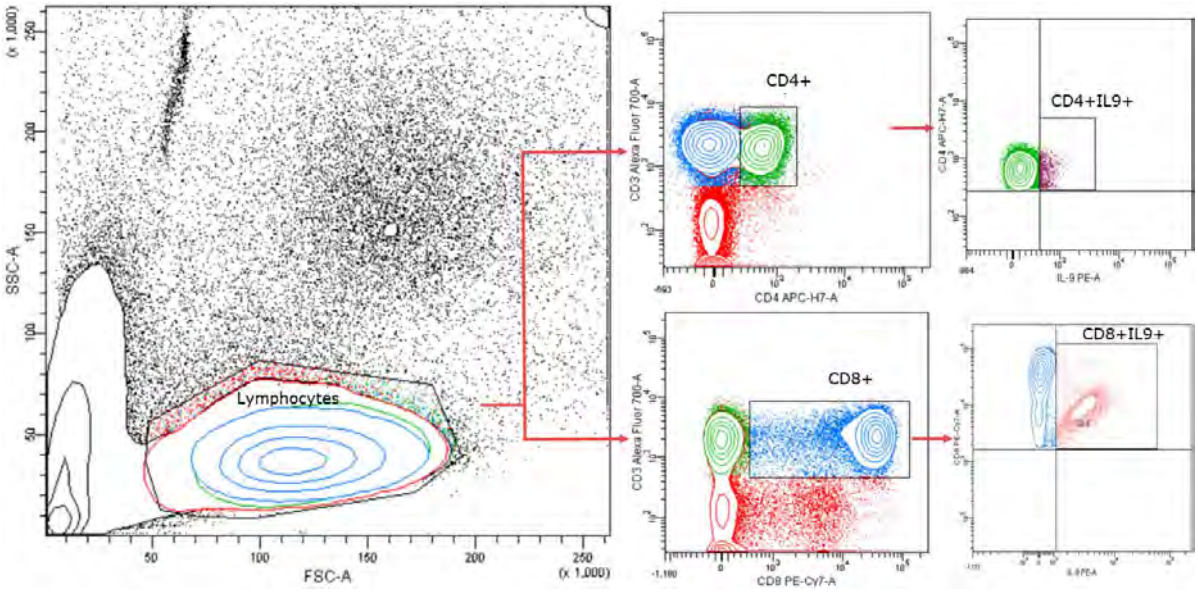


Figure 9.1 An example of the gating strategy used to identify CD4+IL9+ and CD8+IL9+ cells within the lymphocyte population by flow cytometry.

9.3.2 Expression of IL-9 in CD4+ and CD8+ lymphocytes

Median expression of IL-9 by CD4+ lymphocytes (CD4+IL9+) were significantly higher in TB patients compared to presumed LTBI controls in PPD-stimulated cells of BAL (median expression of 2.2% *vs.* 0.1% respectively; p=0.03) and peripheral blood (median expression of 1.8% *vs.* 0.7% respectively; p=0.02) (Figure 9.2A). A similar expression pattern of IL-9 was observed in CD8+ lymphocytes. Expression of CD8+IL9+ cells in TB patients,

compared to presumed LTBI controls, was significantly higher in peripheral blood (median expression of 1.7% *vs.* 0.2% respectively; p=0.047) but not in BAL (median expression of 3.6% *vs*. 0.6% respectively; p=0.3) (Figure 9.2B). Two presumed LTBI controls had very high CD8+IL-9+ levels (>15% expression) in the peripheral blood but not in BAL (<1% expression) (Figure 9.2B). There were no significant differences in IL-9 expression between CD4+ and CD8+ lymphocytes.

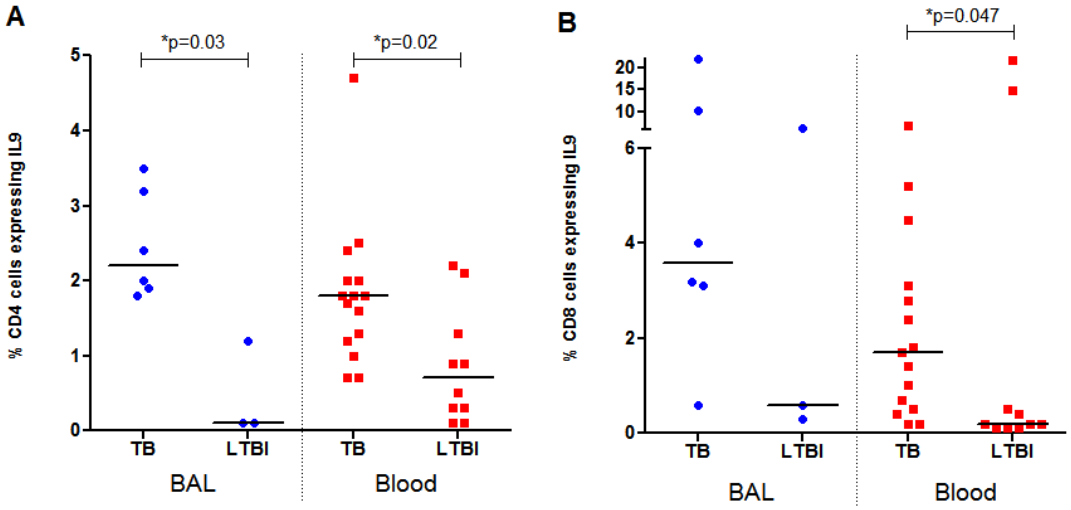


Figure 9.2. Frequency (%) of (A) CD4+ and (B) CD8+ lymphocytes (CD3+) expressing IL-9 in PPD-stimulated cells of broncho-alveolar lavage (BAL; blue circles) and peripheral blood (Blood; red squares) from patients with pulmonary tuberculosis (TB; BAL n=6, Blood n=15) and presumed latently infected controls (LTBI; BAL=3, Blood=10) as measured by flow cytometry. Statistical analyses between groups were performed using the Mann-Whitney test and p<0.05 was deemed significant.

9.3.3 IL-9 expression in peripheral blood cells stimulated with PPD and RD1 antigens

There were no differences in IL-9 expression in CD4+ or CD8+ lymphocytes of matched samples when PBMCs were stimulated with either PPD or RD1 antigens. However, in CD4+ lymphocytes, IL-9 expression was significantly higher in TB patients compared to presumed LTBI controls when cells were stimulated with PPD (median expression of 1.8% *vs.* 0.6%

respectively; p=0.03) but not when stimulated with RD1 antigens (median expression of 2.3% *vs*. 1.5% respectively; p=0.47) (Figure 9.3A). TB patients had higher IL-9 expression in CD8+ lymphocytes, compared to presumed LTBI controls, when cells were stimulated either with PPD (median expression of 1.0% *vs.* 0.2%, respectively; p=0.006) or, to a lesser extent, RD1 (median expression of 0.9% *vs.* 0.2%, respectively; p=0.01) (Figure 9.3B).

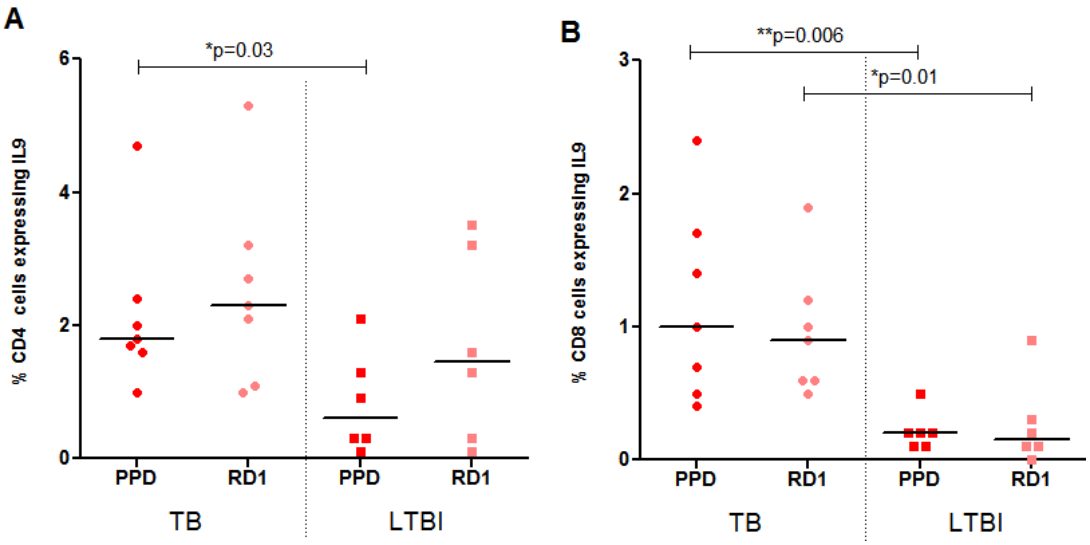


Figure 9.3. Frequency (%) of (A) CD4 and (B) CD8 lymphocytes (CD3+) expressing IL-9 in peripheral blood mononuclear cells stimulated with purified protein derivative (PPD; red) or RD1 (ESAT-6 and CFP-10; pink) antigens from patients with pulmonary tuberculosis (TB; n=7) and presumed latently infected controls (LTBI; n=6) as measured by flow cytometry. Statistical analyses between groups were performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

9.3.4 Gating strategy for determining cytokine expression in IL-9 producing CD4+ and CD8+ cells

The gating strategy used to identify cells co-expressing IL-13, IL-17 and PU.1 within the CD4+IL9+ and CD8+IL9+ populations is shown in figure 9.4. The CD4+IL9+ and CD8+IL9+ populations were identified as described in section 9.3.1 and figure 9.1. The cells expressing, IL-13 and IL-17 were identified within the IL9+ population. Expression of PU.1

was determined in the CD4+IL9+ and CD8+IL9+ populations not co-expressing IL-13 and IL-17 (CD4+IL9+IL13$_{neg}$IL17$_{neg}$PU1+ and CD8+IL9+IL13$_{neg}$IL17$_{neg}$PU1+). The frequency of IL-13, IL-17 and/or PU.1 being co-expressed by IL-9-producing CD4+ and CD8+ lymphocytes were reported as a % of the total CD4+IL9+ or CD8+IL9+ lymphocyte population, respectively.

