Differentiating between the forms of *Mycobacterium tuberculosis* disease through immune markers: a translational study through treatment of active and latent tuberculosis infections

Investigating toll-like receptors and their function, and comparison of two interferon-gamma-receptor assays

A thesis submitted to Monash University in total fulfilment of the requirements for the degree of Doctor of Philosophy

**Dr Sabine Lilamani De Silva**

MBBS (Hons), BMedSci (Hons), FRACP

Centre for Inflammatory Diseases, School of Clinical Sciences
Department of Medicine, Monash University
Melbourne, Australia

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Abstract

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (Mtb), is a global health challenge, accounting for over one million deaths and an estimated 10 million new infections in 2017.¹ It is the leading cause of death from an infectious disease.¹ There is a desperate need for biomarkers to aid in development of improved diagnostic tests, new drugs, a better vaccine and a test of ’cure’ to enable clinicians and global leaders make the “End TB 2035 Strategy” a reality.²

The host responses to Mtb infection involve complex interactions of innate and adaptive immunity, whereby the majority of humans infected never develop symptomatic disease.³ Mtb infections, previously considered two separate entities in humans, latent tuberculosis infection (LTBI) and active tuberculosis, are now widely accepted as a spectrum, influenced by both the host’s and bacterium’s genetic makeup and interactions.³,⁴,⁵ Toll-like receptors (TLRs) are a group of innate immune surface and intracellular receptors through which such signalling is initiated.⁶ In recent years, genetic association studies have shed light on the potential role of these receptors and their downstream function in TB responses in humans.⁷-¹² Clinical and translational studies, however, are limited.

An observational pilot study was undertaken at a tertiary health service in Melbourne to investigate the role of toll-like receptors and a newer interferon-gamma-release assay in patients commencing treatment for active TB and LTBI, as well as monitoring these immune markers through antibiotic therapy.

The observations made in the studies confirm that changes occur on peripheral blood mononuclear cells, such as CD14+ monocytes, natural killer and natural killer T cells, with respect to TLR2, 4 and 7 expression. Furthermore, downstream activation of TLRs result in cytokine changes, including through antibiotic therapy. It is described that TLR2 and 7 expression and functional changes are often in parallel, whilst TLR4 expression is generally the opposite in active tuberculosis infection through treatment. Comparative
evaluations between the two cohorts reveal differences described for the first time following pre- and post-antibiotic therapy in LTBI versus active TB of TLR markers.

This study also confirms that vitamin D levels are often deplete in patients with tuberculosis infections, with improvement of serum levels with supplementation. Also reported are the findings of a weak negative association of vitamin D levels with TLR4 expression and a weak positive association with TLR7 expression through antibiotic treatment for active tuberculosis. No previous translational reports are available regarding the role of vitamin D, TLR7 and active tuberculosis, to the author's knowledge.

Comparison of the new generation QuantiFERON-TB Gold Plus assay to its predecessor through treatment in both these cohorts confirms that it is a highly sensitive test. Evaluations also confirm that IFNγ responses reduce through antibiotic therapy.

The findings reported here allude to the possibility that LTBI and active TB have a specific TLR signatory profile, which alter with antibiotic treatment representing changes in the inflammatory process. These profiles have future potential to be part of a biomarker platform to differentiate active from LTBI, treatment monitoring and possibly towards determining a test of ‘cure’.
Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I declare that the material is less than 100,000 words (excluding tables, figures, and appendices).
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List of Abbreviations

AB  antibody
APC  antigen presenting cell
ApoA-1 apolipoprotein A1
Atg5  autophagy-related gene 5
BAL  bronchoalveolar lavage
BCG  bacilli Calmette-Guerin
CARD  caspase recruitment domain
CBA  cytokine bead array
CCL2  chemokine (C-C-motif) ligand 2 (see also MCP1)
CCL3  chemokine (C-C-motif) ligand 3 (see also MIP1α)
CCR2  chemokine receptor 2
cDNA  complementary DNA
CFH  complement factor H
CFP-10  culture filtrate protein-10
CLR  c-type lectin receptors
CpG  cytosine-phosphate-guanine (CpG oligonucleotide motif)
CXCL10  interferon-γ-induced protein 10 (see also IP-10)
DC-SIGN  dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin
DCs  dendritic cells
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
dNTP  deoxynucleoside triphosphates
ELISA/EIA  Enzyme-linked immunosorbent assay
ES-CBA  Extra-sensitive cytokine bead array
ESAT-6  early secretory antigenic target-6
ESX-1  type VII secretion system
FACS  FACS tubes
FCS  foetal calf serum
fg  femtogram
FVS  fixable viability stain
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
GEE  generalised estimating equations
GMF  geometric mean fluorescence
HKG          housekeeping genes
Hsp70        heat shock protein 70
IFN\(\gamma\) interferon-gamma
IGRA         interferon-gamma release assay
IL-6         interleukin-6
IL-8         interleukin-8
IL-10        interleukin-10
IL-12p70     interleukin-12-subunit p70
IL-17A/F     interleukin-17/F
IP-10        interferon-\(\gamma\)-induced protein 10 (see also CXCL10)
IQR          interquartile
IRAK         interleukin-1 receptor-associated kinase
IRIS         immune-reconstitution syndrome
LAM          lipoarabinomannan
LM           lipomannan
LPS          lipopolysaccharide
LTBI         latent tuberculosis infection
LXA4         lipoxin A4
M1           macrophage phenotype 1
M2           macrophage phenotype 2
mAB          monoclonal antibody
MAIT cells   mucosal-associated-invariant T cells
manLAM       mannosylated lipoarabinomannan
MAP          mitogen associated protein
MAPK         mitogen activated protein kinases
MCP1         monocyte chemo-attractant protein 1 (see also CCL2)
MD-2         myeloid differentiation 2
MDR-TB       multi-drug resistant tuberculosis
mg           milligram
MIP          macrophage inflammatory protein
MIP1\(\alpha\) macrophage inflammatory protein 1\(\alpha\) (see also CCL3)
MR           mannose receptor
mRNA         messenger RNA
Mtb          *Mycobacterium tuberculosis*
MyD88        myeloid differentiation primary response protein 88
NADPH        nicotinamide adenine dinucleotide phosphate
NF-kB        nuclear transcription factor kappa B
             (kappa light chain inducer of B cells)
ng  nanogram
NK  natural killer cells
NKT  natural killer T cells
NLR  NOD-like receptors
NO  nitric oxide
NOD  nucleotide oligomerisation domain
NOS  nitric oxide synthase
NTM  non-tuberculous mycobacteria
P3C  Pam3CSK4 synthetic triacylated lipopeptide
PAMPs  pathogen-associated molecular patterns
PBMCs  peripheral blood mononuclear cells
PBS  phosphate buffered solution
PD-L1  programmed death ligand 1
pg  picogram
PGE2  prostaglandin E2
PIM  phosphatidyl inositol mannose
PPV  positive predictive value
PRRs  pattern-recognition receptors
QFT  QuantiFERON-TB Gold
QFT-Plus  QuantiFERON-TB Gold Plus
R848  imidazoquinoline – synthetic molecule activating TLR7/8 pathway
RANTES  chemokine CC motif receptor 5
RD-1  region of difference-1
RNA  ribonucleic acid
RNI  reactive nitrogen intermediates
ROI  reactive oxygen intermediates
rpm  revolutions per minute
RPMI  Roswell Park Memorial Institute medium
RT-PCR  reverse transcription polymerase chain reaction
SAA  serum amyloid A
SAv-HRP  Streptavidin-horseradish peroxidase conjugate (enzyme reagent)
sCD14  soluble cluster differentiation 14 gene
SD  standard deviation
SNP  single nucleotide polymorphism
TB  tuberculosis
TB1 & TB2  tuberculosis antigen tubes used in QFT-Plus
TCR  T cell receptor
TDM (cord factor)  trehalose dimycolate

xxiii
TDR-TB  totally drug-resistant tuberculosis
TIIDM  type 2 diabetes
TIR  toll/interleukin-1 receptor
TIRAP  toll/interleukin-1 receptor domain containing adaptor protein
TLR  toll-like receptor
TNF  tumour necrosis factor
TNFR  tumour necrosis factor receptor
TRAM  TRIF-related adaptor molecule
TRIF  toll/interleukin-1 receptor domain containing adaptor-inducing interferon-β
TST  tuberculin skin test
VDR  vitamin-D receptor
XDR-TB  extensively drug-resistant tuberculosis
Chapter 1: Introduction

Tuberculosis affects a third of the world's population, is caused by the bacterium *Mycobacterium tuberculosis* (Mtb) and can cause disease in any part of the human body. It is a major cause of morbidity and mortality in the world, having accounted for 20% of adult deaths between the 17th and 19th centuries in Europe and North America.\(^\text{16}\) Tuberculosis (TB) is now the leading cause of death from an infectious disease globally, having previously been second to human immunodeficiency virus (HIV) related deaths.\(^\text{1}\)

The economic and social burden to developing nations is great as a consequence of the sheer numbers, but also the age of those affected. Of importance is that only 10% of those infected with the bacterium go on to develop clinical disease in their lifetime, although this is changing with the advent of advanced age and new immunomodulators.\(^\text{1}\) Other risk factors include poverty, diabetes, smoking, malnutrition, excess alcohol consumption and poor socioeconomic conditions.\(^\text{5,17}\)

The emergence of drug resistant tuberculosis, lack of new treatments, absence of an effective vaccine and the co-existent HIV-tuberculosis pandemic make tuberculosis a global health priority for research. The “End TB Strategy” target to end the global tuberculosis epidemic by 2035 highlights the concerns of the World Health Organization (WHO).\(^\text{2}\)

The host responses to Mtb infection are complex and involves intricate interactions of innate and adaptive immunity, whereby the majority of humans infected never develop symptomatic disease due to these responses.\(^\text{3}\) Mtb infections, previously considered two separate entities in humans (latent tuberculosis infection and active tuberculosis) are now widely accepted as a spectrum of infection, influenced by both the host’s and bacterium’s genetic makeup and interactions.\(^\text{3,4,5}\) Innate immune responses are the gatekeepers of human defence mechanisms, recognising and initiating immune reactions, consequently leading to adaptive immune responses to Mtb exposure.\(^\text{18}\) Toll-like receptors (TLRs) are a group of surface and intracellular receptors through which such signalling is initiated.\(^\text{6}\) Found on immune cells, these receptors are activated by different
molecules present on pathogens and eventually lead to cytokine and chemokine release, in turn instigating further immune reactions.\textsuperscript{6,18,19,20,21} In recent years, genetic association studies have shed further light on the potential role of these receptors and their downstream function in TB responses in humans.\textsuperscript{7-12} Clinical and translational studies, however, are limited. The differences between latent tuberculosis infection (LTBI) and active disease may be at least in part reflected through such innate immune responses, given the lack of ‘memory’ of the latter. Investigation of these could potentially contribute to a better understanding of the complex interactions between innate and adaptive immune responses and also towards the development of biomarkers in TB.\textsuperscript{22,23,24}

The importance of the role of vitamin D in immune responses is becoming better understood and its specific relevance in tuberculosis particularly so, with deficiency associated with development of disease, gene polymorphisms associated with susceptibility to active TB and association with toll-like receptor changes.\textsuperscript{25,26,27,28,29,14,30} Currently, the urgent need to develop a biomarker(s) predominates tuberculosis research, with many different avenues being investigated.\textsuperscript{31,32,24,33,21,34,35,36} However, due to the complex nature of the disease and still incompletely understood immunopathogenesis, the development of suitable biomarkers has been elusive.
Chapter 2: Literature review and study hypotheses

2.1 Epidemiology of tuberculosis

The overall annual global incidence rate of tuberculosis (TB) is 133 per 100,000 persons/year (Figure 2.1). Low and middle-income nations carry the burden of tuberculosis disease globally, particularly nations in sub-Saharan Africa and Southeast Asia. Of the estimated 10 million new infections in 2017, over 66% is accounted for by cases in India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa. An estimated 1.3 million deaths were due to TB in 2017, with approximately 300,000 occurring amongst HIV positive persons. Importantly, the overall incidence and mortality rates due to TB (even when including HIV-TB cases) have been declining worldwide, with an estimated 2% and 3% fall annually of these rates, respectively. These rates need to improve to 4–5% and 10% by 2020 to reach the milestones for the End TB Strategy.

![Estimated TB incidence rates, 2017](image)

**Figure 2.1: Tuberculosis incidence rates 2017**

Adapted from the WHO Global TB Report 2018.
Drug resistant TB is one of the greatest global challenges. In 2017, an estimated 558,000 new cases with rifampicin resistance (of which 460,000 were multi-drug resistant tuberculosis [MDR-TB]) were reported (Figure 2.2). Nearly half of these cases occurred in China, India and the Russian Federation.¹

![Estimated incidence of MDR/RR-TB in 2017, for countries with at least 1000 incident cases](image)

Figure 2.2: MDR-TB and rifampicin resistant TB incidence 2017
Adapted from the WHO Global TB Report 2018.

2.1.1 Local (Australian) epidemiology

Tuberculosis is a notifiable infection in Australia, with a TB program in each state and a comprehensive surveillance program (National Tuberculosis Advisory Committee). In Australia, 1339 cases of tuberculosis were notified in 2014, with 2% estimated to be associated with HIV.³⁷ Ninety-five per cent of these cases were new. The overall Australian incidence rate of TB is 6.1 per 100,000 (although six times higher in indigenous Australians).¹,³⁷ The majority of cases in Australia (86%) occur in people born overseas. Approximately 10 to 20 deaths occur in Australia due to TB annually. Drug sensitivity testing (DST) results were available for 75% of cases notified in 2014, with 17 (1.7%) being MDR and one case of extensively drug resistant (XDR-TB) being reported.³⁷
The Victorian rates of new diagnoses of TB reflect that of the overall Australian rates, partly because Victoria has the highest population after New South Wales. The overall rates of TB cases are increasing in Victoria (Figure 2.3). The majority of people who are diagnosed with active TB are overseas born, with Central and South Asia being the commonest regions. The median time to diagnosis in those born overseas is four years from arrival in Australia, highlighting that the majority of active disease in Victoria is due to LTBI reactivation. It is also important to note the gap between laboratory confirmed cases and reported cases (or those diagnosed and commenced on anti-tuberculous drug therapy).

Figure 2.3: TB notifications and laboratory confirmed cases in Victoria (2005–2010)
From Lavender et al. 2013.

Monash Health is the largest health service in Victoria where 90 to 110 cases of active tuberculosis are diagnosed and managed annually, comprising about 10% of Australia's reported cases.
2.2 Microbiology, clinical infection and laboratory diagnosis of *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* (MtB) is an obligate intracellular pathogen, with humans being the principal host, without animal or environmental reservoirs, although it can infect several animal species. Its origins affecting humans go back thousands of years and recent reports suggest it was likely associated with modern humans migrating out of Africa about 70,000 years ago, suggesting a pre-Neolithic Demographic Transition.\(^5,39,40\) It was first identified as the causative organism of tuberculosis by Robert Koch in 1882. It is now recognised as one of the organisms comprising *Mycobacterium tuberculosis complex* (MtB complex), causing disease in humans including *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti* and *Mycobacterium canetti*.\(^17\) The genome sequence of a common strain of MtB (H37Rv) was determined in 1998.\(^41\)

2.2.1 General microbiology

MtB is an aerobic, acid fast, non-encapsulated, non-spore forming, non-motile bacillus. Whilst able to survive in other environments, it grows best in high oxygen tissues, such as the lungs in humans. Its mycolic acid containing lipid-rich cell wall makes it impermeable to basic dyes, and hence ‘acid fast’, being classified as neither gram positive or gram negative.\(^17\) The cell wall, efflux mechanisms and the presence of a \(\beta\)-lactamase are some of the reasons for MtB to be intrinsically resistant to common antibiotic agents.\(^17,42\) Furthermore, the cell wall renders the bacterium to clump together in culture, which together with its slow growth (division time of 15–22 hours), makes the organism a challenge for laboratory identification and growth.\(^17,43\)

2.2.2 MtB infection and disease

Human to human transmission of MtB occurs through inhalation of infectious aerosols produced by coughing of individuals with active pulmonary disease. Infectivity depends on several factors including the degree of cumulative exposure (to the index pre-treatment) and smear positivity. It is estimated that only one third of sputum culture of positive patients are able to generate aerosols that are infectious.\(^44\) The aerosolised droplets containing the organism are less than 5 \(\mu\)m in diameter and are able to be
inhaled into the lungs of person(s) in close proximity. These inhaled organisms can then be phagocytosed by alveolar macrophages and result in a new infection. A minority of immunocompetent people will eliminate Mtb, whilst the majority will contain it within a 'latent state', with a small risk of reactivation to active disease during their lifetime, whilst a few may develop active disease 'primary infection' (with a consequent risk of further infecting others). The overall risk of reactivation disease is small (10% lifetime risk) and is highest in the first few years following infection acquisition. In areas of low endemicity, such as the United States (US) and Australia, the majority of active cases diagnosed are the result of reactivation. The risk is greater in those who are immunocompromised, particularly those who are living with HIV (5–10% per year). In high endemicity areas, it’s been estimated that HIV-negative patients can be smear positive for up to one to three years prior to formal diagnosis of active tuberculosis. It is also estimated that for every year that a person with pulmonary tuberculosis is untreated, a further 10–15 are potentially infected.

For a third of the world with latent TB infection (LTBI), the bacteria can persist in a state of equilibrium with the host’s immune system. Previously thought to be distinct forms of Mtb infection, evidence is now available to show that it is very much a continuum between the two states of Mtb infection; Mtb DNA and viable bacilli have been isolated from persons who have died in traffic accidents and have macroscopically normal lung tissue. Persons with LTBI are by definition well and asymptomatic. The potential course of TB infection and disease are highlighted in Figure 2.4 (from Drain et al. 2018).

Despite the vast numbers of persons with active and latent TB, the mechanisms surrounding Mtb infection and ultimate control via the host-pathogen interaction is incompletely understood.
Figure 2.4: The clinical spectrum of tuberculosis infections

This graph highlights the challenges of differentiating ‘LTBI’ from traditional ‘active disease’, with a significant portion of patients likely to have incipient or subclinical active disease, but due to lack of symptoms are not diagnosed, hence not treated and potentially be a source of spread of infection. Adapted from Drain et al. 2018.4

Active TB most commonly results in disease of the lungs, but extra-pulmonary TB can affect all systems of the human body, causing significant morbidity and mortality from the central nervous system (CNS), genitourinary TB to eye involvement.17 The diagnosis of active TB relies on patients presenting with symptoms, a high index of suspicion from the clinician and investigations being conducted in a timely manner. Whilst symptoms usually relate to the system(s) involved, non-specific constitutional symptoms such as weight loss, fevers, night sweats and the often indolent and chronic time course can be misinterpreted. Persons with early stages of active TB disease may also be asymptomatic, as reported recently in Vietnam, contributing to spread of infection.3,53

2.2.3 Diagnosis of Mtb infection

2.2.3.1 LTBI diagnosis

There is no gold standard test for the diagnosis of LTBI. Until the late 1990s, the tuberculin skin test (TST) was the only screening test available. It involves measurement of a person’s cellular immunity to antigens injected intradermally.54 The delayed hypersensitivity-type skin reaction resultant from the purified-protein-derivative (PPD) requires a second visit to the clinic / medical services, and correct reading of the
reaction.\textsuperscript{55} In addition to the latter as sources of variability, repeat TST or Mantoux testing and repeat BCG vaccination (Bacille Calmette-Guerin) can result in stronger reactions.\textsuperscript{55,56}

The diagnosis of LTBI, in addition to the detection of cellular immune responses to Mtb antigens, requires the clinical exclusion of active TB in low-incidence settings.\textsuperscript{5} This now commonly involves the use of interferon-gamma (IFN\textsubscript{\gamma}) release assays (IGRAs) in developed countries and currently includes two commercially available tests: QuantiFERON-TB Gold (QFT) / QuantiFERON-TB Gold Plus (QFT-Plus) (Qiagen, Hilden, Germany) and T-SPOT (Oxford Immunotec Ltd, Abingdon, UK) (see Section 2.6). IGRAs and TST are surrogate markers of Mtb infection, measuring cellular immune responses to Mtb antigen sensitisation in an individual, whilst not differentiating between remote or recent exposure, infection and disease state.\textsuperscript{57,58} The use of these tests in special populations (such as people living with HIV, immunocompromised and children) is challenging and discussed in Section 2.6.

2.2.3.2 Active TB diagnosis

Laboratory diagnosis of active Mtb infection involves identification of the organism in samples taken from the site of disease. This most commonly includes sputum or bronchoalveolar lavage (BAL) microscopy and culture. Similarly, identification of the organism in extra-pulmonary TB is also important, as growth of the organism is necessary for phenotypic drug sensitivity testing. This is undertaken by the use of solid and liquid (broth) culture systems, taking up to three to six weeks or 10 to 30 days to grow the organism, respectively.\textsuperscript{42} These methods are highly specific but time consuming, and remain the ‘gold standard’ for active TB diagnosis.\textsuperscript{17} Detection of Mtb DNA by nucleic acid amplification (mostly targeting the IS6110 sequence) has contributed to rapid identification of the organism and genotypic resistance testing through commercially available, standardised rapid DNA assays, including that for rifampicin resistance (used as a surrogate marker for MDR-TB).\textsuperscript{59} Xpert\textsuperscript{®} MTB/RIF (Cepheid\textsuperscript{®}, Sunnyvale, US) is the most commonly used, automated, single platform, cartridge based real time polymerase-chain reaction (PCR) system identifying the presence of Mtb DNA and simultaneously detecting mutations in the rpoB gene sequence commonly associated with rifampicin resistance, which has been endorsed by the WHO.\textsuperscript{1,17} A newer generation of the test
Xpert® MTB/RIF Ultra appears to be more sensitive, particularly in pauci-bacillary samples (such as smear negative sputum, extra-pulmonary TB, HIV positive patients and children).\(^\text{60}\)

### 2.2.3.3 Drug resistance

Drug resistance in human Mtb disease is multifaceted, but mostly due to prolonged mono-therapy selecting spontaneous resistance mutations which occur at different rates for different drugs. The availability of whole genome sequencing (WGS) has enabled better understanding of the different genotypes of the bacterium and possible implications of long-term persistence, selective pressure on genomic loci and drug exposure on genetic variability and diversity.\(^\text{40,61}\)

Rifampicin and isoniazid form the basis of ‘first line’ treatment for drug-sensitive active TB. Resistance to these two antibiotics defines multi-drug resistant (MDR) TB. Drug resistance acquisition is multifactorial and can involve single nucleotide substitutions (e.g. \(\beta\) subunit of RNA polymerase gene [\(rpoB\)] conferring rifampicin resistance), whilst isoniazid resistance is associated with changes in catalase peroxidase (\(katG\)) gene or \(inhA\) gene involved in mycolic acid synthesis.\(^\text{42}\) Stepwise accumulation of gene mutations can contribute to further antibiotic resistance. Importantly, introduction of a single antibiotic to a failing regimen can result in inadvertent sequential mono-therapy and further drug resistance. Risk factors for development of drug resistance include previous treatment for TB, prolonged hospitalisation in TB endemic regions, HIV infection, inappropriate or inadequate drug regimens (dosing, drug choices, duration), lack of directly observed therapy (DOT), delays in diagnosing drug resistant TB and overuse of fluoroquinolones for non-TB respiratory infections.\(^\text{42}\) Drug resistant strains can also be transmitted directly between persons, resulting in primary drug resistant TB occurring in incident TB without prior anti-mycobacterial therapy.

Extensively drug resistant (XDR) TB is defined as MDR plus resistance to second line injectables (amikacin, capreomycin, kanamycin) and fluoroquinolones. XDR-TB is now reported all over the world (8014 cases in 72 countries in 2016) and represents about 6.2% of those diagnosed with MDR-TB globally, with treatment success rates of
approximately 30%. In Australia, 17 cases of MDR-TB (1.7% of total incident cases) and one case of XDR-TB were notified in 2014.

Phenotypic and genotypic drug sensitivity testing offer important ways of providing results to clinicians, each with advantages and disadvantages. Currently, Xpert® MTB/RIF is the most commonly used genotypic assay used worldwide, recommended by the WHO, but does not exclude the need for phenotypic testing for first- and second-line drugs. Molecular (genotypic) tests for second line drug sensitivity testing are also available and recommended as a ‘rule in’ test such as ‘Genotype MTB-DRs1’ (Hain Lifesciences, Germany) alongside phenotypic testing. WGS of direct tissue sampling in the future may enable accurate and timely drug sensitivity profiling. Unfortunately, the costs, infrastructure and expertise are significant barriers to the widespread use of these assays in high-endemicity countries and threaten successful implementation of the End TB Strategy.

2.3 Treatment of tuberculosis infections

Diagnosis and commencement of appropriate antibiotic treatment for active TB infection in a timely manner are crucial to reach the goals set by the End TB Strategy of reducing TB-related deaths by 95% and TB incidence by 90% by the year 2035. Recognition of those at risk of LTBI and treatment is also important to reduce the reservoir of Mtb infection. It is also paramount that there is adherence to the medication by individuals and monitoring of this by clinicians, local health services and governments alike in low, medium and high burden countries. The current recommendations for treatment of active and LTBI are briefly discussed below. There is great variation based on individual patients’ needs, as well as local guidelines, accessible diagnostics and facilities.

2.3.1 Latent tuberculosis infection treatment

The recommendations for identifying and treating persons with LTBI have recently been updated by the WHO. The mainstay of identifying those at risk of TB infection and exclusion of active disease, however, remains unaltered. For close/household contacts of persons with active PTB, screening and treatment for LTBI is recommended for
children under five years of age, those who are immunocompromised (living with HIV, haematological or organ transplant candidates, receiving anti-TNF therapy) and persons with silicosis once active TB is clinically excluded. The other key changes include options of shorter treatment courses with different drugs (and combinations) based on recent clinical studies.

The most commonly prescribed therapy for LTBI treatment is isoniazid monotherapy for six or nine months duration, on the known (or presumption) that the patient has been exposed to isoniazid sensitive TB. Poor adherence, due to many reasons but particularly the duration of therapy has been identified as a key reason for non-completion of treatment. More recently, shorter course therapies (mostly including a rifamycin-based treatment protocol), have been shown to be effective, often with better adherence.

LTBI treatment for those deemed high risk but exposed to rifampicin resistant TB confers a greater challenge and is not discussed here.

2.3.2 Active TB treatment

Treatment of active TB is complex and guided by confirmed diagnosis, drug sensitivity testing (DST) (genotypic and phenotypic where applicable), as well based on patients’ needs and local recommendations. In addition to available genotypic and phenotypic drug sensitivity profiles, treatment choices (such as initial drug choice, planned duration) can be affected by site(s) of disease, underlying medical conditions and other medications a patient may already be on. The latter is particularly important in patients living with HIV, where rifampicin use can significantly affect the metabolism of anti-retroviral drug levels. The site and severity of disease can also alter planned therapy duration (e.g. cavitary pulmonary disease usually requires a nine-month course of therapy, whilst bone and central nervous system involvement requires a longer course of nine to 12 months). The complexity of HIV and TB medications and treatment is beyond the scope of this dissertation. It is important to note, however, for patients who are diagnosed with HIV at the same time of TB, it is recommended that anti-retroviral therapy (ART) be commenced along with or soon after TB drug therapy. An individual’s immune state,
side effect potential and risk of immune-reconstitution inflammatory syndrome also have to be taken into consideration.5,17

Directly observed therapy is now not routinely recommended by WHO for drug-susceptible TB, whilst it is recommended where possible for rifampicin resistant TB.1,2 There is no test of cure following active TB treatment to date.