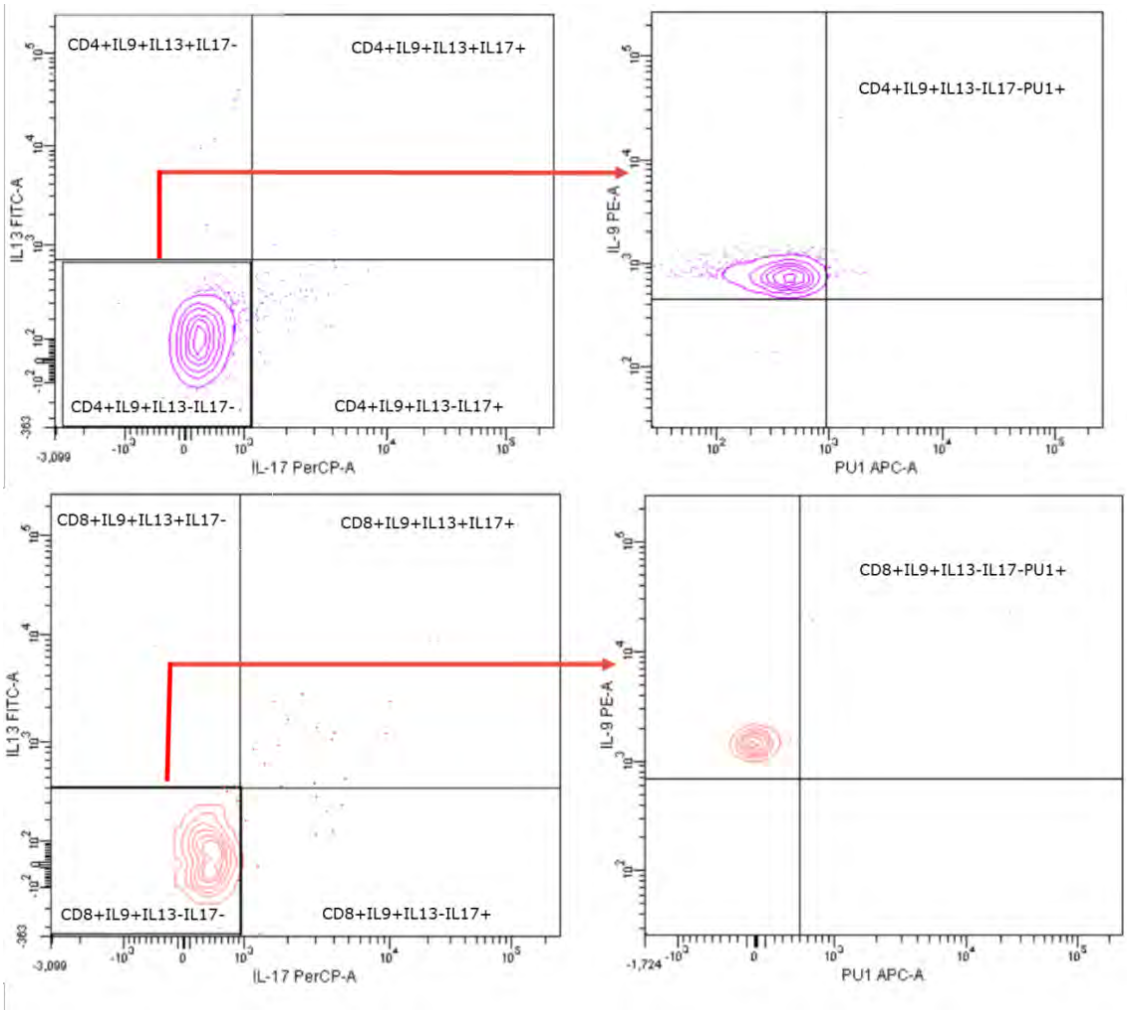


Figure 9.4. The gating strategy used to identify co-expression of IL-13, IL-17 and PU.1 within the CD4+IL9+ and CD8+IL9+ lymphocyte population by flow cytometry.

9.3.5 Co-expression of IL-13, IL-17 and PU.1 by IL-9 expressing CD4 and CD8 lymphocytes in peripheral blood and BAL

Further investigations were performed to ascertain the helper T-cell (Th) and cytotoxic T-cell (Tc) lineage of IL-9-producing CD4+ and CD8+ lymphocytes in PPD-stimulated PBMCs and BAL cells of TB patients. The co-expression of IL-13 (Th2/Tc2), IL-17 (Th17/Tc17) and the transcription factor PU.1 were analyzed and reported as a % of total CD4+IL9+ and CD8+IL9+ cells (Figure 9.5A and B). Co-expression was not assessed in LTBI controls due to the low number of IL-9 producing cells present in this participant group.

A small subset of CD4+IL9+ cells in the peripheral blood and BAL produced IL-13 (Th2 phenotype; median CD4+IL9+IL13+ expression was 3.2% and 6.9%, respectively) and IL-17 (Th17 phenotype; median CD4+IL9+IL17+ expression was 2.9% and 15.8%, respectively). The median expression of IL-13 and IL-17 was generally higher in the BAL CD4+IL9+ cells compared to peripheral blood, but did not reach statistical significance. The majority of CD4+IL-9+ expressing cells exhibited a Th9 phenotype as they did not co-express IL-13 and IL-17 (median CD4+IL9+$IL13_{neg}IL17_{neg}$ expression was 93.1% and 95.2% in BAL and peripheral blood, respectively). The frequency of these cells also expressing the transcription factor PU.1, was higher in BAL compared to blood but still generally low (median CD4+IL9+$IL13_{neg}IL17_{neg}$PU.1+ expression was 8.3% and 1.0% in the BAL and peripheral blood, respectively; p=0.006) (Figure 9.5A).

Similar to CD4+ cells, IL-9-producing CD8+ cells in peripheral blood and BAL produced small amounts of IL-13 (Tc2 phenotype; median CD8+IL9+IL13+ expression was 1.7% and 7.0%, respectively) and IL-17 (Tc17 phenotype; median CD8+IL9+IL17+ expression was 4.5% and 13.5%, respectively). Most CD8+IL9+ cells did not co-express IL-13 or IL-17 and exhibited a Tc9 phenotype (median CD8+IL9+$IL13_{neg}IL17_{neg}$ expression was 93.9% and 91.8% in BAL and peripheral blood, respectively). Of these Tc9 cells, very few expressed PU.1 (median CD8+IL9+$IL13_{neg}IL17_{neg}$PU.1+ expression was 5.0% and 1.6% in the BAL and peripheral blood, respectively). None of these differences in expression between BAL and peripheral blood were significant. (Figure 9.5B)

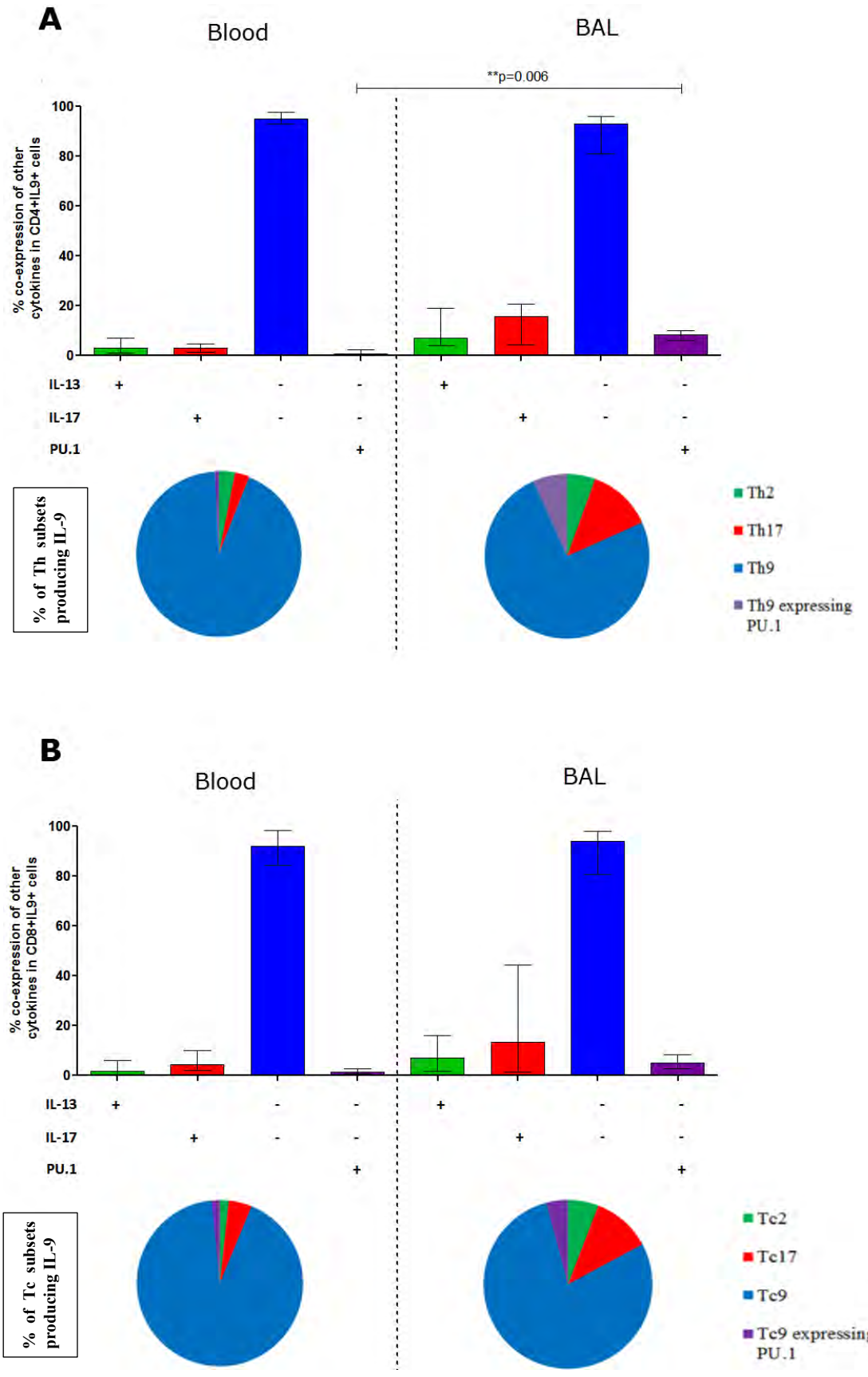

A
Blood
BAL
**p=0.006
% co-expression of other cytokines in CD4+IL9+ cells
100
80
60
40
20
0
IL-13 + - - + - -
IL-17 + - - + - -
PU.1 + +
% of Th subsets producing IL-9
Th2
Th17
Th9
Th9 expressing PU.1
B
Blood
BAL
% co-expression of other cytokines in CD8+IL9+ cells
100
80
60
40
20
0
IL-13 + - - + - -
IL-17 + - - + - -
PU.1 + +
% of Tc subsets producing IL-9
Tc2
Tc17
Tc9
Tc9 expressing PU.1

Figure 9.5 Frequency of cytokines (IL-17 and IL-13) and transcription factors (PU.1) that are co-expressed by (A) CD4+IL9+ cells and (B) CD8+IL9+ cells in the blood and broncho-alveolar lavage (BAL) of TB patients. Bar height represent median values and error bars show the interquartile range. „+" indicates co-expression; „-" indicates no co-expression; „ " indicates that the cytokine/transcription factor was not analyzed for co-expression. The pie charts below each bar chart indicate the contribution of the various CD4+ and CD8+ Th-cell subtypes to the production of IL-9.

9.4 Discussion

In the previous experiments, the frequency and specific lineage of IL-9 producing T-cells in TB patients and presumed LTBI controls was assessed by flow cytometry. CD4+IL9+ cells were significant higher in the PBMCs and BAL cells of TB patients compared to LTBI controls. CD8+IL9+ cells were significantly higher in the blood, but not the BAL, of TB patients compared to LTBI controls. These results were comparable whether PBMCs were stimulated with *M.tb* antigens, RD1 or PPD. In TB patients, the co-expression of lineage-specific cytokines was analyzed in PPD-stimulated PBMCs and BAL cells to determine the Th and Tc phenotype of IL-9 producing CD4+ and CD8+ cells, respectively. In the CD4+IL9+ population, a limited number of cells expressed IL-13 (Th2) or IL-17 (Th17) and most (>90%) exhibited a Th9 phenotype (CD4+IL9+$IL13_{neg}IL17_{neg}$). PU.1 expression in these Th9 cells (CD4+IL9+$IL13_{neg}IL17_{neg}$PU.1+) was higher in BAL cells compared to PBMCs (p=0.006) but still generally very low. A similar expression pattern was observed in the CD8+IL9+ population. Most of these cells (~90%) displayed a Tc9 phenotype (CD8+IL9+$IL13_{neg}IL17_{neg}$) but few produced the transcription factor PU.1.

9.4.1 Increased IL-9 expression in TB

The data presented here show that CD4+ and CD8+ T-cell production of IL-9 was higher in the BAL and blood of TB patients compared to LTBI controls and support the results obtained by Luminex in chapter 8. Increased IL-9, measured either as soluble protein or mRNA, has been observed in *M.tb* antigen stimulated PBMCs of TB patients in previous studies [344, 345]. However, investigations of IL-9 expression at the site of disease have been less conclusive. Herrera *et al* found no differences in IL-9 levels in *M.tb* stimulated

BAL cells of pulmonary TB patients and healthy controls [11]. In contrast, Ye *et al*, using PMA and ionomycin stimulated CD4+ T-cells, observed higher expression of CD4+IL9+ cells in pleural effusions compared to the peripheral blood of TB pleuritis patients [334]. However, this study did not include healthy controls as a comparative group. As such, my study is the first to show increased IL-9 in the CD4+ and CD8+ populations of TB patients compared to presumed LTBI controls at the site of disease and the periphery.

9.4.2 CD4+IL9+ cells express a predominantly Th9 phenotype

The co-expression of other Th lineage specific cytokines was analyzed to determine the Th phenotype of these IL-9 producing CD4+ cells. I found that the majority of CD4+IL9+ cells in TB patients did not co-express IL-13 (Th2) or IL-17 (Th17) (>90% of CD4+ T-cells were CD4+IL9+$IL13_{neg}IL17_{neg}$ in blood and BAL) suggesting that these were in fact Th9 cells. A similar phenotypic profile of Th9 cells has been described in the peripheral blood of both pulmonary allergy and melanoma patients [332, 669]. Th9 cells are a newly described subset of Th cells which develop from naïve T-cells after antigenic stimulation in the presence of IL-4 and TGFβ [49, 50, 322, 323]. These cells are characterized by the production of large amounts of IL-9 but not cytokines from other lineage specific T-cell subsets, including IFN-γ (Th1), IL-17 (Th17), IL-4 or IL-13 (Th2). Th9 cells have been described in allergic airway inflammation [326, 327, 332] and parasitic infections [665-668] but little is known about their role in TB and will be discussed later.

IL-9 production is not restricted to Th9 cells and can also be produced by Th2 [322, 326, 670] and Th17 [339, 642, 664] cells. Th9 cells are closely related to Th2 cells as their development from naïve T-cells is dependent on IL-4 and Th2 cells can differentiate to Th9 cells in the presence of TGFβ [49, 50]. In early stages of Th2 differentiation, Th2 cells can produce both IL-4 and IL-9 [671]. It has been suggested that only a small fraction of fully differentiated Th2 cells continue to express IL-9 and those that lose the ability to express Th2 cytokines develop into Th9 cells [50, 672]. In my study, Th2 expression (CD4+IL13+) was very low and only a small proportion of CD4+ cells co-expressed IL-13 and IL-9 (3% in blood and 7% in BAL). A similar population has been shown in CD4+ T-cells under Th2

polarizing conditions [326, 670]. It is possible that these CD4+IL9+IL13+ cells may represent a proportion of naïve T cells undergoing Th2 differentiation or Th2 cells at an intermediate stage of development into Th9 cells.

Evidence also suggests that Th17 cells are capable of producing IL-9. In a murine autoimmunity model of EAE, large amounts of IL-9 are produced by Th17 cells and Th17 development can be induced by IL-9 and TGFβ [339, 642]. In humans, repeated stimulation under Th17 polarizing conditions can drive Th17 cells to co-produce IL-9 and IL-17, which is further enhanced in the presence of Th17 mediators such as IL1β and IL-21 [664]. Furthermore, these IL9+IL17+ cells are found in greater numbers in diabetic patients compared to healthy controls suggesting that these cells may be associated with inflammatory disorders [664]. In the data presented here, I observed a small but noteworthy population of IL9+IL17+ cells in the BAL of TB patients (16% of CD4+IL9+ produced IL-17). However, it is unknown if these cells have any significance in the context of TB and further investigations will be required to determine if these cells contribute to TB-associated inflammation.

9.4.3 Expression of PU.1 in Th9 cells

Th9 cells are considered a distinct lineage as they do not express other subset-specific transcription factors such as T-bet (Th1), GATA3 (Th2), RORγt (Th17) or Foxp3 (Tregs) at levels comparable to their respective T-cell subsets [324]. However, the identification of a single master regulator, which is thought to define a committed lineage for Th9 cells, has remained elusive. Instead, multiple transcription factors have been instigated in Th9 induction and most of these are mediators in the signaling pathways of IL-4 and TGFβ, the two cytokines required for Th9 development. For example, STAT6 and its downstream target, GATA3, are mainly expressed by Th2 cells but also by Th9 cells, albeit at lower levels [49, 325]. However, these are thought to act indirectly by downregulating T-bet and FoxP3, which inhibit IL-9 production [49, 50, 325]. Two of the more convincing candidates are IRF4 and the ETS transcription factor, PU.1. Both of these transcription factors are induced by TGFβ and directly bind to the *il9* locus [326, 327]. In mice, ectopic expression of

PU.1 increases IL-9 production and OVA sensitized PU.1 deficient mice showed less IL-9 production and reduced allergic lung inflammation [326]. In humans Th9 cells, increased PU.1 expression correlated with IL-9 expression in atopic allergy patients and blocking of PU.1 with siRNA reduced IL-9 expression [326, 328]. In similar experiments, IRF4 displayed the same effect on Th9 differentiation as PU.1 [327]. However, IRF4 also drives Th2 and Th17 development [329, 330] whereas PU.1 represses Th2 cytokine production [673, 674]. Given these data, PU.1 is the only transcription factor identified that is involved in converting a Th2 lineage to a Th9 lineage.

In the data presented here, only a small percentage of Th9 cells expressed PU.1 (CD4+IL9+IL13$_{neg}$IL17$_{neg}$PU.1+). This result is similar to another study which reported PU.1 expression in a proportion of Th9 cells from allergic donors [332]. While PU.1 is necessary for Th9 development, continued PU.1 expression may not be required for fully committed Th9 cells. As such, it is unlikely that PU.1 is the lineage defining transcription factor for Th9 cells. Given the antagonistic relationship of PU.1 in Th2 and Th9 cells, it would have been useful to compare PU.1 expression in these two helper T-subsets. Unfortunately, this was not possible because the overall PU.1 expression (CD4+PU.1+) was generally low. The low PU.1 expression, compared to other human studies, may be explained by the use of isolated Th9 cells [332] or cells differentiated *in vitro* under Th9 and Th2 polarizing conditions [326]. Interestingly, Th9 cells expressing PU.1 in BAL was significantly higher compared to blood. Given that induction of PU.1 also turns off IL-4 production and turns on IL-9 production, it is possible that an upregulation of PU.1 in cells at the site of disease may represent stage of differentiation where Th2 cells „switch" to Th9 cells or naïve T-cells develop a Th9 phenotype.

9.4.4 Function of Th9 cells and possible role in TB

The multifunctional effects of Th9 cells are exemplified by their interaction with different T-cell lineages and their role in different diseases. Th9 cells are pro-inflammatory and, together with Th2 cells, seem to drive allergic inflammation and immunity to parasitic infections. In mice, adoptive transfer of Th9 cells to the lungs resulted in allergic inflammation

characterized by increased eosinophil recruitment, mast cell numbers, mucus production, IgE production and collagen deposition [324, 327, 332, 673, 675]. In humans, IL-9 expression was increased in the lungs of asthma patients and atopic infants [326, 331, 661, 676]. Th9 cells also contribute to parasite clearance in a similar manner to Th2 cells [665, 666]. Th9 cells also function in association with Th17 to drive autoimmunity in murine models of EAE and colitis [49, 333, 339]. In diabetic patients, increased levels of IL-9 produced by Th17 cells were observed compared to healthy controls [664]. In contrast, IL-9, produced by Tregs and Th9 cells, may also have anti-inflammatory functions in skin allograft tolerance by recruiting and promoting Treg suppression at the graft site [342]. However, there is a paucity of data regarding the role of Th9 cells in TB.

Evidence suggests that IL-9 can modulate protective anti-mycobacterial mechanisms and may be detrimental to TB immunity in a similar manner to IL-4. One such mechanism is the downregulation of a protective Th1 response. The addition of exogenous IL-9 to *in vitro* PBMC cultures of TB patients resulted in reduced IFN-γ mRNA expression [344]. IL-9 also inhibited IFN-γ-induced apoptosis in pleural mesothelial cells of patients with TB pleuritis [334] and the production of TNFα and ROIs in LPS stimulated monocytes [343]. Addition of rIL-9 also enhances Treg suppression *in vitro* [642], which can directly subvert mycobacterial containment in monocyte derived macrophages *in vitro* [293]. Furthermore, IL-9 can inhibit the suppressive activity of Tregs on Th2 cells [444] resulting in Th2 polarization [677].