2.3.2.1 Drug-susceptible active TB treatment

The mainstay of drug sensitive therapy is a minimum of six months of daily treatment with 'standard short course therapy' (generally two months of rifampicin, isoniazid, pyrazinamide and ethambutol termed the intensive phase), followed by four months of rifampicin and isoniazid if the patient is responding clinically.77,78 This treatment schedule, even with fixed drug combinations allowing for a lower pill burden in high burden countries, is associated with a moderate defection rate, more commonly in the latter stage of therapy.67 Whilst it is recommended that for persons with smear positive TB, repeat cultures be taken to ensure sputum smear clearance at the end of the intensive phase, this is not always possible.

2.3.2.2 Drug-resistant active TB treatment

The most common drug resistance pattern is isolated isoniazid resistance, usually detected by phenotypic drug sensitivity testing. Treatment for isoniazid mono-resistance is usually nine months of treatment with a rifampicin-based regimen.5 More recently, a six-month course with fluoroquinolones added has been suggested as a treatment option by the WHO.80

Treatment of rifampicin resistant-TB (usually MDR-TB) is complex and involves use of a minimum of four effective drugs including second line injectables. The treatment course is usually 18–24 months. Recent studies using the 'Bangladeshi Regimen' of short course treatment for MDR-TB has been approved by the WHO for persons who have not received second-line drugs prior and in whom resistance to fluoroquinolones and second line injectables is excluded (or thought to be highly unlikely), with a treatment duration of nine to 12 months.42,80,81 The treatment course is still complex and multiple options exist. Even more complex and challenging is XDR-TB and totally drug resistant TB (TDR-TB)
treatment, where treatment regimens are guided by drug exposure as well as drug availability, in addition to previously mentioned considerations.\textsuperscript{5,81,82,83}

\subsection*{2.3.3 New drugs and novel (host-directed) therapies for active tuberculosis}

Few new drugs had been approved in the last decade for TB treatment; the ‘new’ options have simply been repurposed antibiotics or older drugs, often with significant side effect profiles. One of the reasons for this as alluded to previously is the long treatment course and lack of a ‘test of cure’, making drug efficacy challenging to assess.

Fortunately, several drugs are undergoing clinical trials currently, including novel drugs in Phase 1 and 2 trials in 2017 for drug-susceptible and drug-resistant TB.\textsuperscript{84,85,86} Several are similar to drugs already available, but with fewer side effects and interactions. Large clinical trials are also underway to determine if shorter course treatment in drug-susceptible active TB is as effective, including several with newer drugs.\textsuperscript{85,86}

Novel or ‘host-directed therapies’ are also being investigated as adjuvant therapies in active TB.\textsuperscript{23,85,87} Treatments being investigated are in broad categories of therapeutics, from lipid-lowering agents, metformin, steroids, non-steroidal anti-inflammatories to immunomodulatory biologics such as vitamin A and D, microRNA and IL-2. Whilst some such as vitamin D and steroids have already established roles in TB treatment, others are being investigated in clinical trials. The theoretical advantage to the use of such therapies is that they affect the host’s immune responses (rather than the bacterium), therefore reducing the risk of antibiotic resistance development.\textsuperscript{88}

\subsection*{2.3.4 New vaccines for TB infection}

The BCG vaccine has been available for decades and is yet to be superseded, despite being acknowledged as being an ineffective vaccine in reducing TB incidence worldwide. A retrospective study showed vaccine effectiveness for pulmonary TB waning over time with 67\% at nine years, 63\% at 10–19 years and 50\% at 20–29 years.\textsuperscript{89} It does however provide cost-effective protection against disseminated, miliary and CNS tuberculosis in children.\textsuperscript{90} Over 15 vaccine candidates are currently in Phase 1–2 clinical trials and are priming vaccines, prime-boost vaccines or immunotherapeutic vaccines.\textsuperscript{84,89} A Phase 2b
clinical trial of one of these vaccines M72/AS01E, using a recombinant fusion protein derived from two immunogenic Mtb antigens combined with an adjuvant AS01 system, has shown promise of vaccine efficacy of 54% compared with placebo with a two year follow-up in a multi-nation, HIV-negative adult cohort.91

Improved and accessible biomarkers are needed to further enable efficient assessment of future vaccines and novel drugs/ regimens.

2.4 Mycobacteria and immunity

The majority of people infected with Mtb do not develop symptomatic disease.3,46,92 Whilst a few will clear infection when first exposed (Figure 2.4), most will reach a dynamic state between the host’s immune responses and the bacteria. The complex interactions that occur between the host and the pathogen are a combination of innate and adaptive immune responses, which are also influenced by the host’s and pathogen’s genetic makeup.5,4,3

When the bacilli enter the lung alveoli following aerosolisation, they are phagocyted by the local (i.e. alveolar) macrophages and dendritic cells. These cells carry the bacilli to local (and mediastinal) lymph nodes, whereby T cell responses to the infection are initiated. The preference of the aerosolised bacterium is the lower lung, where it has a niche without other commensal organisms which may induce microbicidal reactions.92,93 The initiation of this cascade leads to lymphocytes and macrophages arriving at the primary site of infection (i.e. lung) to form a granuloma.3,92 The granuloma has been historically deemed the hallmark of TB infection control, however as scientific knowledge changes, it is now believed to be a state of flux where it not only renders the bacterium a way to survive whilst avoiding the host’s immune response, but also a means to propagate and instigate further immune responses which can be both beneficial or detrimental to the host.3,4,92,93-95

Despite the crucial role innate mechanisms play in the recognition and defence of Mtb in humans, its primary role appears in assisting the development of an adaptive immune (or T cell) response and managing inflammatory responses, rather than curtailing infection.18 It is likely that evasion of aspects of the innate immune responses, at least in part, enables
the mycobacterium to evade adaptive immune responses which control the infection.\textsuperscript{6,96,97}

Lack of appropriate animal models further contribute to the challenges of determining the immunopathogenesis of Mtb infections in humans.\textsuperscript{3} Whilst non-human primates (such as Rhesus monkeys and Cynomolgus macaques) show ‘latent’ and ‘active TB’ infections when injected with Mtb strains, the mouse model generally does not develop necrotising, caseating granulomas. Zebra fish are another valuable animal model which do develop granulomas, but have other limitations.\textsuperscript{3,95,98-100}

For the purpose of this dissertation, the focus will particularly be on the role of the innate immune responses.

\subsection*{2.4.1 Innate immune responses in Mtb infection}

The importance of the innate immune system in TB infections has been evident for decades, with the accidental injection of Mtb instead of the BCG (bacillus Calmette-Guerin) vaccine in Germany in 1926 of newborn babies, of whom some became gravely ill and died, whilst a few remained unaffected clinically.\textsuperscript{101} The latter also suggests the presence of an inborn variation in susceptibility to TB. Another indicator of the importance of the innate immune response to Mtb is inferred from close-contact studies, where it’s estimated that only 20–30\% of people become infected, suggesting that the host is able to ward off infection.\textsuperscript{3,48,102}

Once inhaled into the lung alveoli, pathogen-associated molecular patterns (PAMPs) (such as glycolipids, lipoproteins and carbohydrates of the bacteria) are recognised by innate immune cells by pattern-recognition receptors (PRRs) and induce a cascade of reactions.\textsuperscript{3,6} Cells such as macrophages, neutrophils, dendritic cells (DCs) and natural killer (NK) cells are involved in this initial process. Recent research suggests that innate-like T cells (mucosal associated invariant T [MAIT] cells and natural killer T [NKT] cells) are also likely to play an important role.\textsuperscript{6,103,104} PRRs such as toll-like receptors (TLRs), NOD-like receptors (NLRs) and c-type lectin receptors (CLR) form the crucial innate signalling paths involved.\textsuperscript{28,3,6,98,105}
Together, macrophages, DCs, neutrophils and NK cells form the predominant innate responses to Mtb via innate immune signalling pathways to elicit cellular immune defences resulting in phagocytosis, apoptosis, autophagy and inflammasome activation\textsuperscript{6,101} (Figure 2.5). The role of autophagy in Mtb control remains controversial, whilst others such as pyroptosis and necroptosis are being investigated.\textsuperscript{99}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2_5.png}
\caption{Major immune cells, cellular functions and pathogen recognition receptors (PRRs) involved in innate immune responses to Mtb infections}
\end{figure}

\begin{flushright}
From Liu et al. 2017.\textsuperscript{6}
\end{flushright}

Once activated in the alveoli, it is postulated that the macrophages (and preceding them DCs) migrate out to draining lymph nodes seven to 10 days after initial infection. Part of this delay is controlled by the bacterium, which enables it to further replicate within the lung before antigen-specific T cell responses can develop and arrive at the site of infection.\textsuperscript{98,95,106} The latter occurs 18–20 days post initial infection, permitting establishment of the granuloma within the lung and bacterial replication during the intervening period.\textsuperscript{98,95} Macrophages have an integral role in granuloma formation (see Section 2.4.4) and can provide a niche for mycobacteria in early and chronic infection but can also be microbicidal (through production of oxygen and nitrogen components,
cytokine release and phagosome formation).\textsuperscript{6,101} Macrophages exist in different sites, can change their function and can be classified into two broad phenotypes: M1 (classical, pro-inflammatory) and M2 (non-classical, anti-inflammatory). The M1 subset is induced by microbial products (such as lipopolysaccharide -LPS) or cytokines (such as IFN\textsubscript{\gamma}, GM-CSF or TNF\textsubscript{\alpha}) and can lead to induction of antimicrobial effects and production of pro-inflammatory cytokines (such as TNF\textsubscript{\alpha}, IL-1\beta, IL-12-p40 and IL-23).\textsuperscript{97} In contrast, the M2 subset is associated with ‘repair’, have poor antigen-presenting capacity and associated with production of IL-10 and IL-4, suppressing cellular immune responses (i.e. Th1).\textsuperscript{6,101} The outcome of these contribute to the concept of ‘permissive macrophages’, whereby initial host responses are partially hampered by Mtb to enable replication but without causing complete necrosis or apoptosis resulting in significant damage to the host either. The overall consequence enables the host to continue to form adaptive but inadequate immune responses to completely control the infection, but similarly preventing the bacteria from causing significant damage to the host or overwhelming infection.\textsuperscript{6,98,93,95}

Neutrophils also play an important role. In close-contact studies, risk of Mtb infection was found to be inversely proportional to the neutrophil count in their blood and in-vitro depletion of neutrophils led to failure of Mtb growth control, suggestive of an antibacterial capacity of neutrophils.\textsuperscript{6,98,107,108,109} Neutrophils are also usually abundant in BAL sampling in patients with active disease.\textsuperscript{110} It is suggested that lack of recruitment to the site of infection can result in increased bacterial burden.\textsuperscript{93,111} It is possible that neutrophils can kill phagocytosed mycobacteria, as well through direct oxidative killing via NADPH oxidase-dependent mechanisms.\textsuperscript{111,93}

Transcriptional profiling studies suggest that neutrophils may also have a role in immune-regulation of Mtb through expression of programmed death ligand 1 (PD-L1).\textsuperscript{112} Another role suggested is that loss of autophagy-related gene 5 (Atg5) in neutrophils can result in sensitisation of these cells to Mtb (in mice models).\textsuperscript{99} Neutrophils also play an important role in the formation of granulomas, as well as facilitating the adaptive immune response by presentation of DCs to CD4+ T cells. Conversely, negative impact on the host can also occur through bacterial manipulation of neutrophils, as well as contributing to dissemination.\textsuperscript{6,113} Neutrophil necrosis can be induced by Mtb, but can prevent apoptosis
through virulence factors encoded on region-of difference (RD-1), possibly by ESAT-6 protein which is secreted through a mycobacterial secretion system (ESX1).\textsuperscript{114,115}

Along with evasion and impeding innate immune processes, other virulence factors enable the bacterium to survive host responses. The cell wall itself (see Section 2.2.1), facilitates the host-immune-response modulation. Its composition, including hydrophobic mycolic acids (up to 50\%) compromises entry of nutrients, but allows greater resistance to degradation from lysosomal enzymes. Other components which render the mycobacteria resistant to chemical damage, antimicrobials and dehydration include the arabinogalactan layer, phosphatidylinositol mannosides (PIMs), lipomannans (LMs), lipoarabinomannans (LAMs), mannosylated lipomannans (manLAMs) and tehalose dimycolate (TDM). These act as PAMPs and have varied effects. For example, manLAMs are an important virulence factor in Mtb and are heterogenous with several forms being found on different strains, associated with varied immunogenic effects.\textsuperscript{101,105} Recognition and outcome of the PAMPs by PRRs such as TLRs are described below (Section 2.4.1.1).

Vitamin D effects on human immune responses have been recognised for several decades and its role in tuberculosis infections has raised a great deal of interest in recent years. At least part of this is due to effects on innate immune cells such as macrophages through the induction of cathelicidin and autophagy.\textsuperscript{27,116} The association with TLRs and the vitamin D receptor (VDR) has also been shown to have significant implications and is discussed in Section 2.5.\textsuperscript{27,116,117}

### 2.4.1.1 Pattern recognition receptors (PRRs) in Mtb infection

The understanding of PRRs and their role in immune responses in tuberculosis has improved significantly in the last few decades. This has been fuelled by the appreciation of the dynamic nature of the innate immune responses and more recently the impact of these on the spectrum of tuberculosis infections. Host cells (including alveolar macrophages, neutrophils and DCs) contain multiple cell surface receptors and are activated when PAMPs are recognised (Figure 2.5 and 2.6).

For the purpose of this dissertation, the focus will be on TLRs and implication on TB infection and control.
2.4.1.1A Toll-like receptor signalling and function

Mammalian TLRs comprise a family of 13 transmembrane pattern recognition receptors. Ten TLRs have been identified in humans. TLRs are expressed predominantly on immune type cells such as macrophages, DCs, the spleen and peripheral blood leucocytes, as well as in organs such as the gastrointestinal tract and lungs.\textsuperscript{20,19} The expression of TLRs vary depending upon the site and type of cells.\textsuperscript{118,119} The intracellular domain of the glycoprotein receptor is a conserved region called toll/interleukin-1 receptor (TIR).\textsuperscript{20} The majority of TLRs occur across the plasma membrane, whilst a few are intracellular receptors across endosomal and lysosomal membranes (TLR3, 7, 8 and 9).\textsuperscript{120} TLR2, 4, 7, 8 and 9 are the best described in their recognition of Mtb.

After specific bacterial components are recognised and bound to the respective TLRs, a cascade of signalling pathways is triggered. The signal transduction pathway is summarised in Figure 2.6, binding first to CD14, TIR-adaptor protein (TIRAP) or myeloid differentiation primary response protein 88 (MyD88), instigating an association with IL-1 receptor-associated kinase 1/4/2 (IRA\textsubscript{K}1/IRA\textsubscript{K}4/IRA\textsubscript{K}2), then TNF-receptor-associated factor 6 (TRAF6) and mitogen activated protein kinase (MAPK) or nuclear transcription factor kappa B (NF\textsubscript{k}B). Downstream gene transcription leads to the production of pro-inflammatory cytokines such as TNF\textalpha, IL-12, IL-6, IL-8, macrophage inflammatory protein (MIP), and nitric oxide (NO) (Figure 2.6). These signalling pathways also lead to activation of Type 1 interferons (IFNs), and eventually commencement of the adaptive immune response (Section 2.4.2). TLR4 appears to work through CD14 or MyD88-adaptor-like protein (MAL) (rather than MyD88).\textsuperscript{121,97,20,19,122,123} The signalling pathway through TLR2 is likely to be important in maintaining the phagocytosed bacteria in a localised compartment.\textsuperscript{124} Whilst most TLR activity is through MyD88, animal studies have shown that MyD88-knockout mice were not highly susceptible to Mtb, suggesting other TLR2-TIR associated, MyD88-independent pathways.\textsuperscript{125}
**Figure 2.6: TLR pathway activation in response to Mtb in humans**

Main signal transduction and molecules involved highlighted in schematic form. Bacterial PAMPs associated with TLRs highlighted in blue rectangles; in red outlines are the eventual cytokine and inflammatory responses which can occur. (Well described signalling shown.)


TLR2 can exist as a heterodimer with TLR1 and 6 and is described as recognising key Mtb cell wall components, including LMs, man-LAMs and PIMs. TLR2 is thought to initiate the innate immune response by its positive effects on TNF production in macrophages. TLR2 knockout mice show abnormal granuloma formation, increased susceptibility to infection on exposure to high doses of Mtb and problems controlling chronic infection. In contrast, high TLR2 expression may result in secretion of anti-inflammatory cytokines and result in poorly controlled infection, as well as allowing the bacterium to survive in a dormant (or latent) state. Interestingly, Mtb antigens such as ESAT6, mycolic acids and lipoproteins have been associated with promotion of apoptosis through TLR2 activation, allowing for restriction or delay in onset of adaptive
immune responses. Activation and interaction with DCs can promote a Type 1 T-helper (Th1) response, as well as contributing to CD4+ T cell expansion. TLR2 ligands also induce activation of the VDR (see Section 2.5).

The role of TLR3 in tuberculosis is unclear and few studies have been undertaken. Limited data suggest that its role in infections is mainly related to viruses. A recent study in TLR3-/- mice infected with BCG reporting lower IL-10 production with potential implication for altered immune responses to Mtb and it has also been found on granulomas of lung tissue in patients with severe pulmonary TB.

TLR4 is activated by proteins including heat-shock protein (HSP) secreted by Mtb (Figure 2.6). TLR4 deficient macrophages infected with Mtb showed lower TNF production, whilst contrasting results have been shown in studies in mice of susceptibility to infection. TLR4 mutant mice infected with Mtb were found to have greater lung damaged infiltrates, as well as reduced capacity to produce Type1 IFN responses upon antigenic stimulation. Another study showed TLR4 agonists (along with NOD2) were found to increase memory CD4+ and CD8+ T cells, reduce Mtb burden in the lungs and reduce dose of antibiotics required for treatment. TLR4 signalling has been shown to be involved with induction of autophagy of macrophages in mice.

The role of TLR5 in tuberculosis is also unclear and has been reported in one study of granulomas in lung tissue of patients with severe pulmonary TB. The role of TLR5 in these patients was unclear with authors suggesting a ‘cooperative’ role with TLR1/2 and/or 9.

Intracellular TLR7 and 8 function are less well described in TB, but it has been shown that there is upregulation of TLR8 protein expression in macrophages after infection with BCG. TLR7 accompanies TLR8 in recognition of ligands and are phylogenetically similar. It is likely that TLR7 has a role in induction of autophagy. Most studies suggesting a link between TLR 7 and 8 arise recently from genetic association studies (see below).

Intracellular TLR9 recognises the CpG motifs in bacterial DNA, including in Mtb and studies have shown that Mtb-induced IL-12 release in DCs are TLR9 dependent. Animal models with TLR2/9 -/- double knockout mice suggest enhanced susceptibility to Mtb.
infection.\textsuperscript{143} It is important, however, to note there are conflicting reports in this area, including that triple knockout mice (TLR 2/4/9) had a milder disease phenotype than MyD88-deficient mice.\textsuperscript{144}

In addition to TLR2 existing as a heterodimer, it is also likely that different TLRs ‘cooperate’ to produce responses to mycobacteria, which can be both host beneficial and detrimental, similar to that to that of studies of individual TLRs.\textsuperscript{19,119,123,143}

**Human studies**

Human studies focussing on TLR expression and function in TB have been limited. The available literature on the subject area is presented in brief here.

Chang et al. (2006) found increased levels of mRNA encoding TLR1, 2, 4 and 6 on peripheral blood mononuclear cells (PBMCs) compared to a lower level in bronchoalveolar specimens in patients with active TB.\textsuperscript{145} The authors found stimulation of myelomonocytic cell lines infected with Mtb (H37Rv strain) with TLR-agonists led to increased levels of mRNA encoding TLRs, suggesting that there was a functional relationship between Mtb infection and TLR expression. The authors reported minimal alternation of TLR7 and TLR9 mRNA expression levels compared to controls. The comparison was to matched controls, rather than patients with latent infection.

A study by Fenhalls et al. (2003)\textsuperscript{119} found TLR1-5 and 9 expression in granulomas of resected lung tissue in nine patients who required lobectomy for haemoptysis. They found that TLR2 and TLR4 were co-expressed in a significant proportion of IL-4 positive granulomas. They also evaluated IL-4 levels in these samples and found a negative association with IL-4 and TLR2. A study in Taiwan comparing HIV negative patients with active TB to healthy controls found increased TLR2 expression on CD14+ monocytes at diagnosis, followed by a decline at two months of treatment.\textsuperscript{146} The authors also reported that a high TLR2 and IL-10 plasma levels initially were associated with shorter survival in their study.

Another study found elevated TLR2 and TLR4 expression (cell surface markers and mRNA expression) in PBMCs of patients with pulmonary TB, which was significantly higher than healthy controls with positive Mantoux tests (i.e. LTBI) and remained so
through active TB treatment. They also found that TLR2 cell surface expression increased through treatment on CD14+ monocytes, whilst there was an increase followed by a decrease on CD3+ lymphocytes. The authors also reported a reduction in TLR4 gene expression through antibiotic treatment, which wasn’t significant on cell surface expression. The authors also reported plasma cytokine measurements and gene expression of cytokines through TB treatment, finding that TNF and IFNγ levels were lower in LTBI compared to start of TB treatment, with a reduction in these levels through treatment.

A more recent study conducted in Mexico compared healthy controls (who had received BCG vaccination) with patients with pulmonary TB and found significantly higher levels of CD14+ monocytes in the latter group, with higher percentage of TLR2 expression and mean fluorescence intensity in this cell line. The authors also describe that although the percentage of TLR4+ cells was similar in the two groups, the group with active TB had a higher mean fluorescence intensity of TLR4+ monocytes. Function was also assessed, and the authors concluded that the death of monocytes in patients with TB was mediated by a TLR2 dependent pathway.

Another group examined soluble serum markers of TLR4 pathway in a group of HIV-negative patients with active TB (n = 19) and compared to a group of six with LTBI and reported a significant difference with higher levels of MD-2 and CD14 and/or LPS between the two groups. They also reported a return to levels similar to LTBI at end of treatment.

A comparison of cytokine measurements in TLR ligand stimulated neutrophils in 15 patients with PTB and 15 healthy donors (Mantoux results unknown) showed significantly higher levels MCP1, MIP1α, TNFα, IL-8 and IL-1β in those with active TB. Pro-inflammatory cytokines were highest in samples following TLR4 ligand stimulation.

A study reported elevated TLR2 expression on peripheral blood CD14+ monocytes and genetic variants of TLR2 in patients with pulmonary TB (n = 50) compared to healthy controls (n = 50).

Another study found higher TLR2 mRNA expression on PBMCs harvested in patients with adults with active TB, as well as close contacts with LTBI (QuantiFERON positive)
compared to close contacts without LTBI (QFT negative) and uninfected healthy volunteers.\textsuperscript{152} They also found that mRNA expression of Coronin-1 and Sp110 correlated with TLR2 mRNA expression, suggesting that TLR2 pathway may be involved in phagolysosome formation and apoptosis of monocytes in response.

A recent study in India evaluated IL-8 levels in TLR ligand stimulated supernatants patients with active TB, treated TB and healthy controls.\textsuperscript{153} The authors report significantly lower levels of IL-8 in patients with active TB (after TLR2, 4, 5 and 9 stimulation). They also describe an impaired TLR9 response in those who had active TB and treated TB infection.

\textit{Genetic association studies}

Though few studies have been undertaken to investigate TLRs in human Mtb infection in detail, genetic studies suggest that there are strong associations with susceptibility. These are mostly single nucleotide polymorphisms (SNPs) and have been reported in a range of ethnic groups from different countries, involving TLR1, 2, 4, 6, 7, 8 and 9. Overall, risk of susceptibility varied between ethnicities, but also between males and females, as well as type of TB disease.\textsuperscript{101,7,8,154,155,130,12,156,9} Several reports, including meta-analyses suggest potential protective association with certain SNPs, mainly with TLR1, TLR6 and TLR8.\textsuperscript{7,8,10,11,156,9} It is important to recognise that the different studies reported on a range of SNPs relevant to each TLR and genetic modelling analyses were variable.

TLR2 in different human populations have been investigated and results suggest correlations between particular gene polymorphisms and susceptibility to TB, including associations with disseminated disease and meningitis.\textsuperscript{157-161} Others have shown a higher ratio of TLR2 and TLR4 polymorphisms in patients with active TB compared to healthy controls.\textsuperscript{162} In contrast, commonly reported TLR2 polymorphisms were not found in a group of Indian patients compared to a control group.\textsuperscript{163} A study in Moroccan patients with TB suggested TLR2 and TLR4 interaction with certain VDR haplotypes may provide protection in pulmonary Mtb infections.\textsuperscript{164} Meta-analyses of TLR2 polymorphisms conclude a higher risk of TB in persons with polymorphisms, often with ethnic variation in risk.\textsuperscript{165}
Meta-analysis of seven TLR9 polymorphisms suggest an association with TB disease risk, with an increase or decrease susceptibility in different ethnic groups depending on the genotype.166,7,8,12

2.4.1.1B Non-TLR innate immune recognition receptors and effectors

Other non-TLR dependent pathways and PRRs are involved in the initial immune response in Mtb26,101,167 (see Figure 2.5). These include, but not limited to NOD-like receptors (NLRs) and C-type lectin receptors (CLR). They provide further complexity to the host-defence responses in TB infection. Similar to TLRs, they induce gene expression of inflammatory chemokines and cytokines following recognition of bacterial molecules.

CLRs are made up of over 17 groups of receptors including collectins, selectins, phagocytic receptors and proteoglycans. CLRs generally have at least one carbohydrate recognition domain, which can bind to carbohydrates, lipids, proteins and inorganic compounds.6,101 Complement receptors (CR3 in particular), integrins (such as DC-SIGN), dectin, along with mannose receptors (MRs) respond to lipoglycans, manLAM, carbohydrates and TDM (for example), to result in chemokine and cytokine production through various intracellular methods, including through NFκB activity. An example is that of the MR which is highly expressed on alveolar macrophages and binding of manLAM can result in production of anti-inflammatory cytokines IL-4 and IL-13, inhibition of IL-12 production and failure to activate oxidative responses. In contrast, binding of manLAM can also result in phagocytosis by macrophages, whilst binding to DC-SIGN (with TLR) can induce IL-10 production in infected DCs.6,101,105

NOD-like receptors (NLRs) are a family of intracellular proteins. These are an important part of the innate immune response which occurs when mycobacteria are phagocytosed into macrophages.6,101,105 Once recognised, they can induce inflammatory cytokines, Type 1 IFNs and inflammasome formation through NFκB activation, through an effector domain (caspase recruitment domain [CARD]).6,105 Importantly, whilst some of these occur with TLR activation, several effectors are independent, with a suggestion that Mtb can be associated with inducing some NLRs to enable persistent infection, rather than virulence.6,168 NLRs have been shown to have a role in autophagy in animal models of Mtb
infection and NLR gene polymorphisms have been associated with a genetic susceptibility to Mtb.$^{6,105}$

### 2.4.1.2 Immuno-phenotypic cell-type variations in tuberculosis

Recent studies have shown differences in monocyte and lymphocyte subsets in patients with Mtb disease and those with LTBI.$^{169-171}$ Veenstra et al. (2006) found correlates between peripheral white blood cell counts and early response to treatment, in particular high counts in a subset of NKT cells ($\text{CD3}^{\text{dim}}\text{CD56}^+$) were associated with earlier sputum clearance of Mtb. This study was undertaken in South Africa and examined patients longitudinally and were compared to healthy controls with LTBI.$^{169}$ The authors found that there was increased intracellular production of IL-4 amongst these cells, suggesting the possibility of ‘an intact immune system’.