Th9 cells may also contribute to the lung remodeling associated with TB. Fibrosis is thought to be driven by TGFβ and the Th2 cytokines, IL-4 and IL-13 [16]. Similarly, IL9 seems to be critical for collagen deposition and extensive lung fibrosis in chronic allergic lung inflammation and blocking IL-9 reduced this airway remodeling [678, 679]. Thus, in a TGFβ rich environment, which is commonly observed in the tuberculous lung [16], it is possible that Th2 as well as Th9 responses may contribute to pulmonary fibrosis in active TB.

Given the clear developmental relationship between Th2 and Th9 cells and their overlapping functions in allergic and parasitic diseases, it is likely that Th9 cells collaborate and even

augment Th2 responses in TB. However, this is only speculative and further investigations are required into the precise regulatory and effector mechanisms of Th9 cells and their interactions with other Th subsets, particularly Th2 cells, to influence TB disease outcome. These experiments were outside the scope of this thesis but will be the objective of future studies.

9.4.5 Expression of CD8+ Tc9 cells and possible role in TB

CD8+ T-cells show similar phenotypic patterns as Th cells and include traditional cytotoxic cells (Tc1), as well as IL-4 (Tc2) and IL17 (Tc17) expressing CD8+ T-cells [219]. Interestingly, I found a distinct CD8+ T-cell subset which expressed IL-9, but not IL-13 or IL-17, and was higher in TB patients compared to LTBI controls. These cells, termed Tc9, develop under Th9 polarizing conditions and express the same transcription factors as Th9 cells [222, 224, 680]. Such non classical CD8+ T-cells are thought to develop under TGFβ rich conditions to support CD4+ mediated inflammatory responses rather than drive cytotoxicity [224]. Tc9 cells are distinct from traditional cytotoxic T cells (Tc1) as they produce much more IL-9 than IFN-γ and cytotoxic molecules such as granzyme B [222, 224, 680]. In an adoptive transfer model of $Rag2^{-/-}$ mice, Tc9 cells are thought to play a supportive role in Th2 mediated allergic airway inflammation by driving recruitment of eosinophils, mucus producing cells and inflammation [224]. Surprisingly, these cells were shown to have greater tumorcidal activity compared to classical Tc1 cells. In an adoptive cell transfer murine model, Tc9 cells were able to target tumor cells and differentiate into effector Tc1 cells, producing IFN-γ and other cytotoxic molecules for effective tumor killing [222, 680]. However, Tc9 cells have never been described in TB. One possible function of Tc9 cells is to augment Th2 activity in TB, similar to allergic airway inflammation. Further investigations are required to elucidate the role of these cells in TB.

9.4.6 Limitations

IL-13 was chosen as a surrogate marker of Th2 cells rather than IL-4 due to the difficulties associated with measuring IL-4 (discussed in chapter 4). However, IL-13 shares receptors

and functions with IL-4 and previous studies used a similar phenotypic profile for describing Th9 cells [332]. As such, the use of IL-13 as a Th2 marker is justified and the data remains valid.

Only the transcription factor PU.1 was analyzed in Th9 cells. However, other transcription factors, such as IRF4, STAT6 and GATA3, have been proposed as regulatory proteins involved in the development and maintenance of Th9 cells. Due to limitations in sample quantity and the number of analytes that could be included in the Th9 flow cytometry panel, it was not possible to measure these additional markers. Future investigations will include these transcription factors to determine their role in Th9 induction in the context of TB.

Only three BAL samples were obtained in the LTBI control group and may explain the lack of significance in the CD8+IL-9+ expression levels between this group and TB patients. Recruitment of participants to undergo an invasive bronchoscopy procedure was difficult and limited cell recovery often meant that immune assays needed to be prioritized based on the amount of alveolar cells that were available. However, additional participants will be recruited from other ongoing studies in the future to increase the LTBI BAL numbers.

9.5 Conclusion

IL-9 expression in CD4+ and CD8+ T-cells was significantly increased in the BAL and blood of TB patients compared to LTBI controls. The majority of these IL-9 producing cells exhibited a Th9 (CD4+IL9+IL13$_{neg}$IL17$_{neg}$) and Tc9 (CD8+IL9+IL13$_{neg}$IL17$_{neg}$) phenotype. These data suggest that a Th9 response may also contribute to dysregulated TB immunity perhaps by augmenting a subversive Th2 response. However, further investigations are required to elucidate the role of Th9 cells in TB.

10. CHAPTER 10: General discussion, implications and future direction

Despite declining global TB rates, the burden of TB in Sub-Saharan Africa, particularly South Africa, remains a major public health threat. South Africa has one of the highest TB incidence rates in the world, driven by a weak primary healthcare system, HIV co-infection and the emergence of drug resistant TB [1].The development of effective vaccines is a key aspect of efficient TB control but current vaccine candidates, such as BCG and MVA85A, have limited protective efficacy [2-4]. These vaccine candidates have been selected based on their ability to induce a Th1-specific IFN-γ response. However, patients who get TB often have strong Th1 responses at the site of disease [9-15]. Successful vaccine design requires an in-depth understanding of the specific immune pathways that not only promotes protective immunity, but also those that are subversive.

One such subversive pathway that may have a corrupting influence on TB immunity involves a Th2 response, driven by the prototype Th2 cytokine, IL-4. A number of studies have reported increased IL-4 in pulmonary TB patients [9, 17-21, 243, 409-413] which correlate with disease susceptibility [22, 23] and immunopathology [9, 17-21]. Furthermore, IL-4δ2, a naturally occurring splice variant and antagonist of IL-4, was found to be associated with a protective phenotype in TB disease [9, 28-30]. Despite these findings, none have attempted to determine if IL-4 and IL-4δ2 have a direct causal relationship on anti-mycobacterial activities and protective host immunity in a human model of TB. This thesis attempts to describe the host immune response of pulmonary TB patients in the context of a Th2 response and to further elucidate the role of IL-4 and IL-4δ2 in an *in vitro* human TB infection model.

There are several limitations to the current study including the difficulty in measuring IL-4 and IL4d2 and the concentrations of IL-4 used in the mycobacterial containment assay. These and other limitations are discussed in the context of the findings below.

10.1 Is IL-4 expression increased in patients with active TB?

There have been several studies that have interrogated the levels of IL-4 and IL-4δ2 in active TB [9, 18, 30, 288, 417]; only one compared expression of these cytokine in the peripheral and disease compartments [9] and ***none*** in a high burden TB setting. Similarly, in the current study, analysis of mRNA expression encoding Th1 (IFN-γ) and Th2 (IL-4 and IL-4δ2) cytokines both at the site of disease and the peripheral circulation using a validated qPCR assay revealed a distinct cytokine profile in TB patients that was specific to the compartment being analyzed (chapter 3). The high levels of IFN-γ mRNA observed in the lung compartment of TB patients likely reflect an accumulation of Th1 effector cells at the site of inflammation [38] and, given the similar levels observed in LTBI controls, suggest that a potent Th1 response is not the only immune component necessary to confer protection.

The higher IL4 expression and lower Th1:Th2 (IFN-γ:IL-4) ratio observed in the peripheral blood of TB patients compared to LTBI controls in the current study confirms results from previous reports [9, 21, 416, 417, 504] and support the notion that a Th2 response is associated with a deficient immune response to *M.tb* (chapter 3). Comparable results were not observed in BAL though, probably due to very low IL-4 expression. The antagonistic Th1/Th2 relationship may explain this observation in the lungs where the accumulation of IFN-γ producing Th1 cells may have subsequently downregulated the Th2 response. However, the technical difficulties associated with measuring low expressing cytokines cannot be ruled out. IL-4 expression is generally low as it is active at concentrations ~1000 fold below that of IFN-γ [24, 25]. Delayed BAL sample processing, which can reduce detectable mRNA levels due to RNase activity [26], may have further contributed to the already low detectable IL-4 mRNA observed in the lungs. These challenges were particularly evident when IL-4δ2 was measured; its expression can be up to ~7 times lower than that of IL-4 expression [377, 378]. This is likely why IL-4δ2 was mostly undetectable across all groups and compartments.

10.2 Does IL-4 have a causal role in TB or is it just an epiphenomenon?

In order to test the *in vitro* effect of IL-4 and IL-4δ2 on mycobacterial containment (chapter 6) and mechanisms of host immunity (chapter 7), it was first necessary to clone and express recombinant IL-4 and IL-4δ2. A baculovirus expression system was chosen for reasons stated in table 4.1. rIL-4 was successfully expressed as a secreted His-tagged fusion protein (chapter 4). The amino acid sequence was confirmed by mass spectrometry and bioactivity was verified by the ability of rIL-4 to induce T-cell proliferation and CD23 expression on B cells (chapter 5). Similar conditions were used for rIL-4δ2 expression but proved to be more problematic as protein yield was very low and purity was sub-optimal. In contrast to rIL-4, His-tagged rIL-4δ2 remained intracellular likely because the protein structure of IL-4δ2, which is slightly altered compared to IL-4, interfered with the protein secretory pathway in insect cells [540-542]. As a result, protein overexpression, under the control of a strong baculovirus polyhedron promoter, and the subsequent intracellular accumulation of protein led to the formation of insoluble aggregates [544]. Furthermore, the higher proportion of intrinsic hisitidine-containing insect cell proteins in the cell lysate, compared to the supernatants, resulted in co-purification of non-specific proteins and low IL-4δ2 purity. Nonetheless, enough IL-4δ2 was obtained to confirm protein function by the ability of IL-4δ2 to inhibit IL-4 induced T-cell proliferation (chapter 5). However, due to the high concentrations required to observe an effect on IL-4-induced functions *in vitro*, rIL-4δ2 protein yield was insufficient to test in the mycobacterial containment model.

Despite increased IL-4 being found in TB patients, it was not known whether IL-4 plays an active role in subverting a protective immune response or is a by-product of TB-associated chronic inflammation. Previous murine studies have shown that IL-4 has a direct cause/effect relationship in TB but the animal model may not be entirely relevant in human tuberculosis [256, 424]. In order to answer this question, a mycobacterial containment assay was performed in this study to assess the effect of rIL-4 on the ability of effector cells to contain H37*Rv* within monocyte derived macrophages (in terms of CFU/ml; chapter 6). Effector T-cells generated in response to PPD and IL-4 had a reduced ability to contain H37*Rv* within MDMs compared to effectors generated in response to PPD alone and this effect was IL-4 dose-dependent. Furthermore, subversion of *M.tb* containment was abrogated if IL-4 was

blocked (using anti-IL-4 antibodies). IL-4 is essential for Th2 development and likely polarizes unprimed T-cells toward a Th2 effector phenotype which subsequently downregulated the Th1 response [574]. A similar subversive effect was observed if IL-4 was added to infected macrophages in the presence of PPD pre-primed effector T-cells but the extent of this subversion was less pronounced. Effector T-cells that develop in response to PPD are predominantly of a Th1 phenotype [603] and may not be as responsive to the Th2 polarizing effect of IL-4 added at such an advanced stage of Th1 differentiation [574]. IL-4 also exerts its effects directly on macrophages by driving alternative activation [351] and downregulating anti-mycobacterial responses [16] and may have also contributed to the reduced *M.tb* containment observed in both of these interventions. To my knowledge, this is the first study to directly evaluate the effect of IL-4 on a human model of TB infection.

The observed IL-4 associated subversion of *M.tb* containment *in vitro* suggests that a Th2 response can impair protective immunity in TB. IL-4 can modulate protective mechanisms of T-cells and macrophages, by downregulating a Th1 response [430, 431], inducing an alternatively activated macrophage phenotype [351, 432] and inhibiting autophagy [44] and production of reactive intermediate species [44]. A role for IL-4 in the development of Tregs, which directly attenuates mycobacterial stasis [293], has also been proposed [301-303, 444, 445] but the data is conflicting. Despite these findings, the interaction of these mechanisms and their direct effect on mycobacterial survival in the context of a Th2 response has not hitherto been explored. In order to investigate these mechanisms, a mycobacterial containment assay was performed where cells were stained for surface and intracellular markers then analysed by flow cytometry (chapter 7). IL-4-mediated subversion of *M.tb* containment was characterised by an increase in Treg (CD4+CD25+FoxP3+) expression and a concomitant decrease in CD4+ T-cell expression of Th1 cytokines (IFN-γ and TNFα). Blocking of IL-4 reverted Treg and CD4+ IFN-γ expression levels to those observed in cells stimulated with PPD alone (no IL-4). An increase in DC-SIGN expression, a marker of alternate macrophage activation that facilitates entry of *M.tb* into these cells [351, 609], was also observed on macrophages in IL-4-containing culture wells. These data implicate IL-4 as a mediator of multiple immune mechanisms in different cells involved in *M.tb* control. IL-4 drives a Th2 response and simultaneously induces Treg proliferation resulting in the

dampening of a protective Th1 response. IL-4 also polarizes alternatively activated macrophages, characterised by increased DC-SIGN expression, to downregulate macrophage-specific anti-mycobacterial mechanisms.

These data provide convincing evidence that IL-4 plays an active role in undermining protective immune mechanisms but whether the *in vitro* findings translate to what is happening within the body during active TB is not entirely certain. The inherent limitation of any *in vitro* model of infection is that it is a one-dimensional representation of the immune response and lacks the complex interaction of cytokines and cellular mechanisms occurring at the site of disease. Nevertheless, existing evidence does support the subversive role of IL-4 *in vivo*. IL-4-deficient mice infected with TB exhibit reduced bacterial proliferation and immunopathology [256]. Biological findings in human TB show that high IL-4 levels correlate with radiological extent of disease [9, 18, 21, 421] and were able to predict progression to active TB [23]. Another concern is whether the concentration of IL-4 used in the mycobacterial containment assay is biologically relevant given that naturally occurring IL-4 is active at much lower concentrations. High rIL-4 concentrations were required in this model to observe a subversive effect on *M.tb* containment because recombinant protein, particularly those expressed in a non-mammalian system, tend to be less active and stable that their natural counterparts [554]. Furthermore, sIL-4R may considerably prolong the half-life of IL-4 protein and enhance the bioactivity of IL-4 *in vivo* [362]. Thus, it is highly plausible that the high IL-4 levels consistently associated with active TB, are also causing immune dysfunction *in vivo*.

10.3 What other Th2 or Th2-like cytokines play a potential role in disrupting protective TB immunity?

The data presented thus far supports the notion that IL-4 can impair TB immunity but other cytokine factors are likely to be involved by either supporting IL-4 function or being a target of IL-4-induced immune dysregulation. Previous studies measuring soluble cytokines in TB have produced disparate results and few have evaluated expression in both the periphery and the site of disease (discussed in more detail in chapter 8). In order to determine what other

soluble mediators may be involved in impaired immunity within the context of a Th2 response, *M.tb* antigen-specific cytokine and chemokine responses were evaluated in culture supernatants of cells from the periphery and the lungs by Luminex assay (chapter 8). A distinct compartment-specific cytokine profile was found in TB patients characterized by decreased Th1 responses in the blood (IFN-γ) and increased IL-9 in the lungs. The ratio of Th2-related cytokines and chemokines (IL-9 and MCP-1) to Th1 cytokines (IFN-γ and TNFα) suggest a mixed Th1/Th2 response similar to the mRNA expression patterns observed in chapter 3. However, IL-4 was undetectable in these antigen driven cultures, likely due the difficulty in measuring soluble IL-4 protein (discussed in chapter 3).

The observation of an increased IL-9 response in the lungs of TB patients warranted further investigation into the cellular sources of this cytokine. Flow cytometric analysis revealed that CD4+ Th9 cells and CD8+ Tc9 cells were the predominant producers of IL-9 in TB. Th9 cells are distinct from Th2 cells but are inherently linked because IL-4 is required for Th9 development [49, 50] and Th9 cells have overlapping functions in Th2-driven allergic inflammatory diseases [324, 327, 332]. Tc9 cells are thought to exhibit a similar paradigm in the CD8+ population but have never been described in TB prior to this study. In both murine and human TB, increased levels of IL-4 and TGFβ are commonly found [16, 439], which provide the developmental conditions for induction of a Th9 response. Thus Th9 cells, and probably Tc9 cells as well, are likely to contribute to dysregulated immunity in TB by augmenting the Th2 response.

10.4 Implication for vaccine design/immunotherapeutic interventions against TB

The efficacy of BCG and MVA85A is based on their ability to prime the immune system by boosting a Th1 response, specifically CD4+IFN-γ producing T-cells. However, these vaccines offer limited protection in adults particularly in high burden settings where they are most needed. BCG efficacy is thought to be dampened in these settings due to a pre-existing Th1 immune response as a result of exposure to environmental mycobacteria [681]. Indeed, other studies and the data presented in chapter 3 reveal that a potent Th1 response is already

present in TB patients. Furthermore, animal studies show that manipulations to increase a Th1 response, above the response induced by BCG alone, failed to provide additional protection [682, 683]. These data suggest that it is the nature, rather than the magnitude, of the Th1 response that is important in inducing protective immunity. But targeting the Th1 response alone may not be the only answer. Chronic helminth infection, common in TB endemic areas, may also contribute to the ineffectiveness of BCG by inducing the development of Th2 and Treg responses [451-453]. However, a Th1 is also induced by NTM and helminth infection and it is rather an unbalanced Th1/Th2 response that may be impairing protective immunity. Evidence of a pre-existing mixed Th1/Th2 response is commonly found in TB patients from developing countries [21, 288, 416-418] and the data presented here (chapter 3 and 8) supports these findings. Furthermore, artificially skewing the immune response to a Th2 phenotype can attenuate mycobacterial containment (chapter 6) by inhibiting protective immune mechanisms (chapter 7).

A large number of vaccine candidates are either in development or at various stages of clinical testing. The high cost of clinical trials and limited field testing sites makes prioritisation of the most promising candidates of paramount importance and there is an urgent need for reliable scientific data to inform vaccine designs. The two most important questions that need to be answered are (i) what antigens to target? and (ii) what type of immune response needs to be induced to confer protection? The evidence presented here and elsewhere suggests that inducing a Th1 response is not enough and targeting the downregulation of Th2 responses and/or immunoregulatory mechanisms, including Th9, may also be required. As such, specific *M.tb* antigens that induce a Th2 or Th2-like response need to be identified so they can be excluded from existing and future vaccine preparations or administered with adjuvants that drive Th2 regulatory responses. One such candidate is *M. vaccae*, which promotes Th1 and simultaneously downregulates Th2 responses by inducing Th2-targeting regulatory cells, and has shown some promise as a multiple dose therapeutic and prophylactic vaccine in clinical trials [465-467]. Similarly, the hsp65 DNA vaccine promoted Th1 responses and inhibited IL-4 in mice and showed significant therapeutic effects [458].

The relevance of these data may also apply to the use of monoclonal antibodies in TB treatment. Neutralizing antibodies to Th2 and Th2-like cytokines or their receptors are commonly evaluated for their therapeutic use in asthma because they are simple to administer and well tolerated [684]. For example, anti-IL4 treatment has been shown to substantially reduce bacterial loads in murine models [685, 686] but its effect in humans is yet to be determined. Anti-IL-9 treatment reduces airway inflammation and hyper-responsiveness in murine models and improved asthma symptoms in humans [687]. However, little is known about their efficacy in TB. In a murine model of TB, injection of a neutralizing antibody to IL-4 in BALB/c mice was largely therapeutic even when introduced late in infection [688]. Blocking of Treg immunoregulatory mediators was successful in tumour clearance in a murine model of mesothelioma [689] and, given the detrimental role of Tregs in TB (chapter 7, [293]), may also prove beneficial in TB in humans.

10.5 Other Limitations

Healthy unexposed individuals were not recruited in this study. The purpose of the study was to determine why some individuals exposed to TB develop progressive disease whereas others remain healthy. As such, healthy, asymptomatic LTBI individuals were chosen as controls because this group best represents exposure to TB but subsequent immune control of the infection. Indeed, it is possible that differences in the cytokine expression profile of unexposed controls, compared to TB patients, may have shown greater significance as was observed in other studies [9, 21, 397]. However, these studies were performed in low TB prevalence countries where exposure to other immune modulating pathogens, including helminthes, is not common [690]. Furthermore, in a high burden setting such as South Africa, it is impossible to definitively distinguish healthy controls from LTBI using the available T-cell sensitization assays (TST and IGRA). Thus, due to the high rates of LTBI in the study setting and sample error, we did not recruit unexposed healthy controls. Nonetheless, it would be informative to determine the immune response to TB in these unexposed individuals, perhaps recruited from a low burden setting, and should be included in future studies.

The higher percentage of males in the TB group and females in the LTBI group may have influenced cytokine expression patterns observed in the study. Indeed, the global TB notification rates are male biased, with a male to female ratio of ~1.8:1 [1]. In this study, the higher percentage of males recruited in the TB group is likely a reflection of the higher incidence of TB in males compared to females. On the other hand, the higher percentage of females in the LTBI group may have been an artifact of the recruitment strategy of the study. LTBI were primarily recruited from a healthcare worker study, which predominantly consisted of female nurses. Although gender differences can play a role in susceptibility to TB, it is unclear whether these sex differences are related to behavior, have a physiological component or a combination of these factors [691]). For instance, men tend to partake in more TB risk associated behavior (smoking, alcohol consumption) than women [691]. Sex hormones are also thought to play a role in the immune response to TB affecting various cell types including macrophages, neutrophils and T cells [691]. For example, estrogen and testosterone seem to have antagonistic effects on macrophage activation via TLR4 expression and programmed cell death via PGE2 and LXA4 production [8, 692, 693]. Furthermore, in mice, IFNγ production is downregulated by testosterone but enhanced by estrogen [694, 695]. In contrast, high levels of estradiol can promote Th2 polarization and Tregs also seem to be influenced with changing levels of hormones, particularly during the menstrual cycle [692]. Given these findings, sex differences will be taken into account in future studies on TB immunity.