NK cell role in mycobacterial infections is one that supports the human immune response through promoting macrophage activation, assisting DC antigen presentation, activation of $\gamma\delta$T cells and inhibition of regulatory T cells (Tregs).$^{172}$ Human studies have shown increased NK cell numbers in pleural fluid of TB patients as well as increased cytolytic activity of NK cells from TB patients when examined ex vivo.$^{173,174}$ NK subsets are also described as having differing functions as well as variation in expression of chemokine receptors and adhesion molecules.$^{173,175}$ ‘Trained immunity’ or adaptive characteristics of NK cells with increased IFN$\gamma$ production on re-exposure to same stimulus (as well as non-related microbial stimuli), has been described, including following BCG vaccination.$^{176}$

A study of NK cell subpopulations found significant differences between LBTI, patients with active TB disease and controls: in particular concluding that high expression of CD3-CD16-CD56+ NK cells alongside CD14+CD16+ monocytes may represent ‘resistance to developing TB’ amongst latently infected individuals.$^{170}$ However, there was no significant follow-up of patients to determine whether the ‘putative resistant’ group with LTBI remained disease free.

A prospective study done in The Gambia found significant differences in granulocytes and lymphocytes amongst patients with LTBI, active TB and household contacts.$^{171,177}$ The authors report lower levels of invariant NKT cells in patients with active TB, which were
restored to LTBI participants' levels after antimicrobial therapy. Conversely another group reported higher levels of NKT cells in patients with active pulmonary TB compared with controls. The authors also noted a greater frequency of IL-4 producing NKT cells compared to IFNγ producing NKT cells. The latter study was undertaken in Thailand, and it is not described whether controls were tested for possibility of Mtb infection (i.e. potential LTBI) which could confound results.

It is thought that innate lymphoid cells (MAIT cells, ßNKT and γδT cells) also act on mycobacterial antigens and contribute to infection limitation through both the innate and adaptive immune responses. The details of the mechanisms of the effector function of these cells are yet to be established, but likely work through the T cell receptor (TCR). A recent study of household contacts found differences in MAIT and γδT cells profiles of IGRA positive compared to those uninfected contacts, suggesting that robust innate T cell responses may be important in resistance to acquisition of Mtb infection.

Innate lymphoid V2γδT cells have been implicated in responding to Mtb antigens differentially between active and LTBI. MAIT cells are likely activated by Mtb on lung epithelial cells but their role is not fully established in primary TB infection, whilst studies have found lower levels of MAIT cells in patients with active TB compared to healthy controls and with impaired cytokine responses.

### 2.4.2 Adaptive immune responses in Mtb infection

Adaptive immunity is what eventually ‘controls’ Mtb infection, with the many caveats alluded to above regarding the fine balance between immune response and evasion. Detailed discussion of the adaptive immune mechanisms are beyond the limits of this dissertation, thus a summary follows.

Primarily the control of Mtb is dependent on Th1 type immunity. Humoral immunity is probably ineffective in controlling mycobacterial infections and Th2 humoral responses are associated with severe disseminated disease in many mycobacterial infections.

The ability of Mtb to cause persistent infection is ultimately due to its ability to establish chronic infection of macrophages. Inhibition of phagolysosomal formation allowing persistence of organisms in immature phagosomes, inhibition of apoptosis of infected
macrophages, and interference with intracellular signalling within the infected macrophage may all have roles in the ability of Mtb to subvert macrophage antibacterial activities.92,98

As mentioned in Section 2.4.1, bacteria enter macrophages and dendritic cells which then migrate to the lymph nodes and spleen. Effective T cell responses require the presence of live bacteria within the presenting DCs. Naïve, Mtb-specific T cell proliferate into predominantly Th1 and Th17 cells.94 They become activated following presentation of Mtb antigens to MHC Class I and II on the surface of antigen-presenting cells (APCs) and then migrate out to the primary area in the lungs, releasing TNF and IFNγ. This usually occurs 18–20 days later, giving the bacterium two weeks to develop within the lung.98 It has been shown that responses to virulent Mtb strains are different to attenuated strains.96 IFNγ can limit the survival of neutrophils and expression of IL-17. This is an important immune response: when the protective immune response is impaired at this stage, neutrophil accumulation continues and is associated with active disease, whereby tissue damage can result.98,110 It is likely that Mtb is able to induce IL-4 expression, which has been implicated in apoptosis inhibition, as well as promoting tissue damage with TNF. Cross-priming allows for induction with CD8+ T cells.96 Overall, a complex interplay between the host and Mtb results in the outcome of a latent but not eradicated infection, characterised by low level Mtb turnover within granulomas, wherein Th1 predominant mechanisms, TNF and nitric oxide contain infection.

The role of B cells (or humoral immunity) is still unclear in TB infection, but recent reports suggest an important role in the risk of development of reactivation, clearance of Mtb infection and responses to vaccines.123,183,184,185 Some authors have reported the use of enzyme-linked immune-assays to specific Mtb antigens as varying in different forms of TB infection and through treatment although these have not been consistent. The use of LAM-Ig subclass assays in patients with HIV-TB compared to those without HIV has been useful, but not in non-HIV or those with HIV without TB.123 A potential functional role of antibody responses in Mtb infection in vitro has been shown with IgG subclasses.123,183,185 Lu et al. described distinct Mtb-specific humoral responses between active TB and LTBI, where those with LTBI had enhanced antibody driven inflammasome activation, macrophage based intracellular Mtb killing and phagolysosomal maturation.185 Altered
humoral B-cell responses which 'normalise' following active TB therapy, as well as lower IgG levels against Mtb antigens in children with disseminated TB compared to those with localised infections have also been reported.\textsuperscript{123, 184} Interestingly, another recent report suggests IgA responses from B cells of persons exposed to Mtb could inhibit mycobacterial infection of epithelial cells in vitro.\textsuperscript{183, 186}

Further evaluation of such responses, including those from recent vaccine studies are likely to further improve understanding of Mtb immune effects in humans.\textsuperscript{183, 187}

### 2.4.3 Key cytokines and chemokines in immune responses to Mtb infection

Efficient Th1 immune response induction is crucial for Mtb defence and the classic cytokines involved are IL-2, IL-1α/β, IL-12, IFNγ, IL-6 and TNF.\textsuperscript{123} The latter three and chemokines of interest in this dissertation are discussed briefly below.

Cytokines are secreted by the interaction of Mtb and the immune system, of which tumour necrosis factor (TNF), interferon-gamma (IFNγ) and the interleukin-1 family of cytokines play a key role. TNF is important in many aspects of the host immune response to TB, from initial inflammatory to adaptive responses, and is produced by many different cell groups, including macrophages, lymphocytes, neutrophils and endothelial cells. One of its key roles is regulation of the inflammatory responses, including stimulation of IL-1 and IL-6 production. Its role in granuloma formation has been highlighted by TNF deficient mice shown to succumb earlier to Mtb.\textsuperscript{188} In recent years, the use of immune modulators such as TNF-inhibitors for a variety of inflammatory conditions has further reinforced its role in Mtb control in humans.\textsuperscript{123, 189, 190, 191} TNF also contributes to the formation of reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) by macrophages.

IFNγ is involved in macrophage activation and assisting MHC class II molecule expression, which consequently results in better antigen presentation to T cells.\textsuperscript{101} This cytokine is released by several T cells, including CD4+ and CD8+ cells, as well as macrophages and NK cells. This is essential in activation of phagocytes and antigen presentation. It also has an important role in promotion of cellular proliferation, cell adhesion and apoptosis. It
also induces production of RNIs and ROIs in macrophages. In addition, through its upregulation of TNFR, it further contributes to the production of ROIs and NO by innate immune cells. In animal models which are NO-synthase deficient or have significant IFN signalling pathway abnormalities, the mice are more prone to severe Mtb infections, fail to develop granulomas and have greater progression to infection.\textsuperscript{175,123} These IFN\(\gamma\) reactions in hosts to Mtb infection form the basis of currently available blood tests (IFN\(\gamma\)-release assays) for identification of persons with Mtb infection (see Section 2.6).

IL-6 effects can exert both pro- and anti-inflammatory responses, and it is an important cytokine in early inflammatory responses. It is produced early in response to Mtb infection and is also involved in the development of both T and B lymphocyte responses but is secreted from a variety of cells (including B and T lymphocytes, phagocytes and endothelial cells). A delayed IFN\(\gamma\) response has been described as occurring in mice without IL-6, as well as an increase in Mtb burden.\textsuperscript{192,193} IL-6 is also important in the IL-17 responses to Mtb infection in mice. Together with IL-12, it has been shown to be important in developing T lymphocyte responses and IFN\(\gamma\)-mediated responses to Mtb antigens (such as culture filtrate protein) following vaccination.\textsuperscript{123} IL-12 subunit (IL-12p40) production is promoted by the Mtb bacteria. IL-12 production is probably dependent on TLR9 and TLR2 in DCs and macrophages and results in the development of Th1 responses.\textsuperscript{142,123}

Chemokines have also been shown to play a key role in TB infection, including promotion of monocytes, DCs, activated macrophages, neutrophils and T lymphocytes in migrating to site of active disease (such as the bronchoalveolar space).\textsuperscript{123} Monocyte chemo-attractant protein 1 (MCP1, also known as CCL2) is a chemokine which has had much interest in TB infections and is now known to be a central activator of macrophages, including involvement through TLR signalling. Direct correlation of elevated MCP1 levels have been reported associated with disease severity. Importantly, MCP1 is also involved in recruitment of both Th1 and Th2 cells, and through IL-4 upregulation facilitates polarisation of naïve T cells to Th2.\textsuperscript{123}

The use of plasma cytokines and chemokines holds promise for future use as biomarkers in TB management, particularly unstimulated sampling in resource-poor settings as point of care tests.\textsuperscript{22} Several groups have used multiplex platforms to investigate the utility of
multiple cytokines/chemokines simultaneously. A study in China evaluated plasma cytokines via a multiplex kit in over 200 patients with confirmed TB, BCG vaccinated and non-tuberculous lung disease. The authors found that median levels of TNF, IL-6, IP-10, IFNγ and MIP-1β were significantly higher in patients with TB compared to those without. They also report differences between MIP-1β between different forms of TB disease (e.g. endobronchial, pleural and pulmonary TB). A large, multinational study evaluated patients with a chronic cough with 22 cytokines/chemokines in plasma including patients living with HIV, using a general discriminant analysis to determine the predictive ability of cytokine combinations. The authors report that whilst many analytes were deemed useful, a combination of seven markers (c-reactive protein, apolipoprotein-1 (ApoA-1), complement factor H (CFH), IFNγ, IP-10, serum amyloid A (SAA) and transthyretin was the best option, with estimated sensitivity and specificity of 87% and 85%, respectively. Importantly, the authors found no significant difference between those with HIV and without. Several other studies have also investigated other combinations of cytokines as biomarkers, including for prediction of fast versus slow responders to treatment or risk of reactivation.

A systematic review by Clifford et al. (2015) described that overall the most investigated cytokines were TNF, IFNγ, IL-6, IL-10 and IP-10 in unstimulated human plasma samples in those with active TB and several reported reductions in cytokines through treatment (or lower than controls), but other studies were contrasting. They also reviewed studies which included LTBI and active TB cytokine responses following stimulation (e.g. with ESAT6, PPD, CFP-10, BCG), where whilst results were also inconsistent, there was a suggestion overall of reduction in TNF, IL-4 and IL-10, and an increase in IL-12. It is critical to note to many of these studies were small and the comparative groups were varied, as were the cytokines measured and the methodologies utilised.

2.4.4 The granuloma

Whilst the granuloma is deemed the hallmark of TB infection and control in humans, it is now recognised as a dynamic interplay of host tissue reactions and Mtb persistence. Similarly, it is also recognised that there are different states of granulomas within a host, with varying implications for both the host and bacterium.
Granulomas also occur in other disorders, both infectious and non-infectious such as sarcoidosis and Wegener’s granulomatosis. Several granulomatous diseases previously thought to be non-infectious, have been shown to be a result of infections (e.g. Whipple’s disease and cat scratch disease). For purposes of this dissertation, the focus is on granulomas in tuberculosis.

The classic granuloma has a centre with different types of macrophages, such as foamy macrophages, multi-nucleated giant cells and epithelioid cells. In addition, other innate immune cells such as NK cells, DCs and neutrophils are present as well (Figure 2.7). Later on, B and T lymphocytes also contribute to the cellular make-up of granulomas. The composition of granulomas within an individual’s lung can vary, as has been described in post-mortem studies.

It has been shown that granuloma formation starts with innate immune responses alone in the zebrafish model, using *M. marinum*. Pro-inflammatory cytokines such as TNF and IFNγ are not essential at this stage, although these can accelerate proceedings. In mouse models, it has been shown that aerosol infection of mycobacteria can instigate granuloma formation. It has also been shown that certain Mtb virulence factors such as ESAT-6 can modulate the granulomatous response. In virulent mycobacteria, the region of difference (RD-1 locus) encodes a secretion system ESX-1, of which ESAT-6 is a substrate, which if deleted results in an attenuated infection with poor granuloma formation.

Macrophages fuse to form multi-nucleated giant cells or can change into foam cells characterised by significant lipid content. Through the delay in adaptive immune responses, Mtb is able to replicate in the macrophages in the lung, recruiting more macrophages whilst other already infected ones are phagocytosed. This enables further bacterial proliferation, with limited tissue damage. When the adaptive immune response commences, this accelerated macrophage recruitment plateaus and an equilibrium is reached. In the centre of the granuloma necrosis can occur, which can be caseating in nature and is ultimately associated with dissemination of the bacterium through aerosolisation via coughing. Caseum is a macroscopic description of ‘cheesy, white’ material which is found in caseous necrosis, resultant from cellular degradation and can be soft (high bacterial load) or hard (paucity of bacteria). It is postulated
that cord factor (also known as TDM) excreted by Mtb is implicated in sensitised persons (based on animal models), with a significant release of TNF as a consequence.\textsuperscript{92}

**Figure 2.7:** Schematic diagram showing the different cells and structure of a tuberculous granuloma

This schematic representation highlights the formation of a mature granuloma, with a necrotic centre of made up of epithelioid macrophages, bacteria and foam cells. Other inflammatory cells, such as neutrophils, dendritic cells and giant cells surround the centre and finally, there is an outer rim of lymphocytes (NK cells, T and B lymphocytes) as well as fibroblasts. This is one of several formations that tuberculous granulomas could be. Adapted from Ramakrishnan 2012.\textsuperscript{95}

2.4.5 The balance between immune control and evasion

The discussion above has highlighted the complex nature of TB infection in humans. Whilst it is believed to be a ‘balance’ between immune responses, the bacterium is able to harness aspects of the human reactions to its benefit. Recent scientific work strongly supports the notion of the bacterium’s ability to manipulate the host’s immune responses to its advantage. The initial delay of migration of DCs to draining lymph nodes and the arrival of sensitised T cells nearly two weeks later is important.\textsuperscript{98,93} Chronic TLR2 signalling is believed to contribute to downregulation of antigen presentation to MHCII and elaboration of immunosuppressive cytokines, resulting in continued survival of Mtb in macrophages.\textsuperscript{97} Mtb can inhibit phagosome-lysosome fusion and consequently
through IFNγ limit autophagy.\textsuperscript{203} The manipulation of inflammation resulting in necrosis rather than apoptosis (mediated through eicosanoids LXA4 and PGE2) is important.\textsuperscript{96,203} Studies have also shown the impact of different strains on the immune responses. An example of using adaptive immunity to its advantage is enabling an adequate CD4+ T cell response, which allows the bacterium to cause progressive disease in the lung whilst limiting disseminated infection and therefore keeping the host alive.\textsuperscript{98,93} These are just a few examples of the intricacies of the interactions that make TB immunopathogenesis incompletely understood.

### 2.5 Vitamin D and its multifaceted role in mycobacterial infections

The role of vitamin D in human immune responses has been known for decades. Vitamin D was used in the treatment of pulmonary TB in the pre-antibiotic era.\textsuperscript{204} In recent years, renewed interest in the non-classical role of vitamin D has advanced scientific understanding of its role in immune responses. In this section I will focus on the role of vitamin D on innate immune responses that are relevant to TB infections, which through VDR signalling contributes to induction of autophagy, release of cathelicidin and phagolysosomal fusion.\textsuperscript{13,116,205,206}

Vitamin D is obtained predominantly from the action of sunlight inducing 7-dehydrocholesterol conversion in the skin to produce vitamin D3 (colecalciferol) and to a lesser extent through dietary sources. This chemical is subsequently hydroxylated to 25-hydroxy vitamin D (25(OH)D), the major circulating form, and then to the active form 1,25 dihydroxy-vitamin D3 (1,25D3) in the liver and further in the kidneys.\textsuperscript{13} Whilst its main role is in calcium absorption and bone mineralisation, it has pleiotropic effects on immune responses, as well as chronic illness.\textsuperscript{205,207,206} Multiple immunomodulatory effects of vitamin D have been described, including effects on both innate and adaptive immunity.\textsuperscript{204,116,117,208,205,206} It is likely that the immune modulation is not simply from vitamin D alone, but also from the effects of ultra-violet radiation on skin.\textsuperscript{209,210} The role of vitamin D has been further suggested by the seasonality of Mtb infections noted in studies.\textsuperscript{204,211} Vitamin D deficiency and vitamin D receptor (VDR) polymorphisms have been linked to tuberculosis susceptibility.\textsuperscript{29,28,212}
Monocyte-macrophage cells from vitamin D deficient subjects show impaired phagocytic killing of mycobacteria, an effect reversed by incubation with vitamin D. A pilot study pre and post-vitamin D supplementation in healthy adult volunteers found an increase in TLR2 expression post supplementation, but not TLR4. The authors also reported lower levels of TNFand IL-6 levels in participants who had replete vitamin D levels post supplementation following ex vivo TLR2 and TLR4 ligand stimulation of PBMCs, suggesting that despite no significant effect on TLR4 expression, it is possible that vitamin D could modulate secretion of pro-inflammatory cytokines. Similar findings were reported in human monocytes supplemented with incremental vitamin D doses, where a dose dependent decrease in cytokine production was reported. Whilst the role of TLR1, 2 and 4 and vitamin D have been investigated, the information of the role of TLR7 and 9 are limited in this area. A study has reported vitamin D modulation of TLR7 expression in relation to liver tumour pathogenesis, whilst TLR9 is considered to be downregulated in inflammatory disorders such as colitis. A study has also reported reduced TLR7 expression in immune cells with increased serum vitamin D levels.

Vitamin D is required for TLR stimulated production of cathelicidin, an antimicrobial peptide with anti-mycobacterial properties (Figure 2.8). Vitamin D also directly contributes to transcription of the gene encoding production of cathelicidin (LL-37), which can consequently induce autophagy. Importantly, a retrospective study showed that low serum vitamin D (25(OH)D) in a cohort of patients with active TB did not correlate with low serum LL-37 levels, suggesting variations in disease state (and therefore potential other cellular mechanisms underlying the discrepancy).

Multiple other immunomodulatory effects of vitamin D have been described, particularly inhibition of T cell responses and inhibition of dendritic cell maturation and functioning, as well as the promotion of the development of regulatory T cells. Several of these aspects are likely related to cytokine production. The 'antimicrobial' role of vitamin D may be in part due to its promotion of autophagy.

Recently, a randomised study in the United Kingdom (UK) of supplementation with high dose vitamin D in patients with active TB showed faster sputum conversion in patients with specific VDR polymorphisms. Another study in Georgia reported no significant effect on sputum conversion in a double-blind clinical trial with high dose vitamin D.
supplementation over 16 weeks (or placebo), along with standard antibiotic therapy in
patients with pulmonary TB.\textsuperscript{220} Epidemiologically, vitamin D deficiency is associated with
both the development of active TB and the acquisition of infection.\textsuperscript{14,15,212} A double-blind, 
randomised control trial where patients who were recent contacts of a person diagnosed
with active TB were given placebo or mega dose of vitamin D found that vitamin D
supplementation resulted in greater restriction of BCG-\textit{lux} luminescence, whilst not
affecting antigen-stimulated \textit{IFN}\gamma responses (measured via IGRA), suggesting that
vitamin D can enhance immunity to mycobacteria independent of \textit{IFN}\gamma cytokine
responses.\textsuperscript{221}

![Figure 2.8: Vitamin D–dependent and TLR associated effects in human innate immune
responses to \textit{Mtb} infections](image)

Shown schematically are the key well-described mechanisms of vitamin D associated \textit{Mtb} effects,
including activation through TLR1 and 2 dependent, and cathelicidin dependent, pathways. Also shown is
the cathelicidin dependent role of autophagy in mycobacterial death in \textit{Mtb} infections.

A recent meta-analysis concluded that vitamin D deficiency was associated with a risk of
tuberculosis rather than being a consequence of \textit{Mtb} infection, and that deficiency was
associated with a greater risk of developing active TB in those with LTBI who were
household contacts.\textsuperscript{13} Authors found significantly lower vitamin D levels in HIV-TB co-
infectected patients (receiving anti-retroviral therapy) who developed TB-immune
reconstitution syndrome (TB-IRIS) compared to those who didn’t develop TB-IRIS. Interestingly, the authors also reported that vitamin D deficiency was not associated with an increased risk of TB in African HIV-infected patients (4 studies included).¹³

Current laboratory measurements are of the major circulating form, 25(OH)D, and recommendations of supplementation relate to calcium and bone homeostasis. Most guidelines and definitions of ‘deficient’ and ‘replete’ levels may not necessarily apply to adequate innate immune mechanism function for TB.²⁰⁵,²²²

2.6 Interferon-gamma release assays (IGRAs)

Recently, the WHO approved updated IGRAs to be used in resource poor countries in diagnosing and managing contacts with LTBI as a means to reduce the worldwide burden of TB.¹,²,²²³ IGRAs and TST are surrogate markers of Mtb infection, measuring cellular immune responses to Mtb antigen sensitisation in an individual, whilst not differentiating between remote or recent exposure and infection.⁵⁷,⁵⁸

2.6.1 Interferon-gamma release assays: the background¹

Currently the two commercially available IGRAs are QFT / QFT-Plus and T-SPOT. They both assess Mtb-specific T cell responses by measuring IFNγ levels through ex vivo stimulation of peripheral blood T cells with Mtb antigens. QFT measures IFNγ using ELISA, whilst T-SPOT.TB uses an enzyme-linked immunospot (ELISPOT) technique. QFT uses peptides eliciting responses of CD4⁺ T cells to ESAT-6, CFP-10 and TB 7.7, whilst T-SPOT uses Mtb antigens representing ESAT-6 and CFP-10. The new changes to QFT-Plus are outlined below.²²⁴-²²⁶

Currently available IGRA sensitivity and specificity profiles provided by the respective companies are summarised in Table 2.1 and Table 2.2.