TB patients recruited into the study had a microbiologically confirmed diagnosis and were on anti-TB treatment for less than 2 weeks. These criteria were chosen because it allowed for a definitive diagnosis of TB early in the disease time course after these patients presented at the clinic. However, the host immune response can vary depending on the clinical stage of TB and subsequently, the measured cytokine profile may be different depending on when samples were collected during the disease time course. For example, IL-4 tends to be highest at the onset of disease and decrease following treatment [9]. Furthermore, bacterial proliferation occurring early in untreated disease may also lead to increased IFNγ responses [8]. However, it is extremely difficult to ascertain exactly when infection was established post-exposure and the time taken for presentation of symptoms as patients usually only seek

medical attention once they have had TB symptoms for at least 2 weeks. Furthermore it was not possible to definitively determine if TB disease in these patients were the result of primary exposure or reactivation of latent infection. These considerations should be taken into account when interpreting the results of this study.

10.6 Future work

The detrimental role of IL-4 on the ability of effector T-cells and macrophages to effectively contain *M.tb* has been established in an *in vitro* model of TB infection. However the role of IL-4δ2, in relation to IL-4, remains unknown. It was not possible to test this protein in my model as insufficient quantities were obtained in a baculovirus system. Once IL-4δ2 expression is optimized, either using an appropriate leader sequence for expression in insects cells or a different expression system altogether, further mycobacterial containment experiments using rIL-4δ2, either alone or together with rIL-4, will be performed to investigate its effect on mycobacterial survival. Given the higher expression of IL-9 in TB patients, similar experiments need to be performed using recombinant IL-9, to determine if it has a similar effect as IL-4 in this model. It will also be important to evaluate the impact of adding these cytokines (IL-4, IL-4δ2, IL-9) to other cell types, particularly those of the innate immune system and on lung resident macrophages, which tend to exhibit different degrees of mycobacterial uptake and anti-mycobacterial activities compared to blood derived macrophages [580, 581].

IL-4 was shown to be associated with the induction of Tregs in the mycobacterial containment assay. Further experiments are required to assess the function, mechanisms of action and primary target of these Tregs and if blocking of Tregs will reverse the effect on mycobacterial containment.

If a function for IL-4δ2 can be elucidated, the next step would be to determine if this protein occurs naturally and is present in TB or other diseases. Currently, detection of mRNA transcript by qPCR is the only reliable method to distinguish IL4 and IL-4δ2. There is limited evidence that IL-4δ2 exists as a protein *in vivo* but commercial antibodies that can effectively

distinguish it from IL-4 are not available. As such, work is currently being performed to generate monoclonal antibodies against these two isoforms.

IL-4δ2 is not the only splice variant of IL-4 that has been discovered. IL4δ3, which lacks exon 3, has been observed in cattle and rabbits [388, 398, 399], and increased levels have been observed in cattle protected against *M. bovis* [399]. However, this cytokine has never been reported in humans. qPCR analysis will be performed to determine if the mRNA transcript of this variant is detectable in humans. If its existence is confirmed, then further experiments will be conducted to assess its function in relation to the other IL-4 isoforms.

HIV infected participants were excluded from the study. However, it is important to determine the immune response to TB in the context of HIV, due to the high co-infection rates, especially in South Africa. High levels of IL-4 have been found in HIV infected individuals and these levels decrease upon commencement of HAART [696, 697]. Furthermore, HIV patients co-infected with TB express higher levels of IL-4 than those who are only HIV infected [415]. Therefore, more experiments are required to determine the extent of the IL-4 subversive effect in cells from HIV/TB co-infected patients.

10.7 Conclusion

TB patients express high levels of IL-4 and exhibit a skewed Th1/Th2 ratio, compared to LTBI controls, despite having a potent Th1 response. It has been shown, for the first time in a human *in vitro* model of TB infection, that IL-4 can subvert anti-mycobacterial mechanisms and subsequently undermine *M.tb* containment in monocyte derived macrophages. This likely occurs by downregulating a protective Th1 response, via induction of Treg activity, and driving alternate macrophage activation. Furthermore, other Th2 and Th2-like cytokines were also found to be associated with active TB. IL-9 in particular, which is produced predominantly by CD4+Th9 and CD8+ Tc9 cells, is increased in TB patients and may augment the subversive activity of a Th2 response.

Collectively, these data indicate a Th2 response plays an active role in undermining host immunity to pulmonary tuberculosis. It is hoped that these data can contribute to the development of vaccines or immunotherapeutic interventions that downregulate or block a Th2 response without compromising induction of a protective response.

11. References

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12. Appendix

12.1 Section A

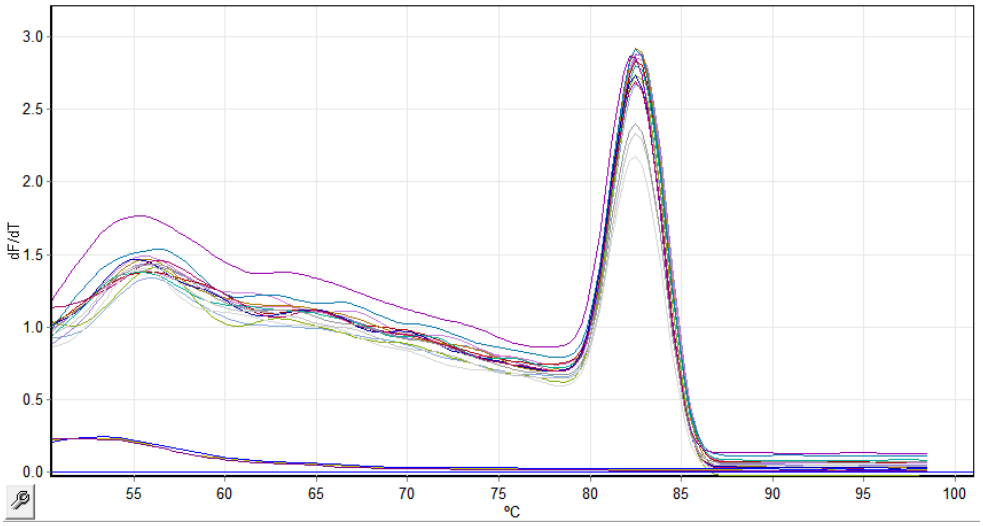

Figure A1. Example of a melt curve analysis to determine the IL-4 qPCR primer sequence specificity using Rotor Gene Q software.

Table A1. Cloning primers used to amplify cDNA regions containing the qPCR targets of interest for IFN-γ and HuPO. The amplified regions were used to generate the plasmid standards.

Gene of Interest	**Sequence** ○ Forward cloning primer 5' - 3' ● Reverse cloning primer 5 '- 3'	**Tm** (°C)
HuPO (NM_001002.3)	○ TTCGACAATGGCAGCATCTACAACC ● AAGGTGTAATCCGTCTCCACAGAC	59.5 58.3
IFN-γ (NM_000619.2)	○ TTGGCTTAATTCTCTCGGAAACGATG ● AGAGTTCCATTATCCGCTACATCTG	57.3 56.2

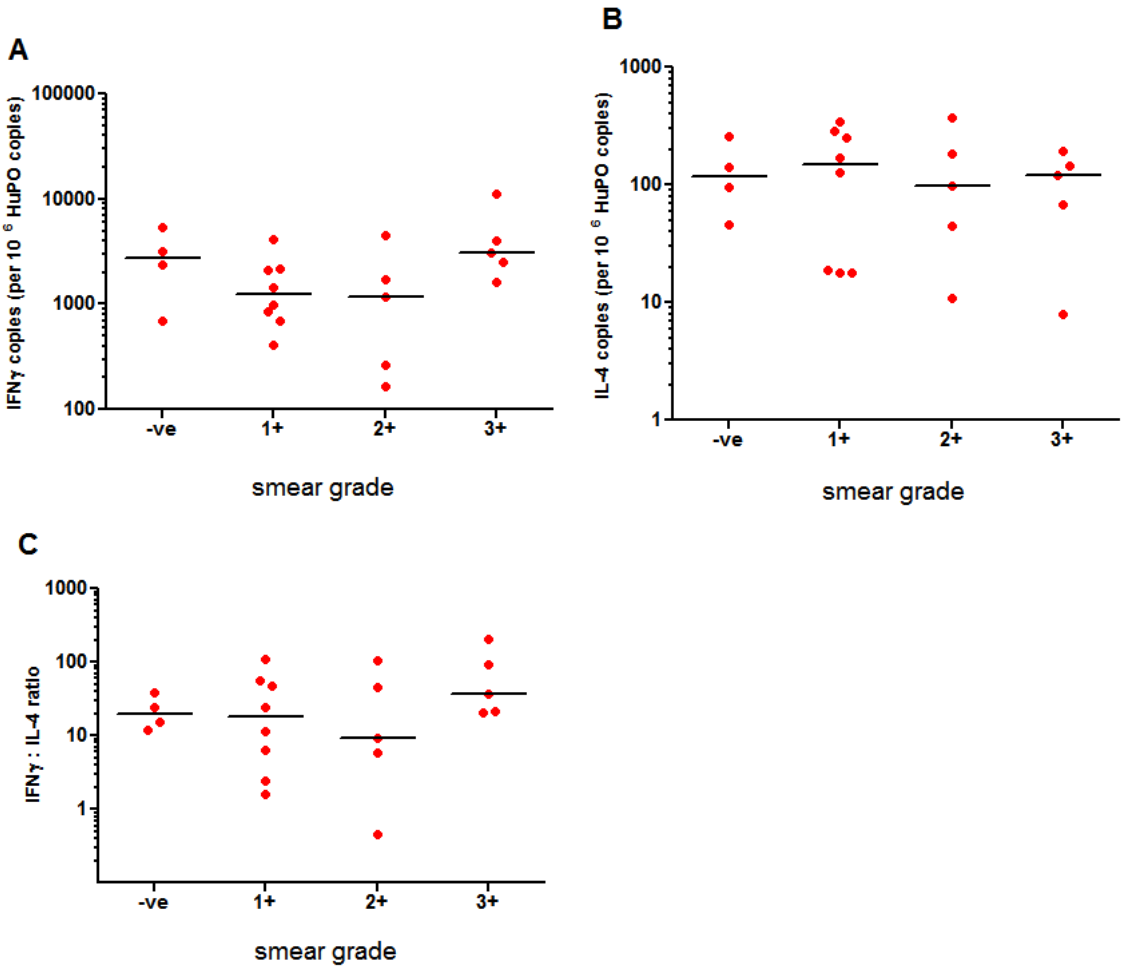

Figure A2. mRNA expression levels of (A) IFN-γ (B) IL-4 and (C) IFN-γ:IL-4 ratio stratified by sputum smear status in peripheral whole blood (Blood; red squares) from patients with pulmonary tuberculosis (TB; Blood n=23) as measured by quantitative real-time PCR. Data is shown on a $\log_{10}$ scale and copy numbers are expressed per million copies of HuPO. Statistical analyses were performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