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¹ A Medline search from 2000 until 15 April 2018 was undertaken: all appropriate reviews and research articles in English were examined and further articles deemed necessary for completeness were accessed and included in the literature review.
Table 2.1: Sensitivity and specificity results of currently commercially available IGRAs

<table>
<thead>
<tr>
<th>IGRA test</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Indeterminates / invalid tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-SPOT.TB</td>
<td>95.6 (91.6–98.1)</td>
<td>97.1 (94.5–98.7)</td>
<td>3.4%</td>
</tr>
<tr>
<td>QFT-GIT</td>
<td>89 (83-93)</td>
<td>99.1 (98–100)</td>
<td>1.3%</td>
</tr>
<tr>
<td>QFT-Plus</td>
<td>95.3 (84–98.3)</td>
<td>97.6 (95.6–98.8)</td>
<td>1%</td>
</tr>
</tbody>
</table>


Table 2.2: Sensitivity and specificity results of the respective antigen tubes in the new QFT-Plus

<table>
<thead>
<tr>
<th>QFT-Plus antigen tube</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB1</td>
<td>93.6 (88.8–96.7)</td>
<td>98.8 (97.2–99.6)</td>
</tr>
<tr>
<td>TB2</td>
<td>94.8 (90.3-97.6)</td>
<td>97.6 (95.6–98.8)</td>
</tr>
</tbody>
</table>

2.6.1.2  IGRA versus TST

A recent systematic review and a meta-analysis by Auguste et al. (2017) compared IGRAs to TST in ability to identify those at risk of progression to active TB from LTBI in three high risk groups: children, those recently resettled from countries of high TB burden and immunocompromised hosts.54 They reported on the strength of association between the tests and cumulative incidence ratios; they found there was no significant difference in IGRA (QFT-GIT) and TST in children (two studies), whilst two other studies in children suggested IGRA (QFT-GIT) was better in identifying LTBI. In immunocompromised hosts no significant difference was found (T-SPOT.TB), whilst in recent arrivals findings were inconsistent between two studies. Overall, through the analysis of 17 studies, they concluded that there was a paucity of prospective research evaluating LTBI progression.54

In another systematic review, Lu et al. (2016) analysed 17 studies comparing TST and IGRAs in TB diagnosis (not necessarily differentiating active TB from LTBI). Of these, nine used QFT-GIT and 12 used T-SPOT.TB.56 The authors concluded that IGRAs were better
than TST in TB diagnosis, but neither of the two IGRAs showed stability in TB diagnosis, which is reflected in previous systematic reviews and meta-analyses.\textsuperscript{56,227,228,58}

A series of studies in Singapore comparing T-SPOT to QFT-GIT in over 200 patients with culture confirmed pulmonary TB reported poor agreement between the two tests, with the former sensitivity of 94.1\% and 83.0\% within two weeks of starting TB treatment, also reporting that whilst levels in both tests fell through treatment course, it was inadequate for treatment monitoring.\textsuperscript{229,230}

An advantage of IGRA over TST is less cross-reactivity with non-tuberculous mycobacterial infections, with the exception of \textit{M. kansasii}, \textit{M. szulgai} and \textit{M. marinum}.\textsuperscript{228,225,231}

\subsection*{2.6.1.2 IGRAs in LTBI and active TB}

The role of IGRAs in diagnosis of active TB is still unclear; a recent position statement by IDSA/CDC does not recommend IGRA in the diagnostic algorithm of active TB.\textsuperscript{232} Several systematic reviews and meta-analyses assessing IGRA in active TB conclude that the variability in results are too great, and the sensitivity and specificity too low for their use.\textsuperscript{57,58,227}

The sources of IGRA variability are many: manufacturing, collection methods, transportation effects, analytical imprecision in the laboratory to immunological effects on the results on both IGRAs.\textsuperscript{233,55} These are discussed in detail in a review by Banaei, Gaur and Pai (2016).\textsuperscript{233} The clinical impact of potential points of variability are suggested in the rates of IGRA conversion and reversion.\textsuperscript{58,234} T-SPOT.TB has been found to be more sensitive than QFT-GIT in extra-pulmonary samples and holds promise for diagnosis in cases where traditional microbiological methods are initially negative (i.e. acid fast bacilli smear, Mtb PCR), but it is still not recommended.\textsuperscript{227,232,235}

The role of IGRA testing as anti-tuberculous treatment monitoring has also been investigated, with a suggestion that there is an overall quantitative decline in IFN\textgamma{} levels. However, two recent systematic reviews have concluded that there was inadequate change and large inter-patient variability for IGRAs to be used in treatment monitoring.\textsuperscript{230,236,237}
2.6.1.3 IGRAs in special groups

Ndzi et al. (2016) summarised that immunosuppression, in the form of advanced malignancy, advanced HIV and other forms of medical immunosuppression can affect QFT results, including increased rates of indeterminate results, or false negative results due to reduced IFNγ production.\textsuperscript{237} A systematic review and meta-analysis of IGRAs in TB diagnosis in HIV infected adults concluded that these tests are sub-optimal.\textsuperscript{238,239,240}

Paediatric patients form an important group in whom diagnosis of active TB can be challenging, due to the pauci-bacillary nature.\textsuperscript{241} A recent study suggested that whilst overall median IFNγ responses in children were not significant, when categorised into age groups (< 5 or > 5 years old), those with active TB had a significantly higher IFNγ response than the children with LTBI.\textsuperscript{242} IGRAs are considered to be highly specific in childhood TB, but not considered to provide greater sensitivity than TST in this group.\textsuperscript{243,241,244}

A report of lower IFNγ response was reported in patients with diabetes, however 44% of those patients with active TB also had HIV, whilst only 9% of the non-TB controls were HIV-infected.\textsuperscript{245}

IGRA is considered an appropriate test in the diagnosis of LTBI in pregnancy, as it is associated with improved adherence (single visit only) than TST and the results do not appear to be affected by pregnancy.\textsuperscript{246}

2.6.1.4 CD4+/CD8+ T cell signatures in immune responses to TB infection

Several studies have described Mtb-specific CD8+ cell responses in patients with Mtb infection (both active TB and LTBI).\textsuperscript{247,248} This has been shown in HIV infected and uninfected individuals.\textsuperscript{249,250,251}

Whilst CD4+ T cells recognise antigen presented to MHC-II expressing APCs, this can be intra or extracellular. It is thought that conversely CD8+ T cells recognise antigens from an intracellular compartment, and therefore may represent responses to burden infection or microbiological load.\textsuperscript{252,253,254} Recent contacts and patients with active TB were found to have higher Mtb-specific CD8+ T cell based IFNγ responses when compared to HCW and BCG-positive healthy controls.\textsuperscript{255} A reduction in CD8+ T cells
responses were also reported through anti-tuberculous treatment\cite{253,254} although this was not consistent.\cite{256} Inclusion of peptides eliciting such CD8+ T cell responses form the basis of the QFT-Plus test, with a reported improvement in the test's sensitivity.\cite{257}

### 2.6.2 QFT-Plus: the next generation of QuantiFERON-TB

The newer QFT is the fourth generation of the QuantiFERON test for tuberculosis. QFT-Plus measures IFNγ levels using peptides of different lengths associating with MHC Class I and II molecules. The new test includes altered peptide lengths of ESAT-6 and CFP-10 to associate with CD4+/MHC-II and CD8+/MHC-I T cells.\cite{226,225}

The published literature involving QFT-Plus is detailed in Table 2.3. Overall, where applicable, authors report a high agreement between QFT, QFT-Plus and/or T-SPOT.TB.\cite{258,259,260,261,262} Whilst most reported similar or improved sensitivity of the newer test in people living with HIV and/or immunocompromised, they also reported lower overall IFNγ measurements in the newer QFT-Plus, whilst one study reported higher levels.\cite{263,257,264}
Table 2.3: Summary of published reports of QuantiFERON-TB Gold Plus research

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Study type and group(s)</th>
<th>TB form (active TB, LTBI)</th>
<th>Numbers (total samples)</th>
<th>Age</th>
<th>Gender</th>
<th>Overall results</th>
<th>Other statistical analyses</th>
<th>Comments</th>
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<tr>
<td>Barcellini et al. 2016&lt;sup&gt;265&lt;/sup&gt;</td>
<td>Cross-sectional, multi-centre Adults (5 HIV infected with active TB, 5 malignancy/steroids with active TB) QFT-Plus (QFT in 73)</td>
<td>Active TB Healthy controls</td>
<td>119 106</td>
<td>NA</td>
<td>Male 61%</td>
<td>Specificity of QFT-Plus 97.17% (95% CI 92.01–99.03%) Estimated sensitivity 88% (all patients) 88.79% (CI 81.43–93.47% with immune-compromised patients excluded)</td>
<td>TB2 IFNγ levels significantly higher in active TB: 2.88 (1–7.89) than in TB1: 2.09 (0.83–6.52) (p = 0.0002 Wilcoxon signed-rank test)</td>
<td>Authors report surrogate CD8+ T cell responses were higher in patients with smear positive PTB, compared to smear negative PTB (TB2-TB1), p = 0.0135 Mann-Whitney test</td>
</tr>
<tr>
<td>Barcellini et al. 2016&lt;sup&gt;268&lt;/sup&gt;</td>
<td>Cross-sectional, prospective Adult contacts of PTB cases with recent TST +ve result QFT &amp; QFT-Plus</td>
<td>119 adults, TST positive, recent contacts</td>
<td>119 Median age 38 (30–79)</td>
<td>Male 52.9%</td>
<td>Agreement between QFT &amp; QFT-Plus high Cohen’s k = 0.8 (95% CI 0.69–0.91)</td>
<td>Univariate logistic regression of difference between TB2 and TB1 &gt; 0.6 IU/mL</td>
<td>Authors suggest QFT-Plus may be a surrogate measure of recent TB exposure when TB2-TB1 &gt; 0.6 IU/mL</td>
<td></td>
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<tr>
<td>Gallagher et al. 2016&lt;sup&gt;266&lt;/sup&gt;</td>
<td>2 adults, 5 samples each</td>
<td>1-Mtb infection 2-No Mtb infection</td>
<td>10 NA NA</td>
<td>Coefficient of variation: QFT 18.25% QFT-Plus 9.60%</td>
<td></td>
<td>In-house validation of QFT-Plus</td>
<td></td>
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</tr>
<tr>
<td>Hoffman et al. 2016&lt;sup&gt;267&lt;/sup&gt;</td>
<td>Single centre in Germany 163 adults for whom clinical testing required QFT, QFT-Plus</td>
<td>Active TB No TB HCW</td>
<td>57 29 77</td>
<td>NA NA</td>
<td>Sensitivity &amp; specificity: QFT-Plus 69.5% (95% CI 81–97), 84.2% (95% CI 66–102) QFT 89.5 (95% CI 81–97), 82.4 (95% CI 62–102)</td>
<td>Sig higher IFNγ in QFT than TB1 (QFT 4.67 ± 3.25 IU/mL TB1 3.1 ± 3.2 IU/mL) two sided t-test p = 0.007</td>
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<tr>
<td>Hofland et al. 2018&lt;sup&gt;268&lt;/sup&gt;</td>
<td>Multi-centre study in Netherlands Adults with active TB, LTBI &amp; healthy controls (HIV excluded) QFT-Plus</td>
<td>Active TB LTBI –not treated LTBI treated Healthy controls</td>
<td>80 (20 in each group) 35.8 ± 13.3 36.0 ± 12.7 36.8 ± 11.0</td>
<td>Male 55% Male 40% Male 35% Male 40%</td>
<td>QFT-Plus does not discriminate between active TB &amp; LTBI</td>
<td>Median levels of IFNγ were significantly higher in TB2 in active TB than untreated LTBI</td>
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<tr>
<td>Author, Year</td>
<td>Study type and group(s)</td>
<td>TB form (active TB, LTBI)</td>
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<tr>
<td>Kamada &amp; Amishima 2017</td>
<td>Single centre in Japan, serial QFT-Plus through anti-TB treatment Adults QFT-Plus</td>
<td>Active TB (immune-competent)</td>
<td>38</td>
<td>66 ± 19.3 (SD)</td>
<td>Male 58%</td>
<td>&lt; 15% had test reversion following TB therapy IFNγ responses were higher in TB2 &gt; TB1 throughout Quantitative sig differences were found between baseline and 3-month timepoint in both TB1 &amp; TB2</td>
<td>Surrogate significantly lower CD8+ T cell response (TB2-TB1 IFNγ levels) found at end of treatment</td>
<td>Authors suggest QFT-Plus may be useful in TB treatment monitoring (pending further studies)</td>
</tr>
<tr>
<td>Knierer et al. 2017</td>
<td>41 adults Serial weekly sampling QFT &amp; QFT-Plus</td>
<td>LTBI, born in high endemicity country or previous TB contact</td>
<td>163</td>
<td>20–36</td>
<td>Male 46.3%</td>
<td>Agreement between QFT &amp; QFT-Plus: 95.1% (κ = 0.89) QFT reversion rate 3.2% (1/31, 95% CI 0.2–18.5), conversion 2.2 (2/91, 95% CI 0.4–8.5) QFT-Plus reversion rate 6.9% (2/29 95% CI 1.2–24.2), conversion rate 4.3% (4/93, 95% CI 1.4–11.3%)</td>
<td>Conversion and reversion rates, IGRA variability</td>
<td></td>
</tr>
<tr>
<td>König Walles et al. 2018</td>
<td>Cohort study of pregnant women in a high endemicity TB &amp; HIV country, unknown TB status (829) QFT-Plus</td>
<td>HIV negative LTBI</td>
<td>780</td>
<td>49</td>
<td>Female (100%)</td>
<td>High agreement between TB1 and TB2 (κ = 0.92) IFNγ responses sig lower in HIV positive compared with HIV negative pregnant women: TB1 0.47 vs 2.16 (IU/mL) p &lt; 0.001 and TB2 0.49 vs 2.24 (IU/mL) p &lt; 0.001, respectively Borderline results in range 0.20–0.70 (IU/mL) and mitogen responses through the different trimesters (Kruskal–Wallis test)</td>
<td>Authors suggest a lower cut-off for evaluating QFT-Plus results, particularly pregnant HIV positive women (0.15 IU/mL)</td>
<td></td>
</tr>
<tr>
<td>Author, Year</td>
<td>Study type and group(s)</td>
<td>TB form (active TB, LTBI)</td>
<td>Numbers (total samples)</td>
<td>Age</td>
<td>Gender</td>
<td>Overall results</td>
<td>Other statistical analyses</td>
<td>Comments</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
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</tr>
<tr>
<td>Losi et al. 2015&lt;sup&gt;267&lt;/sup&gt;</td>
<td>Multi-centre, cross-sectional Adults with active TB (80) &amp; healthy controls (low &amp; medium endemicity countries) (290) QFT &amp; QFT-Six</td>
<td>Active TB &amp; HIV positive 20  Active TB, HIV uninfected 65  Healthy controls 290</td>
<td>38 ± 11.5 (mean ± SD)</td>
<td>Male 65%  Male 60%  Male 42%</td>
<td>Increased IFNγ levels compared HIV neg: QFT 3.88 (0.88–10), QFT-Six 5.01 (1.20–10) p &lt; 0.01; HIV pos: QFT 0.82 (0.22–2.10), QFT-Six 0.99 (0.39–2.26) p &lt; 0.03 (Wilcoxon signed-rank test)</td>
<td>Coefficient of variation 51% (HIV neg), 56% (HIV pos) compared to QFT (14%) of increased IFNγ levels (p &lt; 0.001)</td>
<td>Flow cytometry to evaluate specific CD4+ and CD8+ antigen-specific responses in a small subgroup</td>
<td></td>
</tr>
<tr>
<td>Moon et al. 2017&lt;sup&gt;268&lt;/sup&gt;</td>
<td>Cross-sectional study Adult healthcare workers in low-incidence setting QFT &amp; QFT-Plus</td>
<td>LTBI 989</td>
<td>35 (med. 28–44)</td>
<td>Female 41%</td>
<td>Agreement overall was high; Cohen’s κ = 0.8</td>
<td>Positivity rate QFT 2.1% (95% CI 1.0–3.2); QFT-Plus 3.0 (95% CI 1.7–4.3)</td>
<td>Most discordant results occurred between IFNγ levels of 0.2–0.7 IU/mL. Authors suggest TB1 AND TB2 to be positive for QFT-Plus = positive</td>
<td></td>
</tr>
<tr>
<td>Petruccioli et al. 2017&lt;sup&gt;261&lt;/sup&gt;</td>
<td>Cross-sectional Adult in Italy 179 total</td>
<td>Active TB 69  LTBI 58  Treated TB 33  Healthy volunteers 19</td>
<td>44 ± 18 (mean ± SD)</td>
<td>Female 45%</td>
<td>Agreement between QFT &amp; QFT-Plus was 95% (Cohen’s κ = 0.828)</td>
<td>Sig difference between TB and LTBI in TB1 vs TB2 responders</td>
<td>QFT-Plus: median IFNγ in active TB in TB1 and TB2 was lower than LTBI (p = 0.0007 &amp; p = 0.003). Similar trend in QFT, but not significant</td>
<td></td>
</tr>
<tr>
<td>Pieterman et al. 2018&lt;sup&gt;262&lt;/sup&gt;</td>
<td>Multi-centre, comparative laboratory study in Belgium &amp; Netherlands Adults Comparing QFT to QFT-Plus</td>
<td>Consecutive clinical blood sampling 1031</td>
<td>44 ± 18 (mean ± SD)</td>
<td>Female 40%</td>
<td>Agreement between QFT &amp; QFT-Plus was 95% (Cohen’s κ = 0.828)</td>
<td>Significantly higher IFNγ release in TB2 than TB2 in recent exposure / contact screening &gt; 0.6 IU/mL (p = 0.029)</td>
<td>41 discordant results, further 9 indeterminate results (in one or other of QFT or QFT-Plus)</td>
<td></td>
</tr>
<tr>
<td>Author, Year</td>
<td>Study type and group(s)</td>
<td>TB form (active TB, LTBI)</td>
<td>Numbers (total samples)</td>
<td>Age</td>
<td>Gender</td>
<td>Overall results</td>
<td>Other statistical analyses</td>
<td>Comments</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------</td>
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</tr>
<tr>
<td>Takasaki et al. 2018&lt;sup&gt;279&lt;/sup&gt;</td>
<td>Single centre in Japan Adults Active TB and healthy controls QFT-Plus compared to QFT and T-SPOT</td>
<td>Active TB Healthy controls</td>
<td>99 106</td>
<td>42 (med. 29–55 IQR) 20 (med. 20–21 IQR)</td>
<td>Male 65% Male 18.6%</td>
<td>Sensitivity &amp; specificity (95% CI): QFT-Plus: 99 (0.945–0.998), 98.1 (0.934–0.995) QFT: 98 (0.929–0.994), 99.1 (0.949–0.998) T-SPOT: 96.9 (0.914–0.994), 98.1 (0.934–0.998)</td>
<td></td>
<td>Authors suggest severe diabetes mellitus may contribute to negative test results</td>
</tr>
<tr>
<td>Telisinghe et al. 2017&lt;sup&gt;264&lt;/sup&gt;</td>
<td>Single centre Adults with active pulmonary TB in Zambia QFT-Plus</td>
<td>Active TB – HIV positive 68/108 – previous TB 18/104</td>
<td>108</td>
<td>32 (med. 27–38 IQR)</td>
<td>Male 73.1%</td>
<td>QFT-Plus sensitivity 83% (95% CI 75–90) Sensitivity not significantly different in those with HIV and without, except if CD4+ count &lt; 100 cells μL (p = 0.02) Median IFNγ levels were higher in TB2 than TB1 (irrespective of HIV status)</td>
<td>CD4+ &lt; 100 cells/μL or being underweight were associated with decreased odds of positive QFT-Plus results (OR 0.15, 95% CI 0.02–0.96 p = 0.05 and OR 0.27 95% CI 0.08–0.91 p = 0.02, respectively)</td>
<td></td>
</tr>
<tr>
<td>Yi et al. 2016&lt;sup&gt;271&lt;/sup&gt;</td>
<td>Multi-centre study in Japan Adults Active TB &amp; and healthy controls with low MtB infections risk</td>
<td>Healthy controls Active TB (immune-suppressed included, total 9)</td>
<td>212 162</td>
<td>20 (med. 9–21 IQR) 59 (med. 19–21 IQR)</td>
<td>Male 49.5% Male 79.6%</td>
<td>QFT-Plus lower sensitivity (91.1%) compared to QFT (96.2%) with standard cut-off of IFNγ of 0.35 IU/mL (p = 0.008, McNemar test) ROC comparing QFT vs QFT-Plus Trend in change of IFNγ assessed by Jonckheere–Terpstra test</td>
<td></td>
<td>Authors suggest an altered/cower cut-off for QFT-Plus (to 0.168 IU/mL)</td>
</tr>
</tbody>
</table>
2.7 Clinical challenges in tuberculosis

2.7.1 Latent versus active TB

Given that only 10% of people infected with TB develop active disease, determining the immune correlates that predict such progression is important. Such an assay (biomarker) could predict those most at risk and allow appropriate interventions. Although the recent development of interferon-gamma release assays (IGRAs) with improved specificity and sensitivity compared to the tuberculin skin test (TST) has improved the diagnosis of LTBI, they are still inadequate in several groups, including medically immunosuppressed, elderly, people living with HIV and paediatric populations.²⁷²

It is known that development of active TB is greatest in the first two years post exposure and also in the first five years post resettlement from a country of high endemicity to a low prevalence country.¹ The reason for the discrepancy between the two is uncertain, but likely influenced by environment changes and physical stressors.

Prospective studies to serially examine immune responses in patients with LTBI until development of active TB are not practical because of the magnitude of patient numbers needed to be monitored.

2.7.2 ‘Cure’ and vaccination

Although the current anti-tuberculous drugs are excellent in their effectiveness against the bacterium, the prolonged duration of therapy with multiple agents results in at least moderate non-compliance, making curative treatment challenging. This is particularly so because there is no definitive diagnostic marker of ‘cure’, rather it is based upon ongoing clinical assessment and historical patterns. The rise in drug-resistance to standard first line drugs further threatens disease control and spread.

The current BCG vaccine with at best 50% efficacy in adults has been in use since World War II, and whilst recent clinical trials of new vaccines are promising, a more effective vaccine is desperately needed.
2.7.3 **Drug resistance, testing and treatment choices, including new drug development**

New drug treatments are slow to develop and trial, partly because current microbiological and clinical end points require extended follow-up periods. A biomarker that could predict response would therefore expedite the assessment of the efficacy of new drugs.

Detailed understanding of the immunopathogenesis is vital for establishing the bacterium’s ability to infect and survive by manipulation and evasion of the human host’s immune processes. As described in this literature review, whilst knowledge of this complex interaction is improving daily, it is very much incomplete. The heterogeneity in immune responses within an individual further adds to the complexity. In reality it is likely that a platform of biomarkers will be needed to guide assessments of Mtb infections in humans.\(^{31,24,33,22,35,23,273}\)

### 2.8 Theoretical and conceptual framework

**This pilot project aims to look at the interaction between immune markers with a focus on changes that occur during treatment in patients with different forms of Mtb infection.**

It is primarily because the innate immune system does not possess memory that I propose to focus on it for this longitudinal study, given the high prevalence of LTBI. I am particularly interested in determining whether TLR expression and function have a specific profile in infected versus treated patients. Similarly, I am interested whether these will enable discrimination between active and latent TB infection, as well as be a marker of ‘treatment cure’.

I hope this study will shed light on the changes of TLRs through TB treatment and similarly determine whether these can be used as part of a platform for potential biomarker(s). I also investigated the use of a newer IGRA assay (QFT-Plus) in its use through TB treatment.
2.9 Hypotheses

Primary hypotheses and subsidiary questions

1. It is hypothesised that differences of the innate immune system exist in patients with Mtb infections which will be detectable and variable between those who have active TB disease and those who have been infected but are asymptomatic (LTBI).
   • It is hypothesised that these distinct variations will be detectable in peripheral blood mononuclear cells (PBMCs), particularly with respect to toll-like receptor (TLR) expression (TLR2, 4 and 7) and function (TLR2, 4, 7/8 and 9).
   • It is likely that such variations will fluctuate during the course of treatment in patients with active TB, and likely to reverse by the end of the antimicrobial treatment course.
   • Accordingly, it is postulated that at the end of treatment patients with active disease will have a TLR expression profile similar to that of latently infected patients, and pro-inflammatory cytokine expression in these patients will change over treatment and may reflect patterns manifested in their TLR function profiling.

2. It is hypothesised that the association between vitamin D levels, in particular deficiency and active tuberculosis is at least partly mediated by innate immune mechanisms and therefore
   • likely that replenishment of vitamin D will likewise affect corresponding TLR expression.

3. The newer QuantiFERON-TB Gold Plus will be evaluated in patients with active and LTBI infection through treatment. It is hypothesised that the newer test will be at least as sensitive as the previous test (QuantiFERON-TB Gold) and that levels of IFN\(\gamma\) response will alter at the end of treatment.
   • A descriptive analysis of absolute IFN\(\gamma\) responses of the two tests will be undertaken.
   • It is hypothesised that differences will be seen in patients with mild/moderate and severe active tuberculosis infection in the different tubes of the newer test.
Chapter 3: Study description and methods

3.1. Study population and recruitment

This study was a prospective, observational study conducted through Monash Health in Melbourne, Victoria. Monash Health is Victoria’s largest health service and serves a quarter of Melbourne’s population. Amongst its infectious diseases services are a TB clinic (Monash Medical Centre, Clayton), Refugee Health Clinic (Dandenong Hospital) and busy inpatient units. On average 90–120 patients with TB are diagnosed (and/or treated) through Monash Health annually, which is approximately 10% of the national diagnoses. Tuberculosis is a reportable infection in Australia and requires notification to the state TB program within five days of diagnosis by the clinician. Referrals to the TB clinic arise from several main areas: general practitioners, post admission follow-up, Victorian TB program and the state Migrant Screening Clinics. The TB program and Migrant Screening Clinic are the mainstay of referrals from contact screening and the Australian migration screening programs in Victoria respectively.

This translational study aimed to characterise the differences in aspects of immune responses in patients with active and LTBI. I was also interested determining what the changes were through antibiotic treatment. Innate immune responses investigated include TLR expression and function on PBMCs (MYC study) and IFNγ responses through a newer QuantiFERON-TB assay (QFT study). MYC study commenced in 2011 and the QFT study commenced in 2013; patients could choose whether they participated in one or both studies from 2013. Differences between the studies are described below.

Ethics approval was attained from Monash Health Human Research Ethics Committee in November 2010 for the MYC study (Project Number 10287A) and October 2013 for the QFT study (Project Number 13059A), with reciprocal approval from Monash University.

Volunteers were actively recruited from Monash Health hospital wards and outpatient clinics as detailed above. Those recruited from the inpatient setting were patients with active TB (diagnosed or presumptive), predominantly from Dandenong Hospital and
Monash Medical Centre. All patients enrolled signed the Patient Information and Consent Form on a voluntary basis, with an ability to withdraw from the study if they so wished.

### 3.1.1 Definitions of active and latent TB infection

**Active TB**: patients who were commenced on treatment based on clinical grounds for active TB and notified to the Victorian TB program as a new case. This was based on one or more of: positive TB culture results, histology consistent with TB diagnosis, positive Mtb PCR (MTB PCR IS6110) and/or radiological grounds consistent with active TB. Active TB treatment drug choices were based on WHO guidelines and individual clinical case assessments by the treating clinician, but generally consisted of rifampicin, isoniazid, pyrazinamide and ethambutol (see Section 2.3).\(^77,80,274\)

**LTBI**: defined as a patient who was diagnosed with Mtb infection, where active TB was excluded, on clinical grounds. MYC study requirement included a positive QuantiFERON-TB Gold result, or a break-of contact positive Mantoux on contact screening suggestive of recent TB infection acquisition.\(^274,69,275\)

### 3.1.2 Recruitment

Based on the above, patients were recruited into the two main cohorts of each study:

1. **Active TB**: these patients were recruited prior to commencing anti-tuberculous antimicrobial therapy (or within two weeks of doing so). Specific inclusion criteria: microbiological and clinical evidence of active disease and commencement of ATT (anti-tuberculous therapy).

2. **Latent TB infection (LTBI)**: these patients were recruited predominantly from outpatient clinics and commencement of chemoprophylaxis with isoniazid or rifampicin.

Exclusion criteria applicable to both studies:

- Age (patients under 18 excluded).
- Patients with significant mental health issues or unable to competently provide informed consent.
Exclusion criteria applicable to the MYC study:

- Patients with HIV infection.

**Numbers needed for completion of the MYC study:** formal assessment of power was not undertaken as it was a pilot study and research looking at similar cohorts longitudinally have not been published. I aimed to recruit 80 patients with active TB (MYC A) and 100 patients with LTBI (MYC L). The reason to recruit greater numbers in the MYC L cohort as it was anticipated there would be less adherence and attendance at follow-up appointments. For the QFT study, once again as a pilot study investigating a newer QuantiFERON-TB assay, no power assessment was undertaken. I aimed to recruit 50 patients in each of the cohorts.

All participants had a study number allocated which was available only to study coordinators. All collected tubes were labelled with a study number once in the research laboratory and were stored only with the allocated number to maintain confidentiality. Study coordinators had access to participant data to enable follow-up sampling to have correct allocated numbers.

### 3.1.3 Clinical investigations

All patients had the following blood test done at baseline:

- QFT, vitamin D (25(OH)D), FBE, EUC, LFT, CRP, Ca2+, HIV and hepatitis B and C serology (part of routine investigations for patients with active TB and also recommended for those with LTBI).

Vitamin D levels were repeated when clinically relevant, as were other blood tests.

All other testing was done on clinical basis alone and not altered by the patient’s inclusion in the study.

All patients also had chest radiography (at least a chest X-ray) done at baseline.
3.1.4 General laboratory investigations

Thirty millilitres of blood were drawn from each participant in lithium heparin tubes for research investigations outlined below at four timepoints: baseline (prior to or within two weeks of commencing medication for active or latent TB infection, at one month into treatment, two months into treatment and at the end of treatment). The first two months of treatment in active TB are deemed the ‘intensive phase’ of treatment where patients were usually on a minimum of three active drugs and clinical outcome is often based on its adequacy.\textsuperscript{77,274} One and two timepoints were termed ‘early timepoints’ and results may be collated in cases where only one sample was available. From 2013, if the participant volunteered for both studies (i.e. MYC and QFT), then 40 mL of blood was drawn into lithium heparin tubes and transported to the laboratory at room temperature within four hours.

3.2 Isolation of PBMCs

3.2.1 PBMC isolation (2011–2015)

This was the method used for harvesting PBMCs on all patients in the MYC study throughout the treatment course.

Blood samples collected from participants were processed within four hours. PBMCs were isolated from samples in a Class II safety cabinet in a PC2 laboratory. PBMCs were extracted from 30 ml of blood by Ficoll-plaque density gradient centrifugation (Figure 3.1). Blood tubes were centrifuged at 1500 rpm to enable separation of plasma. The plasma was stored into two or three 1 mL aliquots in 2 mL sterile microtubes (Sardtedt, Germany) and placed in −20°C for 24 hours prior to being stored at −70°C freezer for batched processing of cytokine measurements (see Section 3.5). The cellular components were then diluted with sterile Dulbecco’s phosphate buffered solution (Gibco®, Life Technologies) in a 1:2 ratio in a sterile 50 mL falcon tube (Cellstar®, Greiner Bio-One). The diluted blood was then carefully overlaid with a sterile transfer pipette into falcon tubes containing 15 mL of sterile Ficoll-Paque Plus™ (GE Healthcare, Uppsala, Sweden). The falcon tubes were then centrifuged again at 2200 rpm, for 25 minutes at room temperature with the brake off. The resulted in separation of the different blood...
components and allowed the buffy coat containing the PBMCs to be harvested (Figure 3.1). The collected buffy coat was placed into a fresh falcon tube (maximum of 15 mL per tube) and then topped up to 50 mL with sterile PBS. The tubes were then centrifuged at 1500 rpm for 10 minutes at room temperature with brake on. This completed the first wash. The supernatant was then poured out and the pellet resuspended in 50 mL of PBS and the wash step was repeated again for a total of three washes. After the final wash, the pellet was suspended in 1 mL of RPMI-media (RPMI supplemented with 5% FCS, penicillin-streptomycin and L-glutamine) (RPMI Medium 1640, Gibco®, Life Technologies™) and viability undertaken through trypan blue exclusion (1 in 10 dilution), after which cells were counted with a haemocytometer. Cryovials (Cryo.s™ Greiner Bio-one, Germany) with 500 µL of freezer mix (20% DMSO in FCS) were prepared and labelled. A minimum of 10 million cells/vial were then placed into labelled cryovials with a 1:1 ratio of freezer mix:PBMC-media suspension into a Mr Frosty into a −70°C freezer for 24 hours prior to transferring into liquid nitrogen for storage until laboratory investigations were performed.

![Figure 3.1: Isolation of PBMCs via Ficoll-Paque density gradient centrifugation](image)

**Figure 3.1: Isolation of PBMCs via Ficoll-Paque density gradient centrifugation**

*A:* Pre-centrifugation blood diluted 1:1 sterile PBS and gently overlaid onto Ficoll-Paque containing falcon tube. **B:** Shows separation of components of blood enabling harvesting of PBMCs within the buffy coat.

### 3.2.2 PBMC isolation using SepMate™ (2016–2017)

From 2016 PBMC isolation procedure was done with the use of SepMate™50 (STEMCELL™ Technologies, Vancouver Canada). These changes only applied to PBMC harvesting for investigative work using healthy volunteers (Section 3.8). SepMate™50 tubes were
prepared with 15 mL of density gradient medium (Lymphoprep™). Participant blood was collected in Lithium heparin tubes. The collected volume of blood was then diluted 1:1 with sterile PBS in the Class II cabinet in a 50 mL falcon tube. The diluted blood was then carefully poured down the side, into the SepMate™50 tubes. Tubes were then centrifuged at 1200 \( g \) for 10 minutes with brake on. The PBMC (buffy coat layer) was then poured into a fresh 50 mL falcon tube and topped to 50 mL with sterile PBS. Tubes were then centrifuged at 300 \( g \) for eight minutes and supernatants discarded. The wash step was repeated for a total of three washes. Finally, the pellet was gently resuspended in 1 mL of RPMI-media for use.

### 3.3 Surface and intracellular TLR expression studies

TLR expression studies were batched and performed on individual participant samples together where possible. The TLR receptor being analysed ('T1') was measured and compared as a ratio to that of corresponding isotype control ('T2'). The expression was therefore a ratio of the geometric mean fluorescence (GMF) of receptor measurement to isotype GMF (Figure 3.2 and 3.3). In analysis of the results, the relative changes of TLR expression through treatment were expressed as a fold change compared to the baseline (or prior to treatment commencement). This process is referred to as ‘normalisation’ of results to baseline in this thesis.

One vial of collected PBMCs per timepoint from one participant were rapidly thawed (in a 37°C water bath), cells recounted with trypan blue exclusion and cells were resuspended in 1 mL supplemented RPMI-media. PBMC concentration of 0.5 x 10^6 cells/mL was adequate for flow cytometry. The sample tubes were labelled “T1”, and “T2” represented the isotype control for the corresponding sample.
### Table 3.1: Surface and intracellular TLR expression studies: antibodies utilised

<table>
<thead>
<tr>
<th>Tube label</th>
<th>Marker</th>
<th>Fluorophore</th>
<th>Volume</th>
<th>Antibody type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>Surface TLR</td>
<td>TLR2</td>
<td>APC</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td>Surface TLR</td>
<td>TLR4</td>
<td>PE</td>
<td>1.5 µL</td>
<td>Anti-human</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Intracellular TLR</td>
<td>TLR7</td>
<td>FITC</td>
<td>10 µL</td>
<td>Anti-mouse</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>T cells</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Tube 2</td>
<td>Surface TLR</td>
<td>IgG2a</td>
<td>APC</td>
<td>1.5 µL</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>Surface TLR</td>
<td>IgG2a</td>
<td>PE</td>
<td>2.5 µL</td>
<td>Anti-mouse</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Intracellular TLR</td>
<td>IgG2a</td>
<td>FITC</td>
<td>10 µL</td>
<td>Anti-mouse</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>T cells</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation TLR2</td>
<td>TLR2</td>
<td>APC</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Compensation TLR4</td>
<td>TLR4</td>
<td>PE</td>
<td>1.5 µL</td>
<td>Anti-human</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Compensation TLR7</td>
<td>TLR7</td>
<td>FITC</td>
<td>10 µL</td>
<td>Anti-mouse</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Compensation CD14</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation CD3</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation CD56</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Unstained</td>
<td>Nil</td>
<td>Nil</td>
<td>3 µL</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

#### 3.3.1 Surface staining of TLR receptors (2011 to May 2016)

The monoclonal antibodies used for TLR staining are listed in Table 3.1. The PBMCs were also stained for expression of the monocyte marker CD14, T cells (using CD3) and NK cells (CD56). Further evaluation of NK subsets was done by plotting CD56 and CD3 (Figure 3.2). Antibodies were placed in the quantities listed in tubes T1 and T2, and the compensation tubes with the corresponding mAB (monoclonal antibody), with the exception of the Compensation TLR7 (intracellular receptor mAB). The ‘unstained’ tube remained antibody free. Into each of the tubes (T1, T2 and compensation tubes) 100 µL of PBMC-mix was added. All the compensation tubes, including the unstained, had PBMC-mix from one sample added (whichever sample had the greatest number of viable PBMCs after thawing). The tubes were vortexed (short) and placed into the dark (protected from light) for 20 minutes. Following incubation, 1 mL of non-sterile PBS was added into T1, T2 and all compensation tubes (except the unstained and Compensation TLR7) and centrifuged at 1500 rpm, at room temperature for five minutes, brake on. This completed
the first wash, allowing unbound antibody to be washed away. The supernatant was pipetted off into a waste bottle. The cells were fixed by adding 250 µL of Cytodix-cytoperm™ (BD Biosciences) to every tube (including Compensation TLR7 and unstained). This was done in the fume cupboard. Cells were gently resuspended whilst the solution was added. Tubes were then covered and placed in the fridge at 4°C overnight. The following day the cells were permeabilised with 1 mL of Perm/Wash™ buffer (1 in 10 dilution with distilled water) (BD Biosciences, San Diego, US) in the fume cupboard. The cells were then washed (2500 rpm, five minutes, room temperature, brake on). Then, 1250 µL of supernatant was removed into formaldehyde waste bottle (in fume cupboard) from each tube. Perm/Wash™ buffer (1 mL) was added to every tube and centrifuged to complete the second wash. The supernatant (1 mL) was pipetted off carefully into formaldehyde waste bottle. Once the second wash was completed, the rest of the staining was done on the bench.

3.3.2 Intracellular staining (2011 to May 2016)

TLR7-FITC mAB, and corresponding isotype control was added to T1 and T2 respectively in quantities specified previously in Table 3.1. The tubes were vortexed and placed at 4°C for incubation for 30 minutes (to slow down the reaction and allow specific binding to occur). Following incubation, two further washes were conducted with addition of 1 mL of Perm/Wash™ buffer to all tubes which had TLR7-receptor antibody or corresponding isotype control added.

Levels of TLR expression were then analysed by flow cytometry (BD FACS Canto II Analyser). CD14 positive (CD14+) monocytes, NK cells (CD56+), NKT cells (CD3+CD56+) (and subsets: NK CD56bright and NK CD56dim) were gated and the geometric mean fluorescence for TLR2, TLR4 and TLR7 expression were determined on up to 10,000 cells. The data was acquired using BD FACSDiva™ software and flow plots analysed using FlowJo© Version 10 (Treestar, Oregon, US). Examples of the gating strategies used are shown in Figure 3.2 and Figure 3.3.
Figure 3.2: Gating strategies used for surface and intracellular TLR staining on different cell types

T1 (sample) and T2 (isotype control).
Comparison of gating strategy highlighting the use of Sample and Isotype controls:

1: Lymphocytes and monocytes
2: NK and NK cells
3: CD14+ monocytes in histogram form
4: TLR2
5: TLR4
6: TLR7

Steps 4 to 6 were assessed on all cell types previously described (CD14+ monocytes, NK cells, NKT cells, NKCD56 subsets). The geometric mean fluorescence (GMF) of TLR2, 4, & 7 on specific cell type is determined for every sample and respective isotype control.

Figure 3.3: Gating strategies used for surface and intracellular TLR staining on different cell types, highlighting the differences in gating for T1 (sample) and T2 (isotype control)
3.3.3 Surface and intracellular TLR expression studies 
(from May 2016)

Amendments were made to the TLR staining protocol in May 2016, accommodating the use of a viability stain, as well as compensation beads (instead of cells) (Table 3.2). The following outlines the main changes, otherwise the protocol as listed previously.

Viability stains using FVS 620 (Fixable viability stain) (BD Horizon) or FVS 510 (BD Horizon). Thawed, counted PBMCs were made up in PBS at $1 \times 10^6$ cells/mL into a volume of 2 mL. The RPMI-media was washed off and supernatant carefully pipetted off. In a clean, non-sterile FACS tube, PBMCs were resuspended in PBS according to calculated volumes. FVS stain was added 1 µL/mL into each FACS tube. Then the tubes were incubated in the dark for 15 minutes. Following incubation, unbound antibody was washed off by centrifugation at 1500 rpm, five minutes, room temperature, break on. The supernatant was carefully removed and 1 mL of PBS added for the second wash. Then 200 µL of PBS was added and 100 µL placed in the respective FACS tubes labelled T1 and T2. Antibodies were only added to the respective T1 and T2, as compensation beads were used the following day instead of cells. The protocol followed was as previous and samples placed in fridge overnight in Cytofix-cytoperm. Washes and intracellular staining were done as previous, with the exception of different antibodies and isotype controls, due to manufacturer changes (Table 3.2). BD™ CompBeads anti-Mouse Igκ and negative control were used according to the data sheet (Catalogue # 552843). Into a non-sterile FACS tube, I added 1.5 drops of the negative and positive control. Then I added 500 µL of PBS and mixed by a short vortex. The required volumes of antibodies were placed into the labelled CompBeads FACS tubes (six in total), 100 µL of the prepared CompBeads mixture added into each tube and incubated in the dark for 15 minutes. Following incubation, the excess antibody was washed off with 1 mL of PBS into each tube and centrifugation at 1500 rpm, five minutes, brake on. This had to be repeated and supernatants carefully removed each time. Once the intracellular staining is completed, flow cytometry was performed, with the added gating changes shown in Figure 3.4.
Table 3.2: Surface and intracellular TLR expression studies: antibodies utilised (amendments made from May 2016 onwards)

<table>
<thead>
<tr>
<th>Tube label</th>
<th>Marker</th>
<th>Fluorophore</th>
<th>Volume</th>
<th>Antibody type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>Surface TLR</td>
<td>TLR2</td>
<td>APC</td>
<td>5 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>Surface TLR</td>
<td>TLR4</td>
<td>PE</td>
<td>5 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>Intracellular TLR</td>
<td>TLR7</td>
<td>AlexaFluor</td>
<td>5 µL</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>1 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td>Tube 2</td>
<td>Surface TLR</td>
<td>IgG2a</td>
<td>APC</td>
<td>5 µL</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td></td>
<td>Surface TLR</td>
<td>IgG2a</td>
<td>PE</td>
<td>2.5 µL</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td></td>
<td>Intracellular TLR</td>
<td>IgG2a</td>
<td>AlexaFluor</td>
<td>5 µL</td>
<td>Anti-rat</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>1 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td>CompBeads TLR2</td>
<td>TLR2</td>
<td>APC</td>
<td>1 µL</td>
<td>Anti-human</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CompBeads TLR4</td>
<td>TLR4</td>
<td>PE</td>
<td>0.5 µL</td>
<td>Anti-human</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CompBeads TLR7</td>
<td>TLR7</td>
<td>AlexaFluor</td>
<td>1 µL</td>
<td>Anti-mouse</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CompBeads CD14</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>0.5 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CompBeads CD3</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>0.2 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CompBeads CD56</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>2 µL</td>
<td>Anti-human</td>
<td>Beckman Coulter</td>
</tr>
</tbody>
</table>
Figure 3.4: Amended gating strategies used for surface and intracellular TLR staining on different cell types

From May 2016, using fixed viability stain and compensation beads.
3.4 Stimulation of PBMCs with TLR ligands to evaluate innate immune function

TLR function was assessed by stimulation of PBMCs ex vivo with TLR ligands and measuring cytokines and chemokines in the supernatants. This was performed on participants’ blood samples in the MYC study, through antibiotic treatment. Where possible, an individual’s samples were batched and evaluated together.

Between 2011 and November 2014, preparation of samples were done for measuring cytokines/chemokines via ELISA (see Section 3.4.2.1) and from November 2014 PBMCs were prepared for measurement through cytokine bead arrays (CBA) instead (as this permitted smaller volumes of supernatants to be tested, with a greater number of cytokines/chemokines to be measured concurrently).

3.4.1 Preparation of PBMCs for TLR-function studies

PBMC vials were rapidly thawed as described in Section 3.3.2. In sterile conditions, cells were counted following trypan blue exclusion and the samples made up to $2 \times 10^6$ cells/mL. Samples were processed in sterile conditions in Class II safety cabinet. Where possible, TLR functional assays were combined with TLR surface and intracellular staining.

3.4.2 TLR ligand stimulation of PBMCs

3.4.2.1 TLR ligand stimulation of PBMCs, for measurement of cytokines through ELISA

Thawed PBMCs were prepared as per Section 3.4.1 and placed to rest in the tissue culture incubator whilst ligands were prepared. The number of tubes and volumes of ligands required depended upon the number of samples evaluated. The following describes the process for two samples, but often four to eight samples were processed simultaneously.
Figure 3.5: Preparation of FACS tubes for TLR ligand stimulation for measuring cytokines and chemokines via ELISA (A) and CBA (B)

Samples were prepared so that the final volume is 1000 µL. The final samples were in a ratio of ligand:PBMCs of 1:1 (with 500 µL of ligand and 500 µL of PBMCs in RPMI-media) (Figure 3.5A). For each sample, $1 \times 10^6$ cells per ligand and for unstimulated measurements were required, therefore need a total of approximately $6 \times 10^6$ cells per sample. The ligands were prepared as per volumes shown in Table 3.3. The ligands used were based on TLR structure that helps ‘activate’ specific TLRs by binding to them. The ligands were mixed by gentle pipetting once thawed. When the ligands and PBMCs were prepared, 500 µL of ligand was placed into a labelled, sterile FACS tube, then the PBMCs added (500 µL at $2 \times 10^6$ cells/mL). Therefore, for each sample, there were five FACS tubes (four with ligands, and one unstimulated).

The samples were then placed in the tissue culture incubator for 20 hours. Following incubation, samples were centrifuged at 1500 rpm, brake on, for five minutes. Supernatants were drawn off carefully and placed in labelled sterile Eppendorf tubes (two aliquots of 500 µL each). These samples were then placed in the −80°C freezer for cytokines measurements at a later date.
Table 3.3: Ligands and volumes used in preparation of TLR functional studies for ELISA (2011 to November 2014)*

<table>
<thead>
<tr>
<th>TLR</th>
<th>TLR ligand</th>
<th>2x [ ]</th>
<th>Volume of ligand</th>
<th>Volume of media</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Pam3CSK4 (P3C)</td>
<td>200 ng/mL</td>
<td>30 µL</td>
<td>1470 µL</td>
<td>InvivoGen, US</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>200 ng/mL</td>
<td>30 µL</td>
<td>1470 µL</td>
<td>InvivoGen, US</td>
</tr>
<tr>
<td>TLR7/8</td>
<td>R848</td>
<td>10 µg/mL</td>
<td>15 µL</td>
<td>1485 µL</td>
<td>InvivoGen, US</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG2006#</td>
<td>0.6 µM</td>
<td>30 µL</td>
<td>1493 µL</td>
<td>Geneworks</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>1500 µL</td>
<td></td>
</tr>
</tbody>
</table>

* Example of volumes of ligands & media used for stimulation of ex vivo PBMCs for 2 samples for ELISAs. # CpG2006 stock concentration 129.5 µM.


3.4.2.2 TLR ligand stimulation of PBMCs, for measurement of cytokines through CBA

The main difference between preparation of PBMCs for stimulation for cytokine measurements in future via CBA was the volume that is required (and therefore the total number of PBMCs required were also fewer). The differences to Section 3.4.2.1 were as follows:

Volume of PBMC-media mix was 125 µL, whilst ligand-media mix volume required was 125 µL (Figure 3.5B)

The ratios of final samples were unchanged at ligand:PBMCs of 1:1, and the concentration of PBMCs was still 2 x 10⁶ cells/mL, but only required 750 µL at this concentration. The ligands were prepared as per volumes shown in Table 3.4. The ligands and samples were gently mixed together in a sterile U-bottom 96-well plate (Costar, Round Bottom 96-Well Assay Plate, Corning Inc., New York, US), based on a prepared template and placed in the incubator for 20 hours. Following incubation, samples were centrifuged at 1500 rpm, brake on, for five minutes. First aliquot of supernatants was drawn via multi-channel pipette into the allocated well for corresponding supernatant. The second 125 µL
supernatant aliquot was drawn into a labelled Eppendorf tube for storage. The samples were then placed in the –80°C freezer for cytokines measurements at a later date.

Table 3.4: Ligands and volumes used in preparation of TLR functional studies for CBA (from November 2014)*

<table>
<thead>
<tr>
<th>TLR</th>
<th>TLR ligand</th>
<th>2x [ ]</th>
<th>Volume of ligand</th>
<th>Volume of media</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>P3C</td>
<td>200 ng/mL</td>
<td>30 µL</td>
<td>1470 µL</td>
<td>InvivoGen, US</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>200 ng/mL</td>
<td>30 µL</td>
<td>1470 µL</td>
<td>InvivoGen, US</td>
</tr>
<tr>
<td>TLR7/8</td>
<td>R848</td>
<td>10 µg/mL</td>
<td>15 µL</td>
<td>1485 µL</td>
<td>InvivoGen, US</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG2006*</td>
<td>0.6 µM</td>
<td>8.55 µL</td>
<td>1491.5 µL</td>
<td>Geneworks</td>
</tr>
<tr>
<td></td>
<td>Unstimulated</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>1500 µL</td>
</tr>
</tbody>
</table>

* Example of volumes of ligands & media used for stimulation of *ex vivo* PBMCs for 8–10 samples for CBA.
# CpG2006 stock concentration altered to 105.3 µM.

3.4.3 Measurement of cytokines in supernatants using ELISA (2011 to November 2014)

Enzyme linked immunosorbent assays were utilised for measurement of cytokines (IL-6, TNF), in stimulated supernatants (Section 3.4.2), however the samples were re-evaluated using CBA. Comparisons of results are discussed in Chapter 4.

Procedure was done according to manufacturer’s recommendations (*BD™* OptEIA™ Set Human TNF Catalogue # 555212, and *BD™* OptEIA™ Set Human IL-6 Catalogue # 555220). In summary, templates were drawn up to enable identification of samples and planned dilution(s). The planned dilutions of the supernatants varied depending on the TLR ligand, cytokine measured and also the timepoint at which sample was collected. The required volumes of supernatant and diluent were calculated accordingly (dilutions were done in labelled Eppendorf tubes). All wells were evaluated in duplicate. Based on the total number of wells to be used, calculations were made of the approximate volumes of capture antibody, detection antibody and substrate solution required.

Plates were coated with Capture Antibody diluted with coating buffer, 1:250 dilution (100 µL per well) and placed in a container overnight in the fridge (see Appendix 1). The
following day, plates were washed and aspirated three times using wash buffer and blotted on paper towels. Plates were then blocked by placing 200 µL of assay diluent into each well and incubated at room temperature in humidified box for one hour. During the incubation, standards and samples dilutions were performed. Eight FACS tubes were required for each set of cytokines.

**IL-6:** top standard made at 300 pg/mL with assay diluent and six serial dilutions performed to generate 150 pg/mL, 75 pg/mL, 37.5 pg/mL, 18.8 pg/mL, 9.4 pg/mL, and 4.7 pg/mL. Assay diluent served as the zero standard (0 pg/mL).

**TNF:** top standard made at 500 pg/mL with assay diluent and six serial dilutions performed to generate 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 pg/mL. Assay diluent served as the zero standard (0 pg/mL).

Tube 8 only had assay diluent.

Following incubation, the diluted samples and standards were added to the corresponding allocated wells and incubated in a humidified box for two hours at room temperature. Plates were then aspirated and washed five times to remove unbound antibody; 100 µL of working detector (detection antibody diluted in assay diluent with and Sav-HRP added) was added to all wells with samples or standards. Plates were then incubated for one hour at room temperature. Plates were then aspirated and washed seven times with 30–60 second soaks in between. Finally, 100 µL of substrate solution (Sure Blue™, TMB Microwell peroxidase substrate, KPL, US) was added to the wells and plates incubated for 30 minutes in the dark. Stop solution (50 µL) was then added to stop reaction continuing and plates read on FLUOstar Omega (BMG Labtech) at 450 nM within 30 minutes. Standard curves were generated with linear regression and results reported in pg/mL.

### 3.4.4 Measurement of cytokines/chemokines in supernatants using cytokine bead arrays

Standard BD™ Cytometric Bead Array Human Protein Flex Sets (BD Biosciences, San Diego, US) were used to measure chemokines and cytokines in supernatants from TLR ligand stimulated PBMCs (from November 2014 onwards). Each chemokine/cytokine is
conjugated to its own specific labelled capture bead and fluorophore detection, and because of the unique size, enable multiplex evaluation of up to 30 proteins simultaneously (with the added advantage of requiring small amounts of specimen). Master Buffer Kits for CBA were required containing assay diluent, wash buffer and reagent diluents (Catalogue # 561523 ES 500 tests) Proteins measured included IL-6, IL-8, TNF, MCP1, IL-10, IFNγ, IL-17A, IL-17F, IL-12p70. The amount of fluorescence measured is related to the amount of bound protein measured by flow cytometry. The assay range was 10 pg/mL to 2500 pg/mL. All steps had to be conducted on the day of procedure; all tubes/plates were labelled and calculations were conducted the day prior. Samples and standards were processed on the bench. Not all cytokines/chemokines listed were undertaken each day (see Section 4.2.2). Samples to be used were thawed on the day of procedure at room temperature. When conducted in plates, samples were placed in sterile 96-well U-bottomed microplates (TPP® Tissue Culture Test Plate, Sigma Aldrich).

The provided protocol was followed, with the basic steps being outlined below:

1. Preparation of standards
2. Preparation of samples
3. Preparation of capture beads
4. Preparation of PE-detection reagent
5. Performance of assay and analysis using FCAP-Array

3.4.4.1 Preparation of standards

Standards are provided in lyophilised form, so required reconstitution on the day of use, with serial dilutions. Vials containing each standard to be used in each procedure was opened and pooled into one 15 mL falcon tube (labelled "Top Standard"). Then, 4 mL of assay diluent was added and allowed to equilibrate at room temperature for 15 minutes and gently mixed through pipetting. Eight FACS tubes were labelled 2 to 9, and 500 µL of assay diluent added to these. A serial dilution with fresh pipettes was then undertaken, which allowed for the standard curve to be formed during analysis (1:2 = 1250 pg/mL, 1:4 = 625 pg/mL, 1:8 = 312.5 pg/mL, 1:16 = 156 pg/mL, 1:32 = 80 pg/mL,
1:64 = 40 pg/mL, 1:128 = 20 pg/mL, 1:256 = 10 pg/mL. A final tenth FACS tube containing only assay diluent had a concentration of 0 pg/mL.

3.4.4.2 Preparation of samples

Samples were diluted with assay diluent as predetermined per procedure day. This was determined by which cytokines/chemokines were to be tested and whether supernatants with all TLR ligand stimulations were included. For example, if IL-8 was being performed, dilutions of 1/50 for R448 and LPS were included and 1:10 for unstimulated samples, in addition for neat testing of samples for every supernatant.

3.4.4.3 Preparation of capture beads

Capture beads were prepared whilst samples were being thawed. Each bead used was vortexed well for a minimum of 15 seconds and the required volume placed in a FACS tube labelled “Capture Bead Mix”. From each protein (or flex kit), 0.5 µL of capture beads was required per sample and standard. The volume of capture beads required per protein to be analysed was calculated as follows:

\[
\text{Total volume of Capture Bead Mix per procedure} = 25 \, \mu\text{L} \times \text{number of tests (samples plus standards)}
\]

\[
\text{Capture bead volume (per protein) volume} = 0.5 \, \mu\text{L} \times \text{number of tests (samples plus standards)}
\]

Assay diluent volume required

\[
= \text{total volume} - (\text{capture bead volume x number of proteins evaluated})
\]

3.4.4.4 Preparation of PE-detection reagent

The volumes of detection reagents required were the same as per capture bead for each procedure and placed into a labelled FACS tube covered with foil (as the reagent is light sensitive). The “Detection Reagent Mix” was prepared during the first incubation.
3.4.4.5 Performing assay and analysis using FCAP-Array software

Labelled FACS tubes (for standards 1 to 10, with 0 pg/mL = 1) had 25 µL of standard added accordingly. Sample tubes (or plates) had 25 µL of respective sample added according to templates labelling); 25 µL of Capture Bead Mix was then added into the 10 standards tubes and every sample tube/well was mixed well. All tests were then placed in the dark at room temperature for one hour of incubation.

During the incubation step, PE-detection reagent was prepared. Following incubation, 25 µL of Detection Reagent Mix was added to every test tube/well and incubated in the dark for two hours. During the incubation, the flow-cytometer was set up as required.

Following incubation, 500 µL of wash buffer was added to every test tube and centrifuged at 200 g for five minutes at room temperature. Supernatant was then carefully aspirated and 300 µL of wash buffer was added and tubes vortexed prior to acquisition on flow-cytometer. If plates were used for samples, 200 µL and 100 µL of wash buffer was used respectively for the washes listed above.