12.2 Section B

12.2.1 Primers sequences

This section describes the primers used to construct the vector sequences in chapter 3

(i) These primers were used to construct the pAP01 vector:

AP1_Inv1

5’-(Phos)-TGGCTCGAGTGGCATATTTATCC-3’

AP1_Inv2

5’-(Phos)-AGTACTCCTAGGTAGCTGAGC-3’

(ii) These primers were used to construct the pAP01-IL4-TEV-His vector:

AP2_F

HindIII BamH1

5’-AAGCTTGGATCCATGGGTCTCACCTCCCAACTGCTTCC-3’

AP2_R1

4 His codons linker TEV recognition site

5’-ATGGTGATGGTGGGTACCCTGAAAATACAGGTTTTCGCTCGAACACTT

TGAATATTTCTCTCTCATGATCG-3’

AP2_R2

AvrII stop 6 His codons

5’-CCTAGGCTAATGGTGATGGTGATGGTGATGGTGATGGTGGGTACCCT

GAAAATACAGGTTTTCGCTCG-3’

(iii) These primers were used to construct the pAP01-NSIL4δ2-TEV-His vector:

AP3_Inv1

5’-(Phos)-AACACAACTGAGAAGGAAACCTTCTGCAGGGCTGCG-3’

AP3_Inv2

5’-(Phos)-CTTCTGCTCTGTGAGGCTGTTCAAAGTTTTGATGATCTCC-3’

AP4_Inv1

5’-(Phos)-CATGGATCCAAGCTTTATATTTATAGGT-3’

AP4_Inv2

5’-(Phos)-CACAAGTGCGATATCACC-3’

(iv) These primers were used to construct the pAP01-GST-TEV-NSIL-4δ2 vector:

AP5_Inv2

5' –(Phos)-CACAAGTGCGATATCACCTTACAGG-3'

AP5_Inv2

5' –(Phos)- GCCCTGAAAATACAGGTTTTCCTCG-3'

12.2.2 Nucleotide sequences

This section describes the full sequence of the plasmid vectors used in chapter 3

(i) pAP01-IL4-TEV-His (5720bp)