FACS-Canto II cytometer was used for result acquisition according to recommendations: voltage and compensation were performed as per the BD CBA Flex Set Template. In summary, five non-sterile tubes labelled “A9”, “PE-F1”, “F1”, “F9” and “A1” had 25 µL of the corresponding set-up beads (contained in Master Buffer Kit) added and mixed well with 200 µL of wash buffer. Events were set up to 500 in log scale for set-up beads. The voltage was adjusted as required until FSC-A and SSC-A were a mean of 30,000 for tube A9 and a gate placed around this singlet population. Then, using this singlet population, A9 was used to adjust the APC-Cy7 and APC voltages to a mean of 160,000 ± 20,000 and the PE-F1 tube a mean voltage of 65 ± 5 for PE. The compensation as then calculated using set-up bead tubes F1, F9 and A1. Then the standards (in tubes) were run from least to most concentrated and then the samples (in tubes or plates, with events recorded for a minimum of 300 events for each flex set [or protein]). Data was then acquired using BD-FACS Diva software (Version 6.1.3) and exported and analysed using FCAP-Array (Version 3). Standard curves with an r² minimum of 0.98 were used for subsequent data analysis.
3.5 Plasma cytokine and chemokine measurements

Enhanced-sensitivity CBA flex kits were initially used for cytokine measurement in plasma (2014–2016). Due to changes in the products available in 2017, Magnetic Luminex® Performance kits were used (all samples where possible were repeated and results are discussed in Section 4.2.4). The procedures were both conducted according to product manuals and summarised below.

3.5.1 Plasma cytokine measurement using Human Enhanced Sensitivity CBA Protein Flex Set

The ES-CBA Protein Flex Sets™ (BD Biosciences, San Jose, US) was used to measure IL-6, IL-8, IL-12, IL-4, TNF and IFNγ in stored plasma of participants enrolled in the MYC study cohorts. The BD CBA Master buffer kit was utilised, along with specific ES-CBA Flex Set depending on the cytokines to be measured on the procedure day, with reagents for plasma testing utilised.

3.5.1.1 Preparation of standards

ES-CBA procedure for plasma was similar to the BD CBA for supernatants described previously (Section 3.4.4) with several exceptions summarised below. Assay range was 274 fg/ml to 200,000 fg/mL, with eight standards used to form the standard curve and 1:3 serial dilution (1:3 = 66,667 fg/ml, 1:9 = 22,222 fg/ml, 1:27 = 7,407 fg/ml, 1:81 = 2469 fg/mL, 1:243 = 823 fg/mL, 1:729 = 274 fg/mL and the final being assay diluent alone 0 fg/mL).

3.5.1.2 Preparation of samples

Plasma samples evaluated were placed in the 4°C fridge the night before, to allow quicker thawing on the bench on the day of procedure. All samples were thawed at room temperature on the day and centrifuged at 5000 rpm, brake on, for five minutes to separate any cellular material. Samples were used neat and 37.5 µL of sample placed into allocated well according to prepared template
3.5.1.3 Preparation of capture beads

The main difference between the ES-CBA flex kit and CBA was volumes of capture beads per sample and total volume required per flex per sample. The capture beads were vortexed well. The calculations are below:

\[
\text{Total volume of Capture Bead Mix per procedure} = 15 \, \mu\text{L} \times \text{number of tests (samples plus standards)}
\]

\[
\text{Capture bead volume (per protein) volume} = 0.75 \, \mu\text{L} \times \text{number of tests (samples plus standards)}
\]

\[
\text{Assay diluent volume required} = \text{total volume} - (\text{capture bead volume x number of proteins evaluated})
\]

3.5.1.4 Preparation of detection reagents

The volume of Detection Reagent Mix (Part A) required was the same for each experiment as it was for the corresponding Capture Bead Mix; 0.75 \, \mu\text{L} of detection reagent was required for each flex set (or cytokine/chemokine) and diluted with assay diluent in a non-sterile FACS tube labelled “Detection Reagent Mix Part A” and protected from light until required.

A vial lyophilised of ES-CBA detection reagent (Part B) was reconstituted with 550 \, \mu\text{L} of detection reagent, allowed to stand for 15 minutes at room temperature (in a covered FACS tube). Then, 4.5 \, \text{mL} of detection diluent was placed in a 15 \, \text{mL} falcon tube and 500 \, \mu\text{L} of the reconstituted reagent added (labelled “Detection Reagent Mix Part B”) and protected from light. This allowed for 66 tests.

3.5.1.5 Performance of assay and analysis using FCAP-Array

Labelled FACS tubes (for standards 1 to 8, with 0 \, \text{pg/mL} = 1) had 15 \, \mu\text{L} of standard added accordingly. Sample wells had 37.5 \, \mu\text{L} of respective sample added according to template; 15 \, \mu\text{L} of Capture Bead Mix was then added into the eight standards tubes and every well. Plates were placed on a digital shaker for five minutes (500 rpm) and standards tubes
vortexed. All tests were then placed in the dark at room temperature for two hours of incubation.

During the incubation step, detection reagents Part A and B were prepared. Following incubation, 15 μL of Detection Reagent Mix Part A was added to the standards tubes and wells. Plates were mixed on a digital shaker at 500 rpm for five minutes and standards tubes were vortexed. All tubes and plates were then incubated in the dark for two hours. During the incubation, the flow-cytometer was set up as required.

After incubation, the plates and tubes were aspirated and washed twice using 200 μL of wash buffer. Then, 75 μL of Detection Reagent Mix Part B was added to all wells and standards, mixed on a digital shaker and then incubated in dark at room temperature for one hour; 150 μL of wash buffer was then added to the plate wells and 750 μL to the standards tubes and centrifuged at 200 g for five minutes. The supernatant was carefully aspirated and 100 μL of wash buffer added and vortexed gently to resuspend beads.

The samples were then ready for data acquisition and this is described in Section 3.4.4.5 (FACS-Canto II cytometer set up and procedure).

3.5.2 Plasma cytokine/chemokine measurement using Magnetic Luminex® Performance Assay

Due to changes in ES-CBA flex kit availability, R&D Systems® Human XL Cytokine Discovery Pre-mixed Kit, Magnetic Luminex® Performance Assay (Minneapolis, US) was used to measure cytokines and chemokines in unstimulated plasma from participants in the MYC study through the treatment. This experiment was conducted by Jackie Yu. The following were measured: TNF, IP-10, MCP1, IFNγ, IL-6, IL-8, IL-10, IL-12p70 and IL-17A. The top standard concentrations for the analytes were 8320 pg/mL (TNF), 1770 pg/mL (IP-10), 3070 pg/mL (MCP-1), 4450 pg/mL (IFNγ), 6590 pg/mL (IL-6), 1170 pg/mL (IL-8), 19,330 pg/mL (IL-10), 13,880 pg/mL (IL-12p70) and 8120 pg/mL (IL-17A).

The assay procedure was performed as recommended by the manufacturer (Catalogue FCSTM18), in brief as follows.
Plasma samples were thawed at room temperature, spun at 13,000 g for five minutes and diluted 1:2 with calibrator diluent provided in the kit. Standards, low and high kit controls were reconstituted and allowed to sit for a minimum of 15 minutes at room temperature before use. The top standard was serially diluted 1:3 to create six additional standards and calibrator diluent served as the blank (0 pg/mL, eighth standard).

Briefly, 50 µL of standard, control or diluted sample were added to each well followed by the addition of 50 µL of diluted Microparticle Cocktail. Plates were incubated for two hours at room temperature on a horizontal orbital microplate shaker (Ratek platform shaker) and then washed three times using an automated magnetic microplate washer (Bio-Rad Pro Wash Station).

Then, 50 µL of diluted Biotin-Antibody Cocktail was added to each well, followed by one hour incubation and three washes. Finally, 50 µL of diluted Streptavidin-PE was added to each well and plates incubated for 30 minutes and washed three times before the addition of 100 µL of wash buffer for reading on a Bio-Plex Manager™ Software (Version 6.1.1, Hercules, California, US). Multiple samples from the same patient were run on the same plate. Both samples and standards were assayed in singlet. Reading from the blank was subtracted from each standard and sample reading. Standard curves were created for each analyte to generate a five parameter logistic curve-fit. Concentrations in the samples were multiplied by the dilution factor of two.

### 3.6 Messenger RNA studies

PBMCs which had been stimulated with TLR ligands (Section 3.4) were used for mRNA studies with Ambion RNAqueous®-4PCR Kit (Ambion®, Life Technologies™, US). The samples stimulated in a 96-well plate, following removal of supernatant for TLR studies had 50 µL of PBS added to resuspend the pellet. Then the samples were placed into individual, labelled 1 mL plasma tubes. Further, 950 µL of PBS was added to wash out the FCS contained within RPMI-media. Tubes were then centrifuged at 13,000 rpm (micro-centrifuge) for five minutes, brake on and the supernatant discarded; 200 µL of RNAqueous® lysis buffer was carefully added and pipetted until the solution was no longer viscous in nature. This was done in the fume cupboard (chemical safety cabinet).
Tubes were incubated at room temperature for at least 10 minutes, and were then stored at -80°C.

3.6.1 Ribonucleic acid extraction from PBMCs

Samples to be used were thawed at room temperature on the day; 200 µL of 64% ethanol was added to the lysate and gently mixed in the fume cupboard. The ethanol and lysate suspensions were then drawn through a filter cartridge column, ensuring not to touch the filter. The samples were then centrifuged at 1300 rpm for one minute. The follow-through was carefully discarded and 650 µL of wash solution added and centrifuged at 13,000 rpm for one minute. The follow-through was discarded. Washes two and three were done with 500 µL of wash solution. A further centrifuge at 1300 rpm for 30 seconds was done to ensure all excess fluid was removed. The filter was transferred into labelled new collecting tubes. Then the samples were eluted, by adding 60 µL of heated Elution Solution (75°C on heat block) to the centre of the filter cartridge, avoiding touching the filter itself. Samples were left for one minute and centrifuged at 13,000 rpm for one minute. The elution step was repeated, using 40 µL instead. The eluted samples could be stored after elution at -70°C for later or procedure continued on as below.

3.6.2 DNase1 inactivation of extracted RNA

This step helps reduce DNA activity below limit of RT-PCR detection. “DNase1 cocktail” was made up with DNase1 and DNase buffer (10x) based on following calculations:

10 µL per specimen => 9 µL DNase buffer + 1 µL DNase1

10 µL of DNase1 cocktail was carefully added to each collecting tube, gently mixed and then incubated at 37°C for 20 minutes. Then, DNase1 Inactivation reagent (10 µL) was added. This reagent exists as a slurry, had to be vortexed well and continue to be mixed as it was added. This was then incubated for two minutes at room temperature, then centrifuged at 13,000 rpm for one minute. To determine if the quantity of RNA in the sample was adequate, spectrophotometry was performed on the supernatant. Nano-Drop was used with following settings 260 nM > / = 0.15; ng/µL > 7; A260/280 ratios 1.5–2.5.
Supernatant was collected into sterile RNase/DNase-free Eppendorf tubes and could be stored at −70°C or procedure continued for complementary DNA (cDNA) preparation.

### 3.6.3 Complementary DNA preparation for PBMCs

cDNA was prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). The kit components (except for MultiScribe™ RTase) were allowed to rest at room temperature from −200°C. The MultiScribe™ RTase was kept on ice. Samples were thawed out at room temperature. The sterile Eppendorf tube per sample was labelled and another labelled “RTase Cocktail”. RTase Cocktail was made up (with 10% extra) (Table 3.5). The concentration of the cocktail was dependent on the quantity of RNA: if > 10 ng/µL, use 3x. For the laboratory work reported in Chapter 4, 2x concentrations were used. Equal volumes of sample (or RNA) and RTase cocktail were required. The enzyme (RTase) was placed in the cocktail tube last.

#### Table 3.5: Components of RTase cocktail

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1x</td>
</tr>
<tr>
<td>10 x buffer</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>25 x dNTP (10 mM)</td>
<td>0.8 µL</td>
</tr>
<tr>
<td>10 x RT primer</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>MultiScribe™ RTase</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>RNase Out</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Sterile water</td>
<td>3.2 µL</td>
</tr>
<tr>
<td>RNA (sample)</td>
<td>10 µL</td>
</tr>
<tr>
<td>Total volume per tube</td>
<td>20 µL</td>
</tr>
</tbody>
</table>
The required volume of the sample was placed into the labelled tube and then the RTase cocktail, pipetted gently to mix. The tubes were allowed to sit at room temperature for 10 minutes (to initiate reaction) and then incubated at 37°C (on heat block) for two hours. The tubes were placed in an 85°C heat block for five minutes to inactivate the MultiScribe™. Then 2.0 μL of glycoblu (2 μg/μL) was added to enable visualisation of the pellet. Then 7.0 μL of 100% ethanol was added and cDNA precipitated by placing at −20°C overnight. The following day, tubes were centrifuged at 4°C for 20 minutes at 13,000 rpm. The supernatant was aspirated and the pellet washed with 400 μL of 70% ethanol and vortexed to mix well. The tubes were then centrifuged for 15 minutes at room temperature at 13,000 rpm. Supernatant was aspirated carefully and tubes air dried to enable residual ethanol to evaporate. The cDNA pellet was then dissolved in 200 μL of TE buffer (10 nM Tris Buffer and 1 mM EDTA) and could be used for RT-PCR studies or stored at −80°C for later use.

3.6.4 Performing mRNA expression studies

mRNA expression studies were performed by RT-PCR. cDNA samples were thawed at room temperature, and PCR run in a 7500 Fast Real-Time PCR system (Applied Biosciences) using SYBR® Green reagents. A cocktail consisting of 2.0 μL of 5 μM primers (1.0 μL each of forward and reverse primers), 3.0 μL of sterile water, and 10 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) per well was prepared, and 15 μL of this added to wells in MicroAmp® fast optimal 96-well plates containing 5 μL of sample cDNA. Wells containing TE were included to exclude non-specific amplification from contamination or primer-dimers. Duplicate reactions were analysed for each sample. PCR was performed for 40 cycles using default run method PCR conditions (one cycle each of 50°C for 20 seconds and 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for one minute) and included the melt-curve program. A Ct value (cycle threshold) of less than 35 was deemed acceptable, along with a satisfactory amplification and melt curve. The relative concentration of each gene was determined using the delta-delta Ct method and normalised to that of two housekeeping genes (HKG), β-actin and GAPDH. For each gene and timepoint, the fold-change in expression of stimulated samples was calculated relative to that of unstimulated samples.
Figures 3.6 and 3.7 show examples of an amplification plot and melt curve from the results of this experiment respectively.

Table 3.6: Primer details for mRNA expression in stimulated PBMCs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>NM_000600.4</td>
<td>5’-CAGACAGCCACTCACCTCTTC-3’</td>
<td>5’-CAGGTTGTTTCTGCAGTG-3’</td>
</tr>
<tr>
<td>TNF</td>
<td>NM_000594.3</td>
<td>5’-CATCTTCTCGAACCAGGATG-3’</td>
<td>5’-ATGAGGACAGCCCTCTGAT-3’</td>
</tr>
<tr>
<td>IFNγ</td>
<td>NM_000619.2</td>
<td>5’-GAGTGTGGAGACCATAAGGA-3’</td>
<td>5’-TTTAGCTGCGCGAAGCTTTC-3’</td>
</tr>
<tr>
<td>MCP1</td>
<td>NM_002982.3</td>
<td>5’-GAAAGTTCCTGCGCTCTTC-3’</td>
<td>5’-GCTTCTTTGGGACACTTGCTG-3’</td>
</tr>
<tr>
<td>IP-10</td>
<td>NM_001565.3</td>
<td>5’-GACTCTAATGGCATCAGGA-3’</td>
<td>5’-GATTCAAGCCTCTCTCTCAC-3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>NM_000572.2</td>
<td>5’-GAGATGCGCTCAGAGCTGTA-3’</td>
<td>5’-AAGCTGAGACCCACGACCCGA-3’</td>
</tr>
<tr>
<td>β-Actin (HKG)</td>
<td>NM_001101.4</td>
<td>5’-CATGCGAGGATGAGCTGAC-3’ (Y = C/T)</td>
<td>5’-GCTGACACCACCTCTGCTGAA-3’</td>
</tr>
<tr>
<td>GAPDH (HKG)</td>
<td>NM_002046.6</td>
<td>5’-ATGGGTGTAAGCAGTGAAGT-3’</td>
<td>5’-AGTCCCTCAGATACAAAGT-3’</td>
</tr>
</tbody>
</table>

Figure 3.6: Amplification plot example of mRNA expression of IL-6 in active TB cohort
Figure 3.7: Melt curve example of mRNA expression of IL-6 in active TB cohort

3.7 Measurement of intracellular TNF following stimulation of PBMCs with TLR ligands in different cell types

The role of identifying whether intracellular TNF was being released following TLR ligand stimulation, and which cells were implicated was investigated through the following experiment. This was conducted once, and the results are discussed in Chapter 6. In summary PBMCs from one patient collected at baseline and end of treatment were stimulated with TLR ligands, then surface and intracellular staining was undertaken to identify which cells and whether TNF was released respectively.
PBMC vials were rapidly thawed and prepared as per Section 3.4.1 and TLR ligand stimulation undertaken as per Section 3.4.2.1. In addition to the four tubes with TLR ligands and the unstimulated tube, another tube was prepared with LPS for use in compensation. The volumes of ligands required are as per Table 3.3, with extra LPS prepared for the compensation tube as mentioned above. Once tubes were prepared with 0.5 mL of ligand mix and 0.5 mL of cells, Golgi plug (1 µL/million cells, to prevent cytokines from exiting the cells) (GolgiPlug™, BD Biosciences, US), was added into tubes labelled “G0” and all tubes placed in the tissue culture incubator for two hours. At two hours, 1 µL of Golgi plug was added into tubes labelled “G2”. All samples were incubated again for further four hours. Tubes were then centrifuged at 1500 rpm for five minutes and all but 100 µL of supernatant drawn off. From the compensation LPS tube, about 300 µL of supernatant was removed. Cells were gently resuspended and the staining process commenced with fresh non-sterile FACS tubes being labelled T1 per sample (similar to Section 3.3.1).

Antibodies were added to the corresponding FACS tubes (except for intracellular staining and unstained), as listed in Table 3.7. Then, 100 µL of sample was placed into respective FACS tubes; 100 µL of sample from compensation LPS tube was added into each compensation FACS tube and unstained tube. All tubes were vortexed and placed in the dark for 20 minutes incubation. Following incubation, 1 mL of PBS was added to all tubes except the unstained and intracellular stain compensation tube, to wash off excess unbound antibody. Tubes were then centrifuged at 1500 rpm for five minutes, brake on. Then, 1 mL of supernatant was carefully drawn off and 250 µL of Cytofix-Cytoperm™ (BD® Biosciences, US) added to all tubes in the fume cupboard, whilst resuspending cells. Samples were then tightly covered and placed in the fridge overnight. The following day, samples were processed as per intracellular staining in Section 3.3.1: Perm/Wash™ buffer was prepared and all tubes had 1 mL added in the fume cupboard, then were centrifuged at 2500 rpm for five minutes; 1100 µL of supernatant was then removed and the second wash performed. Once the second wash was completed, intracellular staining with TNF-FITC (as listed in Table 3.7) was added to all sample tubes and respective compensation tube. All tubes were then placed in the fridge to slow the reaction down for 30 minutes. Following incubation, tubes with intracellular staining were washed twice with Perm/Wash™ buffer as previously described. Finally, 100 µL of PBS was added and
samples evaluated using BD FACSDiva™ software and flow plots analysed using FlowJo (Version 9, Treestar, Oregon, US). Examples of the gating strategies used are shown in Figure 3.8.

Table 3.7: Antibodies utilised in intracellular TNF measurement studies

<table>
<thead>
<tr>
<th>Tube label</th>
<th>Marker</th>
<th>Fluorophore</th>
<th>Volume</th>
<th>Antibody type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>Monocytes</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>T cells</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>mDC</td>
<td>BDCA1</td>
<td>APC</td>
<td>4 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>B cells</td>
<td>CD19</td>
<td>PE</td>
<td>5 µL</td>
<td>Anti-human</td>
</tr>
<tr>
<td></td>
<td>Intracellular TNF</td>
<td>TNF</td>
<td>FITC</td>
<td>5 µL</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>Compensation CD14</td>
<td>CD14</td>
<td>APC-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation CD3</td>
<td>CD3</td>
<td>Pacific Blue</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation CD56</td>
<td>CD56</td>
<td>PE-Cy7</td>
<td>3 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation BDCA1</td>
<td>BDCA1</td>
<td>APC</td>
<td>4 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation CD19</td>
<td>CD19</td>
<td>PE</td>
<td>5 µL</td>
<td>Anti-human</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Compensation TNF</td>
<td>TNF</td>
<td>FITC</td>
<td>5 µL</td>
<td>Anti-mouse</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Unstained</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>
Figure 3.8: Gating strategies used for intracellular TNF staining on different cell types on TLR ligand stimulated PBMCs in a patient prior to and at the end of treatment for active tuberculosis

Step #3 done on lymphocytes isolated on step 32; steps #4 and #5 performed on monocytes isolated from step #2.
3.8  

**Stimulation of healthy, non-TB-infected PBMCs with plasma from Mtb infected participants**

Based on results from laboratory work conducted, the following experiment was designed to evaluate the possibility of inhibitory effects in the plasma of patients with active TB that prevents immune control of their infection.

The overall method was to harvest PBMCs from healthy volunteer(s), stimulate these PBMCs with TLR ligands and Mtb infected plasma, and measure cytokine/chemokines.

PBMCs were harvested from healthy volunteers who were not TB-infected (QFT negative, no history of treatment for TB in past) and were otherwise well on the day of blood collection. Blood was drawn and collected into Lithium heparin tubes and PBMCs harvested using SepMate™50 (see Section 3.2.2). Mtb plasma previously stored in 1 mL vials was thawed on the day from the –80°C. These plasma samples were ones from patients who had volunteered for the MYC study but did not have complete sample collections (i.e. three or four timepoints) and therefore didn't have plasma or PBMCs used for previously described laboratory work.

3.8.1  

**Stimulation of healthy PBMCs using Mtb plasma to cell ratio of 50:50**

The TLR ligand stimulation (TLR 2, 4 7/8 and 9) protocol for measuring cytokines through cytokine bead array (Section 3.4.2.2) was followed, using 96-well plates (Costar, Round Bottom Assay Plate, Corning Inc., New York, US). Plasma from baseline timepoints of two MYC study participants with active TB and one with LTBI were used for this experiment; 250 µL of final volume was made up with 62.5 µL of PBMCs, 62.5 µL of ligand-mix and 125 µL of plasma. In addition, healthy control PBMCs were evaluated unstimulated and stimulated with control plasma and media. In addition, plasma alone (patient plasma and healthy control plasma) and media alone were also placed into wells for evaluation of cytokine levels.
3.8.2  Stimulation of healthy PBMCs using Mtb plasma to cell ratio of 80:20

The experiment as per protocol as above, using three healthy controls and three patients’ plasma with active TB. Volumes used: 250 µL of final volume was made up with 25 µL of PBMCs, 25 µL of ligand-mix and 200 µL of plasma. All else is as described above in Section 3.8.1.

3.8.2.1 Measurement of cytokines/chemokines in healthy control PBMCs stimulated with TLR ligands and Mtb plasma through Cytokine Bead Arrays

Standard BD Cytometric Bead Array Protein Flex Sets™ (BD Biosciences, San Jose, US) were used to measure chemokines and cytokines in the supernatants of the stimulated healthy PBMCs. Cytokines/chemokines measured were IFNγ, TNF, MCP1 and IL-6. Assay procedure was conducted as per manufacturer’s recommendations, as described in Section 3.4.4.

Dilutions were performed for all stimulated and unstimulated supernatants in labelled Eppendorf tubes as per templates, prior to addition of sample to respective well(s) for the assay procedure. Stimulated supernatants were used neat, 1/10 dilution and 1/100 dilution for this experiment.

Cytometer set up and data acquisition was as described previously in Section 3.4.4.5.

3.8.3  Stimulation of healthy PBMCs using variable Mtb plasma to cell ratios

This experiment was designed to further evaluate the possibility of the presence of an inhibitory effect contained in plasma of patients with active TB infection. Four healthy controls, without any form of TB infection, had blood drawn and PBMCs harvested using SepMate™50 (Section 3.2.2). Unstimulated, and TLR ligand 2 and 9 stimulated Healthy control PBMCs were evaluated. The key difference from Section 3.8.1 was the use of variable ratios of PBMCs to plasma. The following plasma to cell ratios were undertaken:

Table 3.8: Ligand Mix #1 volumes used in preparation of TLR ligand stimulation of healthy control PBMCs with variable plasma to cell ratios

<table>
<thead>
<tr>
<th>TLR</th>
<th>TLR ligand</th>
<th>2x [</th>
<th>Volume of ligand</th>
<th>Volume of media</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>P3C</td>
<td>200 ng/mL</td>
<td>40 µL</td>
<td>960 µL</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG2006</td>
<td>0.6 µM</td>
<td>11.4 µL</td>
<td>988.6 µL</td>
</tr>
</tbody>
</table>

To enable appropriate ratios of ligand, cells and plasma, each TLR ligand was made into three concentrations, using the following dilutions from “Ligand Mix #1” (Table 3.8). “Ligand Mix #2” was made with a 1:4 dilution from Ligand Mix #1 (375 µL and 825 µL of media), whilst “Ligand Mix #3” was made with a 1:7 dilution from Ligand Mix #1 (200 µL of Ligand Mix #1 and 1200 µL of media). Once the different ligand mixes were made, the following quantities of plasma and PBMC and ligand mix were used. This allowed for one concentration of PBMCs to be made up at 10 x 10^6 cells/mL for each healthy control. The final volumes used are as follows:

**Plasma:cells 80:20** (labelled Ligand Mix #1)
200 µL plasma + 25 µL PBMCs and 25 µL of Ligand Mix #1

**Plasma:cells 50:50** (labelled Ligand Mix #2)
125 µL plasma + 25 µL PBMCs and 100 µL of Ligand Mix #2

**Plasma:cells 20:80** (labelled Ligand Mix #3)
50 µL plasma + 25 µL PBMCs and 175 µL of Ligand Mix #3

**Media only and healthy control**
125 µL media + 25 µL PBMCs and 100 µL of Ligand Mix #2

The plasma (or media) and ligand mix was placed in wells as per template and, finally, 25 µL of respective PBMCs were put into well(s) and gently mixed and placed in CO2 incubator for 20 hours overnight. The following day, plates were centrifuged at 1500 rpm for five minutes and supernatants carefully drawn off into a fresh plate for measurement of cytokines through CBA.
3.8.3.1 Measurement of cytokines/chemokines in healthy control PBMCs stimulated with TLR ligands and Mtb plasma through Cytokine Bead Arrays

Standard BD Cytometric Bead Array Protein Flex Sets™ (BD Biosciences, San Jose, US) were used to measure chemokines and cytokines in the supernatants of the stimulated healthy PBMCs. Cytokines/chemokines measured were TNF, MCP1 and IL-6. Assay procedure was conducted as per manufacturer’s recommendations, as described in Section 3.4.4.

Dilutions were performed for all stimulated and unstimulated supernatants in labelled Eppendorf tubes as per templates, prior to addition of sample to respective well(s) for assay procedure. Stimulated supernatants were used neat and 1/20 dilution for this experiment.

Cytometer set up and data acquisition was as described previously in Section 3.4.4.5.

3.9 QuantiFERON-TB Gold Plus: laboratory methods

Recruitment commenced in October 2013 and was completed in August 2016. Patients commencing treatment for active TB or LTBI were offered participation in the study. Informed, written consent was gained from those who volunteered to participate. Participants were allocated a sequential number and personal details were only available to study coordinators to enable follow-up blood samples to be collected. Laboratory processing of samples for QFT study was done by Jackie Yu. I aimed to recruit 50 patients with active TB and LTBI.

A minimum of three blood samples were planned to be collected:

- Baseline (TP0) (within 14 days of treatment commencement)
- Early (TP1 or TP2) (at one and/or two months into treatment)
- Final (TPF) (end of treatment sample, usually at six, nine or 12 months of treatment)

An amendment to the methodology was made in May 2015 whereby patients were able to consent to provision of a single (i.e. baseline only TP0) blood sample.
3.9.1 Blood sample collection

Whole blood samples were collected for QFT and QFT-Plus in lithium heparin tubes and transported to St Vincent’s Hospital Immunology Research Centre, where samples were processed. Blood samples were transferred into a total of five assay tubes: QFT, QFT-Plus (TB1 and TB2), mitogen (positive control) and nil (negative control) within six hours of collection. Once tubes were filled according to the manufacturer’s description, they were shaken firmly to coat the tube and dissolve the antigen lining the respective tubes. The samples were then incubated and plasma harvested by centrifugation at 2000 g for 15 minutes. Plasma samples were then frozen at −80°C until ELISA was performed in batches, according to the manufacturer’s recommendations.226

3.9.2 ELISA assays for QuantiFERON-TB

The plasma samples were thawed at room temperature and analysed using standard QFT ELISA kits for IFNγ production according to manufacturer’s recommendations.225,226 Each ELISA plate included both 4- and 8-point standards in duplicate. Multiple samples from the same patient were analysed on a single plate. Further dilutions of plasma between the range of 1:10 and 1:100 were performed and ELISA repeated where IFNγ concentrations in the neat samples were measured > 10 IU/mL. Results generated from the 4-point standard curves were used for data analysis.

3.10 Statistical analyses

All statistical analyses for the following chapters were undertaken by myself, except where specified.

3.10.1 Statistical analyses of MYC study (Chapters 4, 5, 6 and 7)

TLR expression and functional assays results were analysed using Microsoft® Excel for Mac, Version 16.15 (Microsoft Corporation, California, US) and Prism 7.0b (GraphPad Software, California, US). Fold changes in TLR expression and function were used to accommodate any variation within the individuals from baseline (at start of treatment) to the other timepoints when comparing individuals within one cohort (active patients or LTBI patients). This process is referred to as ‘normalisation’ of results in this thesis.
Wilcoxon rank-sum test (for non-parametric data) with an alpha of 0.5 was used in evaluation of changes in the cohorts between each timepoint through treatment for active or latent TB infection.

Column graphs when shown are depicted with standard deviation (SD) of the mean. Outlier tests were used with a ROUT 1% are described where necessary. ROUT is a statistical method used for identifying outliers in non-linear regression analyses; this was done in Graph Pad Prism).

Correlation evaluations were done in Prism, showing Spearman’s r coefficient with 95% confidence intervals, evaluating the results of cytokine measurements through ELISA and CBA, ES-CBA and Magnetic Luminex assays.

Fold changes in mRNA expression of cytokine/chemokine results discussed in Chapter 4 are shown as ratio to the individual unstimulated level for each timepoint.

When comparing results of TLR expression and function, as well as plasma cytokine results, between study cohorts, that is, between patients with active and latent infection, Mann–Whitney U tests (two-sided) were used, with a p value of ≤ 0.05 considered statistically significant (p values to four decimal points are shown in all investigative chapters, except those undertaken by Dr Sara Vogrin, which are shown to three decimal points). Two-way Anova was applied in evaluating whether an inhibitory effect of Mtb plasma occurred in Chapter 6.2.

Chapter 7 statistical analyses were done using Stata 15.1 (StataCorp, College Station, Texas, USA) by Dr Sara Vogrin. Correlation was assessed with Spearman’s r. The natural logarithm of TLR expression was used to determine association between vitamin D levels and TLR expression. Disease burden was assessed against stimulated cytokine results with Wilcoxon signed-rank test. Generalised estimating equations (GEE) were utilised to see the effect of clinical variables (separately) on TLR expression distribution through treatment.
3.10.2 Statistical analyses of QuantiFERON study (Chapter 8)

Clinical data was collated and analysed through Microsoft® Excel for Mac, Version 16.15 (Microsoft Corporation, California, US) and laboratory data using Stata 15.1 (StataCorp, College Station, Texas, US).

The following statistical analyses were performed by Dr Sara Vogrin. Sensitivity was determined using microbiological, anatomical pathology and molecular diagnostic results, with the prevalence based on the cumulative diagnostic results.

Agreement between QFT and QFT-Plus was examined using Cohen’s kappa. Bland-Altman plots were constructed to evaluate systematic differences in IFNγ responses between the two tests. The agreement between the tests and mean difference, with 95% limits of agreement in QFT and QFT-Plus antigen tubes was evaluated, along with linear regression analyses to determine the correlation of IFNγ responses between the new TB-antigen tubes.

Changes in absolute IFNγ measurements over treatment course were evaluated using GEE for each outcome separately (QFT, TB1, TB2, difference between QFT-Plus antigen tubes and ratio in QFT-Plus antigen tubes). All outcomes required a transformation into its natural logarithm. Results are presented as exponentiated coefficients with 95% confidence intervals.

Wilcoxon rank-sum test was used to assess the associations between categorical variables (factors of TB severity, recent exposure, active vs latent) and levels of QFT and QFT-Plus responses while Pearson’s correlation was used for continuous variables (age). All results are presented as median (interquartile range) or frequency (percentage). Results with a p value of $\leq 0.05$ were considered statistically significant.
Chapter 4: Toll-like receptor expression and function in active and latent tuberculosis infections

Toll-like receptors (TLRs) play a significant role in initiating the human immune response to mycobacterial infections, along with other pathogen recognition receptors (PRRs). These transmembrane receptors occur on immune type cells and are found on the cell surface and intracellularly. TLR2,4,7,8 and 9 in particular have been shown to have roles in induction of the innate immune response to Mtb infection.6,20,123,207 Through their activation, these TLRs can trigger a cascade of signal transduction, resulting in downstream gene transcription, which leads to the production of pro-inflammatory cytokines and chemokines. TLRs, in combination with other innate immune and cellular processes, lead to Type 1 IFN activation and instigation of the adaptive immune response, resulting in control or maintenance of a dynamic equilibrium within the host.4,123,172 Studies in humans have shown cellular and functional changes relating to TLR signalling in recent years145,148-150,152, whilst genetic association studies have further illustrated the role of TLR polymorphisms to disease susceptibility.8,10-12

Active tuberculosis (TB) and latent tuberculosis infection (LTBI) are two forms within a spectrum of Mtb infection in humans. Active TB diagnosis is made based on the presence of symptoms and/or diagnostic tests identifying the presence of the organism in samples from the disease site. The current gold standard for active TB diagnosis is a positive culture result. LTBI is diagnosed by clinicians where the patient is asymptomatic and has evidence of a cellular immune response to Mtb antigens. There is no gold standard test for LTBI infection currently. Treatment for active TB involves multiple antibiotics for six to 12 months for drug-susceptible TB and four or nine months of a single antibiotic for LTBI.69,77 Timely identification of those with active TB (and commencing drug treatment), as well as those with LTBI who may be at high risk of developing active TB, is crucial to reach the Global End TB Strategy.2

Evaluation of TLR responses in patients with active TB and LTBI through antibiotic treatment may shed further light on the differences between these two forms of Mtb infection. Because of the lack of ‘memory’ in innate immune responses, it is postulated
that there are differences in TLR expression and function between those with active TB and LTBI and that these will vary through treatment. I aim to identify patterns in TLR responses in these two clinically differentiated groups of patients with Mtb infection.

This chapter entails the findings of innate immune marker investigations detailed in Chapter 3. The roles of TLR 2,4,7,8 and 9 are investigated in patients with active tuberculosis and LTBI. Specifically, the results of the surface and intracellular TLR expression on PBMCs, TLR function (evaluated through cytokine/chemokine measurements in supernatants of TLR ligand stimulated PBMCs) and plasma cytokine/chemokine measurements undertaken in the MYC active TB and latent TB (MYC LTBI) cohorts are discussed. These immune studies were undertaken through the antibiotic treatment course for the two forms of TB infection.

4.1 MYC cohort participant recruitment

A total of 80 patients were recruited between February 2011 and August 2014 into the MYC-active TB cohort (with a hiatus between August 2011 to March 2013 due to maternity leave). Of these, a total of five withdrew and three were excluded. The reasons for withdrawal in this cohort were change of mind (two), refused final blood samples collected (two) and one did not provide a reason. Three were excluded because they had not commenced anti-tuberculous therapy. Many participants did not have final blood samples collected and/or were lost to follow-up. In the following work described, the results are based on 53 patients who provided a minimum of three blood samples, including baseline, at least one of timepoint 1 (TP1) and/or TP2 and TP final (with the exception of MYC A3, whose laboratory results have been included, but did not have the final blood sample collected).
Participants for the **MYC LTBI cohort** were recruited between 2011 and 2014 if they met criteria outlined in Chapter 3. A total of 39 participants were recruited, one withdrew (no reason provided) and three were excluded because the baseline QFT negative. Fourteen participants provided at least three blood samples (including baseline and end of treatment samples). Samples of 22 participants have been used for the laboratory work described in this thesis (14 with at least three samples through treatment and eight at baseline alone).

**Figure 4.1:** Age and gender distribution of MYC active TB cohort (2011–2015)

**Figure 4.2:** Age and gender distribution of MYC LTBI cohort (2011–2015)
4.2 Demographics and clinical details of active TB participants

Twenty-nine participants were male (55%), and the mean age was 38.5 (SD ± 16.4 years) (see Figure 4.1 and Table 4.1). The majority were born in TB endemic countries, with India (n = 20, 38%) and Vietnam (n = 5, 9%) being the two countries most commonly represented. Three participants were born in Australia. Participants’ region of birth is described in Table 4.1 (those born in Australia are listed separately, but otherwise would be included in the Western Pacific Region).

Approximately 43% of participants had been in Australia for less than five years at the time of diagnosis. Five had been treated for active TB in the past. The majority reported symptoms at the time of diagnosis (85%). Over 70% of participants had pulmonary TB, whilst 20% had more than one site of disease involved (Table 4.2).
Table 4.1: Baseline demographics of MYC active TB cohort

<table>
<thead>
<tr>
<th>Demographic characteristics (total 53)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>29 (55)</td>
</tr>
<tr>
<td>Female</td>
<td>24 (45)</td>
</tr>
<tr>
<td>Mean age (years ± SD)</td>
<td>38.5 (SD 16.4 years)</td>
</tr>
<tr>
<td>Region of birth*</td>
<td></td>
</tr>
<tr>
<td>SE Asia</td>
<td>26 (49)</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>14 (26.4)</td>
</tr>
<tr>
<td>Africa</td>
<td>4 (7.5)</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>4 (5.7)</td>
</tr>
<tr>
<td>Europe</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>Australia</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>Time in Australia prior to diagnosis</td>
<td></td>
</tr>
<tr>
<td>&lt; 5 years</td>
<td>23 (43.4)</td>
</tr>
<tr>
<td>5–15 years</td>
<td>13 (24.5)</td>
</tr>
<tr>
<td>&gt; 15 years</td>
<td>9 (17)</td>
</tr>
<tr>
<td>Australian born</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (9.4)</td>
</tr>
<tr>
<td>Previous TB treatment</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>48 (90.6)</td>
</tr>
<tr>
<td>Yes</td>
<td>5 (9.4)</td>
</tr>
<tr>
<td>BCG vaccination</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18 (34)</td>
</tr>
<tr>
<td>Childhood vaccination</td>
<td>17 (94.4)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>No</td>
<td>6 (11)</td>
</tr>
<tr>
<td>Data not available</td>
<td>29 (55)</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>34 (64.2)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>Hepatitis / liver dysfunction</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>COPD / asthma</td>
<td>6 (11.3)</td>
</tr>
<tr>
<td>Cardiac / vascular disease</td>
<td>4 (7.5)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>0</td>
</tr>
<tr>
<td>Immunosuppression*</td>
<td>1</td>
</tr>
</tbody>
</table>

* Region of birth according to WHO grouping: Australia listed separately; would otherwise be included in Western Pacific region.1

# One participant was on systemic immunosuppression for rheumatoid arthritis: prednisolone and methotrexate.
Table 4.2: Clinical disease and microbiological findings in MYC active TB cohort

<table>
<thead>
<tr>
<th>Clinical and microbiological characteristics (total 53)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms (present)</td>
<td>45/53 (85%)</td>
</tr>
<tr>
<td>TB disease†</td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>38 (71.7)</td>
</tr>
<tr>
<td>non-cavitary</td>
<td>27 (71)</td>
</tr>
<tr>
<td>cavitary</td>
<td>11 (29)</td>
</tr>
<tr>
<td>LN disease</td>
<td>19 (35.8)</td>
</tr>
<tr>
<td>Pleural</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>Abdominal/cardiac</td>
<td>2 (3.8)</td>
</tr>
<tr>
<td>Spinal/bone</td>
<td>4 (7.5)</td>
</tr>
<tr>
<td>Skin/eyes</td>
<td>1 (1.9)</td>
</tr>
<tr>
<td>&gt; one site</td>
<td>11 (20.8)</td>
</tr>
<tr>
<td>CXR</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>11 (20.8)</td>
</tr>
<tr>
<td>Cavity</td>
<td>11 (20.7)</td>
</tr>
<tr>
<td>Consolidation and/or effusion</td>
<td>26 (49)</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>3 (5.7)</td>
</tr>
<tr>
<td>&gt; 1 lobe involvement</td>
<td>15 (28)</td>
</tr>
<tr>
<td>Smear positive (sputum / BAL sampling)**</td>
<td>19/38 (50)</td>
</tr>
<tr>
<td>Culture</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>44 (83)</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (17)</td>
</tr>
<tr>
<td>MTB PCR and/or histology consistent with TB*</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>Drug sensitivity</td>
<td></td>
</tr>
<tr>
<td>Pan-sensitive</td>
<td>38/44</td>
</tr>
<tr>
<td>Isoniazid resistance only</td>
<td>3/44</td>
</tr>
<tr>
<td>MDR-TBΦ</td>
<td>3/44</td>
</tr>
</tbody>
</table>

# TB disease according to site.
** Respiratory samples only tested for acid fast bacilli (AFB) included. Bronchoalveolar lavage (BAL) sampling involved collection for culture / MTB PCR and cytological examination.
∞ MTB PCR IS6110 results where applicable; anatomical pathology results showing necrotising granulomatous changes / caseating granulomas or non-necrotising granulomas.
Φ MDR-TB defined as per WHO Guidelines.\(^1,\)\(^80\)
4.2.1 Toll-like receptor expression in active tuberculosis

Gating strategies for the work below is described in Chapter 3. In summary, monocyte and lymphocyte gates were applied and consequently CD3, CD56 and CD14 gates applied to differentiate cell types further. Finally, TLR2, 4, 7 gates were applied and compensation undertaken. Geometric mean fluorescence (GMF) for each sample and isotype control were calculated using FlowJo® (Treestar, US) and results analysed using Prism 7.0 (GraphPad Software, US).

The results discussed in the following section refer to the changes of TLR expression using normalised data, performing Wilcoxon signed-rank tests between timepoints as two-tailed, non-parametric analyses. Given the inherent variability within and between individuals with respect to TLR expression and function, ‘normalised’ geometric fluorescence is shown unless specified (as previously discussed in Chapter 3). Figure 4.3 highlights the graphical differences in using GMF and GMF ratio (i.e. evaluating the fold differences between baseline and treatment timepoints). Each data point represents a participant at the respective timepoint, lines at mean with standard deviation (SD) are shown in graphs, p values of ≤ 0.05 are shown.
Figure 4.3: Comparison of effect of normalising GMF to enable comparison of TLR expression with individuals in a group in MYC active TB, through antibiotic treatment*

A & B: TLR2 expression on NK cells with and without normalisation respectively.
C & D: TLR7 expression on CD14+ monocytes with and without normalisation respectively.
TP = timepoint; TP0, 1, 2 and T final represent results at baseline, one month, two months and end of treatment; each data represents one participant at each timepoint.
* Normalisation refers to using ratios (normalised baseline = baseline / baseline; T1(n) = timepoint 1/baseline; TP2(n) = timepoint 2/baseline; T final(n) = timepoint final / baseline). Lines represent mean ± SD.
TLR2 expression was significantly different between timepoints on NKT and NK cells. There was a significant increase between TF(n) and T2(n) on NKT cells (p = 0.0369, Figure 4.4A). TLR2 expression difference found between TP1(n) and normalised baseline on NK cells (p = 0.0351) is shown in Figure 4.4C. The effect of excluding outliers is shown in Figure 4.4B and D. Outlier exclusion has been based on Outlier Test with a Q1% in ROUT (see Section 3.10). The outliers have been included in further analyses and discussion (except where indicated), as the Wilcoxon signed-rank test accommodates the effects of the outliers.

![Graphs A, B, C, D]

**Figure 4.4:** Significant differences of TLR2 expression on NKT and NK cells through antibiotic treatment in MYC active TB cohort with and without outliers*

TP = timepoint; TP0, 1, 2 and T final represent results at baseline, one month, two months and end of treatment; each data point represents one participant at each timepoint.

* Normalisation refers to using ratios (normalised baseline = baseline / timepoint baseline; T1(n) = timepoint 1/baseline; TP2(n) = timepoint 2/baseline; T final(n) = timepoint final/ timepoint baseline. Lines represent mean ± SD.
TLR4 expression did not vary in active TB in this cohort when evaluating CD14+ monocytes, nor the majority of evaluations on NKT, NK and NK cell subsets. The only significant change was TLR4 expression decrease between TF(n) and T1(n) on NK cells (p = 0.0451, Figure 4.5).

TLR7 expression altered in several different cell lines through anti-tuberculous treatment (Figure 4.6). These significant increases in normalised GMF ratios were seen on CD14+ monocytes and NK cells.

![Figure 4.5: TLR4 changes on NK cells during treatment in MYC active TB*](image)

TP = timepoint; TP0, 1, 2 and T final represent results at baseline, one month, two months and end of treatment; each data point represents one participant at each timepoint.

* Normalisation refers to using ratios (timepoint baseline / baseline, timepoint 1/baseline, timepoint 2/baseline, timepoint final/baseline) in analyses to enable comparison as a group. Lines represent mean ± SD.

A summary of significant changes discussed thus far is detailed in Table 4.3. It highlights that while TLR expression evaluated through active TB treatment changes, the commonest timepoint for differences overall was between end of treatment and TP1(n). This is an important consideration as it was observed on several cell types, with an increase in TLR2 and 7 expression, but a decrease in TLR4 expression. Interestingly, no significant changes were found between TP2(n) and other timepoints on any cell type of TLR 2, 4 or 7 expression.
Figure 4.6: Significant differences of TLR7 expression through antibiotic treatment in MYC active TB cohort on CD14+ monocytes (A) and NK cells (B)

TP = timepoint; TP0, 1, 2 and T final represent results at baseline, one month, two months and end of treatment; each data point represents one participant at each timepoint. Lines represent mean ± SD.

Table 4.3: Significant changes on different cell types found in TLR expression in MYC active TB cohort

<table>
<thead>
<tr>
<th>Cell type</th>
<th>TLR</th>
<th>T1(n) vs baseline(n)</th>
<th>T2(n) vs baseline(n)</th>
<th>T final(n) vs baseline(n)</th>
<th>T final(n) vs T1(n)</th>
<th>T final(n) vs T2(n)</th>
<th>T2(n) vs T1(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14+ monocytes</td>
<td>TLR7</td>
<td>NS</td>
<td>NS</td>
<td><strong>p = 0.0140</strong></td>
<td><strong>p = 0.0017</strong></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR2</td>
<td><strong>p = 0.0351</strong></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NK cells</td>
<td>TLR4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td><strong>p = 0.0451</strong></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR7</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td><strong>p = 0.0486</strong></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NKT cells</td>
<td>TLR2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td><strong>p = 0.0369</strong></td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: not significant.

As a way of contrast, Figure 4.7 shows grouped TLR expression through active TB treatment (of cells which had significant differences when normalised data was analysed). This highlights the overall range of GMF and variation depending on the cell type and TLR expression measured in the MYC active TB cohort. Overall, TLR7 expression was highest in NK cells and TLR4 expression was the lowest measured on the same cell type.
4.2.2 Toll-like receptor function in active TB: measuring cytokines/chemokines following PBMC stimulation with TLR ligands

Results of measurement of cytokines/chemokines following stimulation by TLR ligands is described here. As detailed in Section 3.4, cytokines were initially measured via ELISA (2011–2014), then consequently through cytokine bead arrays (CBAs). There were differences in the overall results when absolute values were assessed but given the intra and interindividual variability in TLR function, these results are normalised.

IL-10, IFNγ, IL-17A, IL-17F, IL-12p70, results are not shown as minimal cytokine levels were detectable (the number of patients evaluated in these tests were 32, 31, six, three and six, respectively). Raw data of these cytokine measurements are presented in Appendix 2. The implications for this are discussed in Section 4.3.

4.2.2.1 ELISA and CBA results comparison

Given the changes in laboratory methods for measurement of cytokines post stimulation, a comparison of results was undertaken. Spearman’s correlation (two tailed) revealed a poor correlation between ELISA results from 2011 when compared to stimulated samples from 2017. Cytokine measurements (IL-6 and TNF) were measured in supernatants simultaneously via ELISA and CBA (Appendix 3). Correlation between CBA
results of 2016/2017 and ELISA 2017 was high: TNF combined evaluation (unstimulated and TLR9 ligand stimulated samples) with Spearman’s r coefficient 0.988 (95% CI 0.976 to 0.993) p < 0.0001, IL-6 of same samples Spearman’s r coefficient 0.744 (95% CI 0.553 to 0.860), p < 0.0001. However, the comparison of ELISA 2011 results to ELISA 2017 for combined TNF and IL-6 was poor (Spearman’s r 0.074, 95% CI –0.360 to 0.482 and Spearman’s r 0.297, 95% CI –0.248 to 0.699), as shown in Figure 4.8.

Given the differences in these findings, whilst ELISA results from 2011 have been included in normalised data in tabular form to highlight the effect on the overall findings, further discussions will relate to CBA results only.

Table 4.4 shows significant results when normalised data were analysed using CBA only (ELISA and CBA combined results are in Appendix 4). Figure 4.9 compares the differences in significant results to highlight ELISA and CBA combined results and CBA only in graphical form as examples. When ELISA and CBA results are combined, a total of 53 participants samples are shown, and up to 39 when CBA results alone are analysed.
Figure 4.8: Correlation between supernatant cytokine measurements between ELISA 2017 and CBA 2016/17

A & B: ELISA 2017 comparison to CBA 2016/17, TNF and IL-6 respectively (unstimulated and TLR9 ligand stimulated samples combined).

C & D: ELISA 2017 compared to ELISA 2011, TNF and IL-6 respectively (unstimulated and TLR9 ligand stimulated samples combined). NS = not significant.
Figure 4.9: Comparison of cytokine results of CBA only and ELISA and CBA in supernatants following TLR ligand stimulation in active TB cohort (normalised)

A & B: TNF CBA results alone and ELISA and CBA results (unstimulated).
C & D: IL-6 in CBA alone and ELISA and CBA combined respectively (post TLR4 ligand stimulation).
Lines represent mean ± SD.
Table 4.4: Summary of significant differences in cytokine/chemokine measurements in supernatants through active TB treatment in MYC cohort following TLR ligand stimulation of PBMCs using cytokine bead array

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>TLR ligand</th>
<th>T1(n) vs baseline(n)</th>
<th>T2(n) vs baseline(n)</th>
<th>T final(n) vs T1(n) vs T2(n)</th>
<th>T final(n) vs T1(n)</th>
<th>T final(n) vs T2(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>Unstimulated</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>p = 0.0026</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR2</td>
<td>NS</td>
<td>NS</td>
<td>p = 0.0323</td>
<td>NS</td>
<td>p = 0.0359</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>p = 0.0125</td>
<td>p = 0.0066</td>
<td>p = 0.0344</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR7/8</td>
<td>p = 0.0044</td>
<td>p = 0.0107</td>
<td>p = 0.0116</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR9</td>
<td>NS</td>
<td>p = 0.0017</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6</td>
<td>TLR2</td>
<td>NS</td>
<td>p = 0.0073</td>
<td>p = 0.0104</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>p = 0.0024</td>
<td>p = 0.0038</td>
<td>p = 0.0216</td>
<td>p = 0.0274</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR7/8</td>
<td>p = 0.0130</td>
<td>p = 0.0012</td>
<td>p = 0.0168</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MCP1</td>
<td>TLR4</td>
<td>p = 0.0019</td>
<td>p = 0.0005</td>
<td>p = 0.0025</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR7/8</td>
<td>p = 0.0044</td>
<td>p = 0.0018</td>
<td>p = 0.0046</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR9</td>
<td>NS</td>
<td>NS</td>
<td>p = 0.0293</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-8</td>
<td>TLR4</td>
<td>NS</td>
<td>p = 0.0087</td>
<td>p = 0.0448</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TLR7/8</td>
<td>p = 0.0095</td>
<td>p = 0.0023</td>
<td>p = 0.0200</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Wilcoxon signed-rank test between each timepoint, p < 0.05 shown. NS: not significant.

4.2.2.2 Cytokine and chemokine measurements using BD Cytokines Bead Array Flex Sets

TNF levels in supernatants from stimulated PMBCs were affected by antibiotic treatment for active TB. These changes were seen without and after stimulation with TLR2, TLR4, TLR7/8 and TLR9 ligands. Figure 4.10 shows TNF measurements following TLR ligand stimulation. From a clinical perspective, both one and two months of treatment are considered to be in the ‘induction’ or ‘intensive’ phase of treatment, and therefore potentially comparable to each other.5,17 GMF ratios were higher between all timepoints to baseline with TLR4, which reduced through treatment (Figure 4.10C). There were
increased TNF levels in TLR7/8 and TLR9 stimulated samples at different timepoints (Figure 4.10D and E, respectively).

Figure 4.11 highlights significant changes found between treatment timepoints in IL-6, MCP1 and IL-8 measurements of the stimulated PBMCs. IL-6 levels were generally higher through treatment following TLR2, 4 and 7/8 ligand stimulation compared to baseline (Figure 4.11A to C, respectively). Following TLR2 ligand stimulation, levels generally increased through therapy, whilst with TLR4, levels were reduced (Figure 4.11A and B, respectively). TLR7/8 ligand stimulation resulted in higher levels through treatment compared to baseline, but generally with a fall through antibiotic therapy (Figure 4.11C). The only significant difference between end of treatment and early timepoints (T1) was following TLR4 ligand stimulation (p = 0.0274) (Figure 4.11B).