Boxed – start and stop codons
Signal peptide sequence
Exon 1 of IL-4
Exon2 of IL-4
Exon 3 of IL-4
Exon 4 of IL-4
TEV recognition sequence
Decahistidine tag
Restriction enzyme sites

```
1       GGAACGGCTC CGCCCACTAT TAATGAAATT AAAAATTCCA ATTTTAAAAA ACGCAGCAAG
61      AGAAACATTT GTATGAAAGA ATGCGTAGAA GGAAAGAAAA ATGTCGTCGA CATGCTGAAC
121     AACAAGATTA ATATGCCTCC GTGTATAAAA AAAATATTGA ACGATTTGAA AGAAAACAAT
181     GTACCGCGCG GCGGTATGTA CAGGAAGAGG TTTATACTAA ACTGTTACAT TGCAAACGTG
241     GTTTCGTGTG CCAAGTGTGA AAACCGATGT TTAATCAAGG CTCTGACGCA TTTCTACAAC
301     CACGACTCCA AGTGTGTGGG TGAAGTCATG CATCTTTTAA TCAAATCCCA AGATGTGTAT
361     AAACCACCAA ACTGCCAAAA AATGAAAACT GTCGACAAGC TCTGTCCGTT TGCTGGCAAC
421     TGCAAGGGTC TCAATCCTAT TTGTAATTAT TGAATAATAA AACAATTATA AATGCTAAAT
481     TTGTTTTTTA TTAACGATAC AAACCAAACG CAACAAGAAC ATTTGTAGTA TTATCTATAA
541     TTGAAAACGC GTAGTTATAA TCGCTGAGGT AATATTTAAA ATCATTTTCA AATGATTCAC
601     AGTTAATTTG CGACAATATA ATTTTATTTT CACATAAACT AGACGCCTTG TCGTCTTCTT
661     CTTCGTATTC CTTCTCTTTT TCATTTTTCT CTTCATAAAA ATTAACATAG TTATTATCGT
721     ATCCATATAT GTATCTATCG TATAGAGTAA ATTTTTTGTT GTCATAAATA TATATGTCTT
781     TTTTAATGGG GTGTATAGTA CCGCTGCGCA TAGTTTTTCT GTAATTTACA ACAGTGCTAT
841     TTTCTGGTAG TTCTTCGGAG TGTGTTGCTT TAATTATTAA ATTTATATAA TCAATGAATT
901     TGGGATCGTC GGTTTTGTAC AATATGTTGC CGGCATAGTA CGCAGCTTCT TCTAGTTCAA
961     TTACACCATT TTTTAGCAGC ACCGGATTAA CATAACTTTC CAAAATGTTG TACGAACCGT
1021    TAAACAAAAA CAGTTCACCT CCCTTTTCTA TACTATTGTC TGCGAGCAGT TGTTTGTTGT
1081    TAAAAATAAC AGCCATTGTA ATGAGACGCA CAAACTAATA TCACAAACTG GAAATGTCTA
1141    TCAATATATA GTTGCTGATC AGATCTGATC ATGGAGATAA TTAAAATGAT AACCATCTCG
1201    CAAATAAATA AGTATTTTAC TGTTTTCGTA ACAGTTTTGT AATAAAAAAA CCTATAAATA
1261    TAAAGCTTGG ATCCATGGGT CTCACCTCCC AACTGCTTCC CCCTCTGTTC TTCCTGCTGG
1321    CATGTGCCGG CAACTTTGTC CACGGACACA AGTGCGATAT CACCTTACAG GAGATCATCA
1381    AAACTTTGAA CAGCCTCACA GAGCAGAAGA CTCTGTGCAC CGAGTTGACC GTAACAGACA
1441    TCTTTGCTGC CTCCAAGAAC ACAACTGAGA AGGAAACCTT CTGCAGGGCT GCGACTGTGC
1501    TCCGGCAGTT CTACAGCCAC CATGAGAAGG ACACTCGCTG CCTGGGTGCG ACTGCACAGC
1561    AGTTCCACAG GCACAAGCAG CTGATCCGAT TCCTGAAACG GCTCGACAGG AACCTCTGGG
1621    GCCTGGCGGG CTTGAATTCC TGTCCTGTGA AGGAAGCCAA CCAGAGTACG TTGGAAAACT
1681    TCTTGGAAAG GCTAAAGACG ATCATGAGAG AGAAATATTC AAAGTGTTCG AGCGAAAACC
1741    TGTATTTTCA GGGTACCCAC CATCACCATC ACCATCACCA TCACCATTAG CCTAGGTAGC
1801    TGAGCGCATG CAAGCTGATC CGGGTTATTA GTACATTTAT TAAGCGCTAG ATTCTGTGCG
1861    TTGTTGATTT ACAGACAATT GTTGTACGTA TTTTAATAAT TCATTAAATT TATAATCTTT
1921    AGGGTGGTAT GTTAGAGCGA AAATCAAATG ATTTTCAGCG TCTTTATATC TGAATTTAAA
1981    TATTAAATCC TCAATAGATT TGTAAAATAG GTTTCGATTA GTTTCAAACA AGGGTTGTTT
2041    TTCCGAACCG ATGGCTGGAC TATCTAATGG ATTTTCGCTC AACGCCACAA AACTTGCCAA
2101    ATCTTGTAGC AGCAATCTAG CTTTGTCGAT ATTCGTTTGT GTTTTGTTTT GTAATAAAGG
2161    TTCGACGTCG TTCAAAATAT TATGCGCTTT TGTATTTCTT TCATCACTGT CGTTAGTGTA
2221    CAATTGACTC GACGTAAACA CGTTAAATAG AGCTTGGACA TATTTAACAT CGGGCGTGTT
2281    AGCTTTATTA GGCCGATTAT CGTCGTCGTC CCAACCCTCG TCGTTAGAAG TTGCTTCCGA
2341    AGACGATTTT GCCATAGCCA CACGACGCCT ATTAATTGTG TCGGCTAACA CGTCCGCGAT
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2401   CAAATTTGTA GTTGAGCTTT TTGGAATTAT TTCTGATTGC GGGCGTTTTT GGGCGGGTTT
2461   CAATCTAACT GTGCCCGATT TTAATTCAGA CAACACGTTA GAAAGCGATG GTGCAGGCGG
2521   TGGTAACATT TCAGACGGCA AATCTACTAA TGGCGGCGGT GGTGGAGCTG ATGATAAATC
2581   TACCATCGGT GGAGGCGCAG GCGGGGCTGG CGGCGGAGGC GGAGGCGGAG GTGGTGGCGG
2641   TGATGCAGAC GGCGGTTTAG GCTCAAATGT CTCTTTAGGC AACACAGTCG GCACCTCAAC
2701   TATTGTACTG GTTTCGGGCG CCGTTTTTGG TTTGACCGGT CTGAGACGAG TGCGATTTTT
2761   TTCGTTTCTA ATAGCTTCCA ACAATTGTTG TCTGTCGTCT AAAGGTGCAG CGGGTTGAGG
2821   TTCCGTCGGC ATTGGTGGAG CGGGCGGCAA TTCAGACATC GATGGTGGTG GTGGTGGTGG
2881   AGGCGCTGGA ATGTTAGGCA CGGGAGAAGG TGGTGGCGGC GGTGCCGCCG GTATAATTTG
2941   TTCTGGTTTA GTTTGTTCGC GCACGATTGT GGGCACCGGC GCAGGCGCCG CTGGCTGCAC
3001   AACGGAAGGT CGTCTGCTTC GAGGCAGCGC TTGGGGTGGT GGCAATTCAA TATTATAATT
3061   GGAATACAAA TCGTAAAAAT CTGCTATAAG CATTGTAATT TCGCTATCGT TTACCGTGCC
3121   GATATTTAAC AACCGCTCAA TGTAAGCAAT TGTATTGTAA AGAGATTGTC TCAAGCTCGG
3181   ATCGATCCCG CACGCCGATA ACAAGCCTTT TCATTTTTAC TACAGCATTG TAGTGGCGAG
3241   ACACTTCGCT GTCGTCGAGG TTTAAACGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC
3301   GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA
3361   TCAGGGGATA ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT
3421   AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
3481   AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT
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3601   TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG TAGGTATCTC
3661   AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC
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3781   TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT
3841   ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC
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4141   TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC
4201   AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC
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4741   TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT
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5221   GCGGCGGGTG TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC
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(i) pAP01-IL-4δ2-TEV-His (5672bp)

Boxed – start and stop codons
Signal peptide sequence
Exon 1 of IL-4
Exon 3 of IL-4
Exon 4 of IL-4
TEV recognition sequence
Decahistidine tag
Restriction enzyme sites

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421     TGCAAGGGTC TCAATCCTAT TTGTAATTAT TGAATAATAA AACAATTATA AATGCTAAAT
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901     TGGGATCGTC GGTTTTGTAC AATATGTTGC CGGCATAGTA CGCAGCTTCT TCTAGTTCAA
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2401    TTGGGCGGGT TTCAATCTAA CTGTGCCCGA TTTTAATTCA GACAACACGT TAGAAAGCGA
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2761    AGCGGGTTGA GGTTCCGTCG GCATTGGTGG AGCGGGCGGC AATTCAGACA TCGATGGTGG
2821    TGGTGGTGGT GGAGGCGCTG GAATGTTAGG CACGGGAGAA GGTGGTGGCG GCGGTGCCGC
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4681   GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG
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(i) pAP01-GST-TEV-NSIL4δ2 (6257bp)

Boxed – start and stop codons
GST tag
TEV recognition sequence
Exon 1 of IL-4
Exon 3 of IL-4
Exon 4 of IL-4
Restriction enzyme sites

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5761   GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT
5821   CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG TCAAGCTCTA
5881   AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC GGCACCTCGA CCCCAAAAAA
5941   CTTGATTAGG GTGATGGTTC ACGTAGTGGG CCATCGCCCT GATAGACGGT TTTTCGCCCT
6001   TTGACGTTGG AGTCCACGTT CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC
6061   AACCCTATCT CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC GGCCTATTGG
6121   TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT TTAACAAAAT ATTAACGTTT
6181   ACAATTTCCC ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC
6241   TCTTCGCTAT TACGCCA
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12.3 Section C

12.3.1 Preliminary optimization of mycobacterial containment assay

Preliminary experiments were performed to determine the effect of adding IL-4 at different concentration and at different stages during the assay. Additionally, cells were harvested 48 hours and 120 hours post co-culture to determine the optimal time for harvesting of cells. In addition to the controls and interventions mentioned in Section 6.2.1, Figure 6.1 and Table 6.1, the effect of pre-stimulating monocytes with IL-4 on mycobacterial containment was also performed (see below for description of experimental setup).

IL-4 prestimulation of monocytes

rIL4 was added to monocytes on Day 0 at concentrations of 5, 20 and 100 ng/ml. MDMs were then generated, infected and washed as described in 1.2.1.1. Concurrently, PBMCs were stimulated with PPD (12µg/ml) for 6 days to generate PPD effector cells. On day 6, these PPD effectors were co-cultured with the infected MDMs.

12.3.2 Results: Preliminary optimization experiments

Preliminary assays were performed on presumed LTBI controls (n=4) in order to determine the optimal time to add rIL-4 and time to harvest post co-culture and are shown in Figure C1. rIL-4 was added at 3 different concentrations (5, 20 and 100ng/ml) to PBMCs together with PPD to generate PPD/IL4 effectors (4 in Figure 6.1 and Table 6.1), added together with PPD effectors to infected MDMs (5 in Figure 6.1 and Table 6.1) or added to monocytes prior to infection with H37Rv (discussed above). Infected cells were harvested at 48 hours or 120 hours post co-culture. At 48 hours post co-culture, the „PPD effectors" control (32.1 x 10^3 CFU/ml) showed a 50% decrease in absolute CFU/ml compared to the „MDM only" control (60.4 x 10^3 CFU/ml). The most noteworthy effects of IL-4 were observed in the „PPD/IL-4 effectors" and „Exogenous IL-4" interventions. In the „PPD/IL-4 effectors", the absolute CFU/ml increased with increasing concentrations of rIL-4 from 32.7x10^3 CFU/ml (5ng/ml rIL-4) to 62.1x10^3 CFU/ml (100ng/ml rIL-4). Conversely, in the „Exogenous IL-4" intervention, there was a decrease in CFU/ml from 68x10^3 CFU/ml (5ng/ml rIL-4) to 28.8x10^3 CFU/ml (100ng/ml rIL-4). There was no observable difference in CFUs in the „IL-4

pre-stimulation of monocytes" intervention at any concentration ($13.2x10^3$ at 5ng/ml rIL-4; $9.5\ x10^3$ CFU/ml at 100ng/ml rIL-4) or compared to the PPD effector control ($32.1x10^3$ CFU/ml). Furthermore, CFU counts decreased by a mean of 75% when harvesting was performed 120 hours post co-culture compared to 48 hours post co-culture. Results were not statistically significant. Based on these results, and due to limitations in the amount of blood that could be obtained from TB patients, only the „PPD/IL-4 effectors" and „Exogenous IL-4" interventions were performed in subsequent *M.tb* survival assays on TB patients and additional presumed LTBI controls. Furthermore, harvesting was done at 48 hours post co-culture.

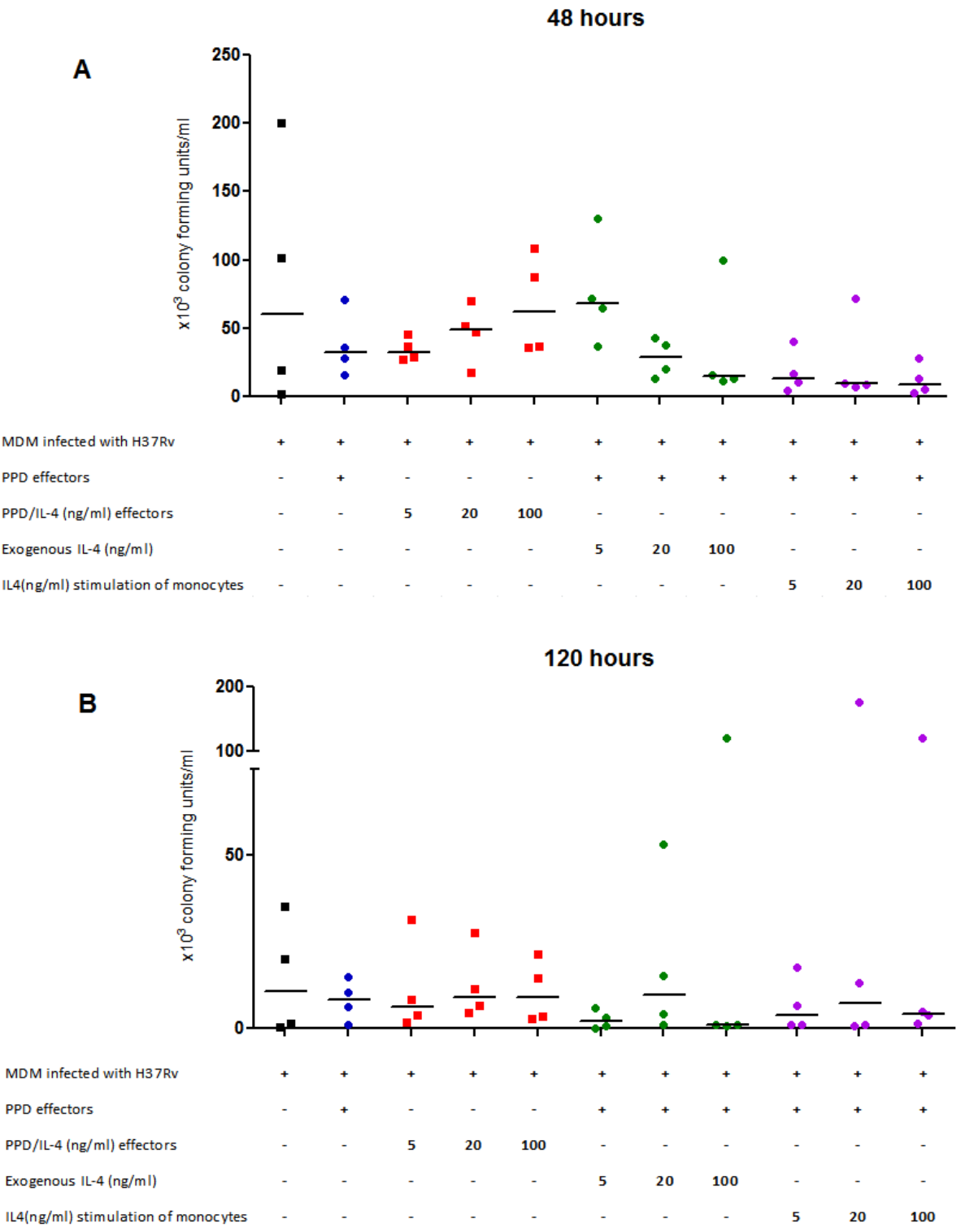
48 hours
A
250
200
150
100
50
0
x10³ colony forming units/ml
MDM infected with H37Rv + + + + + + + + + + +
PPD effectors - + - - - + + + + + +
PPD/IL-4 (ng/ml) effectors - - 5 20 100 - - - - - -
Exogenous IL-4 (ng/ml) - - - - - 5 20 100 - - -
IL4(ng/ml) stimulation of monocytes - - - - - - - - 5 20 100
120 hours
B
200
100
50
0
x10³ colony forming units/ml
MDM infected with H37Rv + + + + + + + + + + +
PPD effectors - + - - - + + + + + +
PPD/IL-4 (ng/ml) effectors - - 5 20 100 - - - - - -
Exogenous IL-4 (ng/ml) - - - - - 5 20 100 - - -
IL4(ng/ml) stimulation of monocytes - - - - - - - - 5 20 100

Figure C1. The effect of IL-4 on mycobacterial containment in monocyte derived macrophages (MDMs) when added at different time points in the mycobacterial containment assay. H37*Rv*-infected MDMs were co-cultured with purified protein derivative (PPD) pre-primed peripheral blood mononuclear cells from latently infected healthy controls (n=4). Recombinant IL-4 (rIL-4) is added at different concentrations (5, 20 and 100ng/ml) and at various points in the mycobacterial survival assay (as described in Figure 6.1). Mycobacterial growth was assessed in cells harvested (A) 48 hours and (B) 120 hours after co-culturing of cells. Median values are expressed as colony forming units/ml. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.