Chemokine MCP1 responses were significantly different between the end of treatment and baseline following TLR4, 7/8 and 9 ligand stimulation (p = 0.0025, 0.0046 and 0.0293 respectively) (Figure 4.11D to F, respectively). Significant changes were also found between early timepoints and baseline following TLR4 and 7/8 ligand stimulation (Figure 4.11D and 4.11E). Once again, chemokine levels were higher through treatment compared to baseline, whilst dropping through treatment.

IL-8 measurements found to be different through TB treatment following TLR ligand 4 and 7/8 stimulation are shown in Figure 4.11G and 4.11F, with similar patterns to that of other cytokines.

The results discussed so far show the comparative changes within the active TB cohort when each of the cytokines/chemokines are measured. Figure 4.12 highlights measurements of different proteins following TLR ligand stimulation in absolute levels. It shows that levels of cytokines vary considerably between TLR ligands and the protein being measured: for example, TNF levels were highest after TLR7/8 (Figure 4.12B), whilst IL-6 levels were highest following TLR4 and TLR7/8 stimulation (Figure 4.12B).
Figure 4.10: TNF levels in supernatants following PBMC stimulation with TLR ligands

TP = timepoint; TP0, 1, 2 and T final represent results at baseline, one month, two months and end of treatment; each data point represents one participant at each timepoint. 
p values < 0.05 shown with Wilcoxon signed-rank test (two-tailed). Lines represent mean ± SD.
Figure 4.11: IL-6, MCP1 and IL-8 levels in supernatants following PBMC stimulation with TLR ligands

A-C: IL-6 measurement ratios through active TB. D-F: MCP1 measurement ratios through active TB. G & H: IL-8 measurement ratios through active TB.

TP = timepoint; TP0, 1, 2 and T final represent results at baseline, one month, two months and end of treatment; each data point represents one participant at each timepoint. Normalisation refers to using ratios (timepoint baseline / timepoint baseline, timepoint 1/baseline, timepoint 2/baseline, timepoint final/baseline) in analyses to enable comparison as a group (i.e. evaluating fold changes through antibiotic treatment). Lines represent mean ± SD.
Figure 4.12: Comparison of cytokines and chemokines following selected TLR ligand stimulation in active TB measured through CBA

A: IL-6 and IL-8 levels in TLR ligand stimulated supernatants. B: TNF levels in TLR ligand stimulated supernatants. C: MCP1 levels in TLR ligand stimulated supernatants. Grouped summary data showing mean with SD.
4.2.3 Messenger RNA expression of cytokines/chemokines of stimulated PBMCs

Messenger RNA expression studies were carried out to determine if the cytokine/chemokine changes were transcriptional or post-transcriptional. The methodology is outlined in Section 3.6, and results of two patients with active TB through treatment are presented here.

Table 4.5 shows the results of IL-6 and TNF mRNA expression following TLR ligand stimulation in PBMCs through treatment in two patients with active TB. Figures 4.13A and 4.13B show these results in graphical form, highlighting the comparison relative to the unstimulated mRNA expression of each cytokine at the specific timepoint.

Table 4.5: mRNA expression of IL-6 and TNF levels relative to the unstimulated result for each timepoint, using delta-delta Ct method and housekeeping genes (HKG)

<table>
<thead>
<tr>
<th>IL-6</th>
<th>US</th>
<th>CPG</th>
<th>LPS</th>
<th>P3C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A50.0</td>
<td>A50.2</td>
<td>A50.6</td>
<td>A48.0</td>
</tr>
<tr>
<td>US</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CPG</td>
<td>21</td>
<td>20</td>
<td>110</td>
<td>16</td>
</tr>
<tr>
<td>R848</td>
<td>208</td>
<td>1642</td>
<td>1458</td>
<td>1579</td>
</tr>
<tr>
<td>LPS</td>
<td>117</td>
<td>1258</td>
<td>747</td>
<td>578</td>
</tr>
<tr>
<td>P3C</td>
<td>19</td>
<td>485</td>
<td>246</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TNF</th>
<th>A50.0</th>
<th>A50.2</th>
<th>A50.6</th>
<th>A48.0</th>
<th>A48.1</th>
<th>A48.6</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CPG</td>
<td>2.7</td>
<td>3.2</td>
<td>2.7</td>
<td>1.7</td>
<td>2.9</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>R848</td>
<td>3.0</td>
<td>4.9</td>
<td>3.0</td>
<td>2.2</td>
<td>8.7</td>
<td>2.1</td>
<td>4.0</td>
</tr>
<tr>
<td>LPS</td>
<td>3.6</td>
<td>5.5</td>
<td>3.4</td>
<td>1.8</td>
<td>4.9</td>
<td>1.8</td>
<td>3.5</td>
</tr>
<tr>
<td>P3C</td>
<td>3.7</td>
<td>5.2</td>
<td>4.1</td>
<td>1.2</td>
<td>2.1</td>
<td>1.3</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Figure 4.13: Cytokine IL-6 (A) and TNF mRNA (B) expression following stimulation of PBMCs with TLR ligands

Each data point represents a participant at different sample points (i.e. two patients at three different timepoints). Lines represent mean with SD. Results shown are relative to the unstimulated level of mRNA expression of the cytokine at each timepoint.

Comparison of cytokines measured in supernatants of stimulated PBMCs in the two patients above with the mRNA expression of IL-6 and TNF are shown in Figure 4.14 and Figure 4.15, respectively. The graphs show that overall, mRNA expression of the cytokines is relatively similar to the protein measurements. To enable this comparison, cytokine measurements were taken as ratios relative to the respective timepoint unstimulated protein level.

In IL-6 evaluation, the mean mRNA expression appears to correlate with the respective TLR-ligand function, with TLR9 ligand stimulated protein levels being the lowest, and TLR7/8 ligand stimulated being the highest (Figure 4.14). Whilst the correlation was high between mRNA expression and cytokines in stimulated supernatants, this only reached significance on comparison of one individual’s results (MYC A 50) (Spearman’s coefficient 1, p = 0.0167) (Figure 4.14).
Figure 4.14: IL-6 mRNA expression compared to protein measured in stimulated PBMC supernatants

Box plots represent mRNA expression (line at mean). Data points and lines represent the relative measurement of the cytokine to the respective unstimulated sample, at different timepoints in stimulated supernatants of PBMCs. US: unstimulated; CPG: TLR9 ligand; R848: TLR7/8 ligand; LPS: TLR4 ligand; P3C: TLR2 ligand. Spearman’s coefficient of mRNA expression compared to cytokine levels was 0.9 and 1 (p = 0.0833 and 0.0167 respectively) for the two individuals evaluated.

TNF mRNA expression patterns also similarly corresponded to the respective protein measurements (relative to the unstimulated at the timepoint) following TLR ligand stimulation and were also highest following TLR7/8 ligand stimulation and lowest with TLR9 (Figure 4.15). Protein measurement following TLR2 ligand stimulation was more varied. Spearman’s coefficient was 0.9 for MYC A48 and 0.6 for MYC A50 when evaluating correlation between mRNA expression and cytokine responses in stimulated PBMCs (p = 0.3500 and 0.0833 respectively) (Figure 4.15).
Figure 4.15: TNF mRNA expression compared to protein measured in stimulated PBMC supernatants

Box plots represent mRNA expression (line at mean). Data points and lines represent the relative measurement of the cytokine to the respective unstimulated sample, at different timepoints in stimulated supernatants of PBMCs. US: unstimulated; CPG: TLR9 ligand; R848: TLR7/8 ligand; LPS: TLR4 ligand; P3C: TLR2 ligand. Spearman's coefficient of mRNA expression compared to cytokine levels was 0.9 and 0.6 (p = 0.0833 and 0.3500 respectively) for the two individuals evaluated.

4.2.4 Plasma cytokines in active tuberculosis through treatment

Plasma cytokines were measured initially through ES-CBA between May 2014 and March 2017. In 2017, when final analyses were to be undertaken, several previously used antibodies were unavailable, and as such the plasma cytokines were measured through Magnetic Luminex® Performance Assay (see Section 3.5.2). Presented below are comparisons of results from ES-CBA Protein Flex Sets™ and Luminex® Performance Assay. The latter results are used in discussions and conclusion in this dissertation.

Correlation analyses of ES-CBA and Luminex of plasma cytokines measurements (TNF, IL-6 and IL-8 are graphically represented in Figure 4.16). Spearman's r coefficient for TNF was 0.014 (95% CI: -0.378 to 0.402), whilst IL-6 and IL-8 correlation was high: Spearman’s r 0.903 (95% CI: 0.740 to 0.966) and 0.884 (95% CI: 0.757 to 0.947), respectively.
Figure 4.16: Correlation graphs of ES-CBA and Luminex measuring cytokines in plasma

A: TNF measurements in active TB, samples at baseline and end of treatment. Spearman's r 0.014 (95% CI -0.378 to 0.402) p = not significant.
B: IL-6 measurements in active TB, samples at baseline and end of treatment. Spearman's r 0.903 (95% CI 0.740 to 0.966), p < 0.0001.
C: IL-8 measurements in active TB, samples at baseline and end of treatment. Spearman's r 0.884 (95% CI 0.757 to 0.947), p < 0.0001. Outliers have been included in these analyses.

Cytokines measured via Luminex method will be detailed and used in the discussion to follow. The cytokines measured are TNF, IL-6, IL-8, MCP1, IL-10, IP-10, IFNγ. IL-17A and IL-12p70 were also measured, but more than 90% of sample results were below the range and hence not reported here. It can be seen that plasma cytokine levels of IL-6 decrease through antibiotic treatment in active TB with both methods, but not seen in TNF using ES-CBA (Figure 4.17).

Figure 4.17: Grouped cytokine measurements in plasma at baseline and end of treatment comparing two methodologies

Enhanced Sensitivity Cytokine Bead Protein Flex Set™ and Magnetic Luminex® Assay.
Mean with SD shown.
The significant changes in cytokine/chemokines measured in plasma through active TB treatment can be seen in graphs in Figure 4.18 and 4.19. Unlike previous comparisons when normalised data (i.e. fold changes) were evaluated, in this section absolute measurements were evaluated because it was plasma sampling.

**Figure 4.18: IL-6 (A), IL-8 (B) and IL-10 (C) measured in plasma through active TB treatment in MYC cohort**

Timepoint early refers to either one or two month blood samples. Each data point represents one participant at each timepoint. Line at mean ± SD.

The key changes occurred in several of the cytokines evaluated: IL-6, IL-8 and IL-10 all declined through treatment, with significant changes between end of treatment and baseline being found in all three. IL-8 and IL-10 plasma levels between end of treatment and early in treatment (i.e. one or two months into treatment) declined significantly (p = 0.00429 and p < 0.0001), and decreased between end of treatment to baseline (p = 0.0037 and p = 0.0362, respectively) (Figure 4.18B and C, respectively). TNF and IP-10 levels also declined through treatment, with significant changes between early timepoints and baseline, as well as between end of treatment and early timepoints (Figure 4.18A and C, respectively). MCP1 levels increased through treatment, including between intensive phase (i.e. early timepoint) and baseline. IFNγ levels did not vary significantly through treatment in this cohort of patients (Figure 4.19D).
Figure 4.19: TNF (A), MCP1 (B), IP-10 (C) and IFNγ (D) measured in plasma through active TB treatment in MYC cohort

Each data point represents one participant at each timepoint. Line at mean ± SD shown. Timepoint early refers to either one or two month blood samples.

4.3 Demographics and clinical details of participants in LTBI cohort

The results of 22 participants’ TLR expression and function are described in this section. Fourteen of these participants had at least three samples collected (i.e. baseline timepoint, early (one or two months into treatment) and final (end of treatment). A further eight had baseline samples which were included in laboratory work shown below. Basic age and gender distribution are described in Figure 4.2. Due to the changes in methodology and sample availability, not all sections have 14 sets of results. Plasma
cytokine and chemokine measurements using Magnetic Luminex® Performance Assay was only done on baseline samples from the LTBI cohort and therefore are not presented here. These results are presented and discussed in Chapter 5 (Section 5.3).

The majority of patients diagnosed with LTBI were from countries of high TB endemicity; nearly 40% were referred by the Victorian TB Program as recent contacts of an index with active pulmonary TB.

Table 4.6: Demographic and clinical information of LTBI (MYC) cohort (2011–2016)

<table>
<thead>
<tr>
<th>Demographic and clinical characteristics (total 22)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>14 (64)</td>
</tr>
<tr>
<td>Female</td>
<td>8 (36)</td>
</tr>
<tr>
<td>Mean age (years ± SD)</td>
<td>29.6 (SD 8.0 years)</td>
</tr>
<tr>
<td>Region of birth*</td>
<td></td>
</tr>
<tr>
<td>SE Asia</td>
<td>7 (31.8)</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>6 (27.3)</td>
</tr>
<tr>
<td>Africa</td>
<td>5 (22.7)</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Europe</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Americas</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Australia</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Time in Australia prior to diagnosis</td>
<td></td>
</tr>
<tr>
<td>&lt; 5 years</td>
<td>9 (40.9)</td>
</tr>
<tr>
<td>5–15 years</td>
<td>6 (27.3)</td>
</tr>
<tr>
<td>&gt; 15 years</td>
<td>3 (13.6)</td>
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<td>BCG vaccination</td>
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<td>9 (40.9)</td>
</tr>
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<td>CXR</td>
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<tr>
<td>Normal</td>
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</tr>
<tr>
<td>Calcified nodule/ scarring</td>
<td>8 (36.4)</td>
</tr>
<tr>
<td>Recent contact with a person with pulmonary TB</td>
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</tr>
<tr>
<td>Yes</td>
<td>8 (36.4)</td>
</tr>
</tbody>
</table>

* Region of birth according to WHO grouping: Australia listed separately; would otherwise be included in Western Pacific region.1
4.3.1 Toll-like receptor expression in latent tuberculosis

TLR expression in LTBI infection was assessed with the same methodology described and utilised for the active TB MYC cohort. The numbers analysed are small, with 14 end of treatment samples. Samples were analysed at baseline (T0), T early (one or two months into LTBI treatment) and end of treatment (usually nine months after isoniazid treatment, or four months if participant was on rifampicin). The geometric mean fluorescence was normalised and analysis with Wilcoxon rank test reported. Significant changes were found on NKT and NK cells, with TLR4 and TLR7/8 (Figure 4.20A-C). There was a significant decrease between early timepoint and baseline with TLR4 expression on NK cells (p = 0.0295, Figure 4.20A). A decrease was also found between end of treatment and baseline on NKT cells of TLR4 expression, whilst there was a significant decrease between end of treatment and early timepoint of TLR7 expression in the same cells (p = 0.0266 and p = 0.0396, Figure 4.20B and C, respectively).

![Graph A: TLR4 expression on NK cells in LTBI](image1)
![Graph B: TLR4 expression on NKT cells LTBI](image2)
![Graph C: TLR7 expression on NKT cells LTBI](image3)

**Figure 4.20: TLR expression on NKT and NK cells in LTBI (MYC cohort)**

Normalized data shown. Each data point represents a participant at each timepoint. Lines are at mean ± SD shown.

4.3.2 Toll-like receptor function in latent TB: measuring cytokines following PBMC stimulation with TLR ligands in LTBI

IL-6, TNF, IL-8 and MCP1 levels were measured with and without TLR ligand stimulation in supernatants via CBA. Results originally available following measurement through ELISA are not reported (done in 2011, and variable correlation with results from ELISA 2011 and CBA 2016/2017; see Section 4.2.2).
The significant findings are reported in Figure 4.21. MCP1 levels in supernatants following TLR4 and TLR7/8 ligand stimulation of PBMCs were significantly higher between early in treatment and baseline (p = 0.0391 and p = 0.0234, respectively) (Figure 4.21A and 4.21B). IL-8 levels were also significantly higher following stimulation with the same ligands, but between end of treatment and baseline (p = 0.0234 and p = 0.0391, respectively) (Figure 4.21C and 4.21D).

**Figure 4.21:** Cytokine measurements post TLR ligand stimulation of LTBI PBMCs through antibiotic treatment

Normalised data shown. Each data point represents a participant at each timepoint. Lines are at mean ± SD shown.
4.4 Discussion

4.4.1 Active TB cohort innate immune study results

4.4.1.1 Active TB TLR expression and function studies

TLR expression varied between cell types and treatment course in the MYC active TB cohort.

Changes were seen on both surface and intracellular TLR expression investigated. The keys trends in TLR expression through active TB treatment were that TLR2 and TLR7 overall increased at end of treatment compared to TLR4, which declined.

TLR2 expression altered through treatment in this active TB cohort, with significant differences occurring between varied timepoints, with an increase and decrease on NK and NKT cells, respectively (Figure 4.4A and C, respectively). The findings of an increase of TLR2 surface expression on lymphocytes (CD3+, not specified further) was reported in a study by de Oliveira et al. (2014).147 Their other findings contrasted those of the present study with respect to TLR4 surface expression through TB treatment, where they reported an increase through treatment, whilst I found a decrease on lymphocytes (Figure 4.5, Table 4.3). It is important to note that my findings relate to NK cells. There was a trend to a decrease in TLR4 expression through active TB treatment on CD14+ monocytes in their study, which was not replicated in the MYC active TB cohort. It is important to note that authors reported percentage of cells which were positive for the TLRs (and mRNA expression relative to HKG), but the former were not normalised to baseline. The pattern of gene expression of TLR2 and TLR4 appeared to correlate with CD14+ monocyte TLR expression more than CD3+ lymphocytes.147 The decline in TLR4 pathway markers was also reported by Feruglio et al. (2013), where a significant reduction in plasma markers of the TLR4 pathway (LPS, MD-2 and soluble CD14) occurred through antibiotic therapy.149 There was no evaluation of which cells may have contributed to these changes. A study evaluating TLR2 and TLR4 in patients with active TB found higher CD14+ monocytes in peripheral blood, in addition to increased TLR2 and 4 expression on these cells (mean fluorescence as well as percentage) compared to healthy controls (healthy defined as unrelated volunteers with previous BCG
vaccination). The authors did not test for LTBI. Another study found TLR2 expression on CD14+ monocytes to be higher in patients with active TB compared to healthy control, whilst also reporting higher TLR2 intensity and elevated IL-10 plasma levels were associated with shorter survival. The authors also reported a decline in TLR2 expression after two months of treatment in those who had sputum culture conversion.

In the MYC cohort, TLR7 expression changes were significant on NK and CD14+ monocytes, with higher expression between end of treatment and early or start of treatment (Figure 4.6). Only TLR7 expression in CD14+ monocytes was significantly higher between end of treatment and baseline, as well as end of treatment and one month into therapy (Figure 4.6A). The overall trends of TLR7 expression appear similar to TLR2, with a decrease from baseline at one month and an increase at end of treatment. Of interest was that there were no significant changes found on NK subsets (i.e. CD3-CD56+dim and CD3-CD56+bright) when comparing these active TB results. A study evaluating TLR1,2,4,6,7 and 9 mRNA in 10 patients with active TB compared to (uninfected) controls found elevated levels in unstimulated whole blood of TLR1, 2, 4 and 6, but not TLR7 or TLR9. The authors also reported an increase in TLR7 expression on CD3+ subpopulations (but these were not further classified).

**TLR function varied during antibiotic treatment in active TB, reflected by altered cytokine measurements in stimulated PBMC supernatants.**

Cytokines and chemokines measured in supernatants following TLR ligand stimulation of PBMCs changed through antibiotic treatment, although the pattern(s) were not necessarily those predicted from the observed TLR expression changes (where applicable). This has previously been described in innate immune studies, including non-infectious conditions.

TLR2 ligand stimulation resulted in altered TNF and IL-6 measurements in the active TB cohort. Significantly increased TNF levels were seen in the intensive phase of treatment (between T2 and T1) and between end of treatment and baseline, whilst IL-6 increased between end of treatment, T2 and baseline (Figure 4.10B and 4.11A, respectively). The overall trend of an increase in TNF and IL-6 through antibiotic treatment after TLR2 ligand stimulation in the MYC cohort reflects the increase in TLR2 expression. The levels
of TNF were higher following TLR4 compared to TLR2 ligand stimulation, which is similar to a report from India. However, it is important to note that the latter study assessed neutrophils stimulated ex vivo.

Interestingly, IL-6, IL-8 and MCP1 declined through treatment between treatment timepoints and baseline following TLR4 ligand stimulation, although generally baseline levels were lower than any treatment timepoints (Table 4.4). These TLR4 functional changes were significant between end of treatment as well as early timepoints, which has not been described extensively in the literature in PBMCs. As mentioned previously, Feruglio et al. (2013) evaluated TLR4 pathway markers through treatment, finding a decrease through active TB treatment.

TLR7/8 ligand stimulation resulted in significant TNF, IL-6, IL-8 and MCP1 changes throughout antibiotic treatment. In most analyses, these cytokines and chemokines were higher through treatment compared to baseline and never returned to baseline, whilst generally declining through therapy (Figure 4.10D and 4.11C, E, H). Absolute measurements of most cytokines were highest following TLR7/8 stimulation (Figure 4.12), which is consistent with reports in the literature (relating to other immune or infectious disease responses). TLR9 ligand stimulation resulted in significant changes in TNF levels between intensive phase and baseline, whilst MCP1 levels were significantly higher between end of therapy and baseline. IL-8 changes were significant between all treatment points compared to baseline. There are few human studies evaluating TLR7 and TLR9 expression and function as discussed in Chapter 2 (Section 2.4.1A). Chang et al. (2006) found no difference in TLR7 or TLR9 mRNA expression in PBMCs in patients with active TB compared to controls. TLR9 expression was, however, found frequently in granulomas from lung tissue where IL-4 levels were high in another study (along with TLR1 and TLR5).

Contrasting results to the current study were reported of IL-8 levels following TLR2, 4, 5 and 9 stimulation in patients with active TB compared to healthy controls and patients post TB treatment in India. The authors found significantly lower IL-8 levels in supernatants in active TB patients compared to treated TB and healthy controls. Interestingly, the authors concluded that low IL-8 levels following TLR9 ligand stimulation suggested impaired TLR9 responses in active TB.
The impact of TLR ligand stimulation is confirmed in this study, with unstimulated comparisons only significantly different in TNF levels between end of treatment and TP2 (Table 4.4, Figure 4.10A). Whilst limited sampling was undertaken, mRNA studies showed good correlation of results through PBMC stimulation (Section 4.2.3). Although human studies may be limited, previous studies in mice have shown the significance of TLR2, 4 and 9, as well as the role of the adaptor protein, MyD88. However, results are conflicting with some authors reporting MyD88 not being essential for immune responses and/or granuloma formation\textsuperscript{125}, whilst others report increased susceptibility to fulminant infection.\textsuperscript{129,124,144} TLR9 knockout mice have been shown to develop granulomas but with a Th2 predominant response\textsuperscript{281}, whilst others have shown that TLR9 helps regulate Th1 responses through cooperation with TLR2 in mice.\textsuperscript{143} A study of TLR 2/4/9 and MyD88-deficient mice revealed that adaptive immune responses can be induced without these receptors, although MyD88 is essential for triggering an appropriate macrophage response.\textsuperscript{144} It is important to note that murine immune responses to Mtb infection differ to those of humans (see Section 2.4).\textsuperscript{98,95,100} Extensive genetic association studies in recent years have, most importantly, further contributed to the acknowledgement of TLRs in Mtb infections (see Section 2.4.1.1A). The risk of susceptibility varies depending on gene polymorphisms studied, between ethnic groups, type/severity of disease based on many reports, whilst others have found contrasting results.\textsuperscript{7,8,10-12,101,140,154,155,158}

The observations in the current study reinforce the importance of TLRs in active TB, as well as providing new information regarding the changes in cytokines and chemokines which occur. In addition, the findings allude to changes which occur through antibiotic treatment, several aspects of which have not been described previously. The observations support the hypothesis that changes would be detectable in PBMCs and some would return to baseline following ‘clinical cure’ (i.e. end of treatment). Overall, the general findings of increased pro-inflammatory cytokines and chemokines following TLR ligand stimulation in active TB with treatment commencement, with a trend to decrease through antibiotic course warrants further investigation. This is particularly so with the differences in IL-6 and TNF with TLR2 stimulation, which tend to increase with antibiotic course, contrasting the other TLR ligands.
It is plausible that TLR expression and function change as a consequence of antibiotic therapy, and the pro-inflammatory state of active TB is altered. Together, these findings from the MYC active TB cohort may have implications for future research as a biomarker investigation for classification of different phases of treatment.\textsuperscript{22}

4.4.1.2 Active TB plasma cytokine and chemokine studies

Unstimulated plasma cytokines and chemokines varied significantly following anti-tuberculous therapy, but were mostly lower at the end of treatment.

As detailed in Section 4.2.4, not all planned cytokines and chemokines were able to be measured due to technical and/or laboratory kit availability. Of the measured proteins in plasma via Magnetic Luminex assays, TNF, IL-6, IL-8, IL-10, IP-10 and MCP1 changed significantly through active TB antibiotic treatment (Figure 4.18 and 4.19). All cytokines except IFN\(\gamma\) and TNF reduced significantly between the start and completion of treatment. Chemokine MCP1 levels increased through treatment, whilst TNF levels were significantly lower at end of treatment and after one or two months of antibiotics. The median levels of IP-10 and IFN\(\gamma\) were comparable at baseline to that of study by Wang et al. (2012)\textsuperscript{282}, whilst TNF in the MYC cohort were lower than the latter study. It is important to note that in the aforementioned study, cytokines were measured in plasma collected in QFT-tubes (i.e. the patients’ blood was sensitised to the Mtb antigens with the QFT tubes). The levels of TNF and IFN\(\gamma\) were comparable to both baseline and through treatment of active TB reported by de Oliveira et al. (2014).\textsuperscript{147}

Overall, MYC active TB plasma findings reflect previous reports of decline in both pro- and anti-inflammatory cytokines and chemokines in plasma through TB therapy, whilst contrasting other studies.\textsuperscript{146,147,196,199} A detailed systematic review by Clifford et al. (2015) concluded that although TNF, IL-6, IL-8, IL-10 and IP-10 overall reduced through treatment in unstimulated plasma, results were inconsistent.\textsuperscript{199} The observations made in the MYC active TB cohort further contribute to the available literature. These results further support the likelihood that a single plasma cytokine or chemokine is unlikely to be adequate as a biomarker to assess treatment efficacy. Combination measurement of multiple cytokines/chemokines have been evaluated as potential biomarkers in the scientific literature, with promising results but yet not validated for clinical use.\textsuperscript{194-196,198}
4.4.2 LTBI cohort innate immune (TLR expression and function) results

Treatment of LTBI was associated with variations in TLR4 and 7 expression on NK and NKT cells and functional changes in cytokine and chemokine measurements post TLR4 and 7/8 ligand stimulation.

The significant changes from TLR surface and intracellular studies were of TLR4 and TLR7 on NKT cells, where there was a reduction at end of treatment compared to baseline (TLR4), and early treatment (i.e. one or two months) compared to baseline (TLR7) (Figure 4.20B and C, respectively). On NK cells, TLR4 expression declined early into treatment compared with baseline (Figure 4.20A). The finding of significant changes on NK and NKT cells are of interest in this cohort, given no changes were found on CD14+ monocytes; it would be worthwhile to further evaluate the counts and percentages of these cells to determine if a specific profile or pattern occurs as was reported by Barcelos et al. (2008).170

The changes seen on TLR functional studies in LTBI were also only of TLR4 and 7, although the patterns varied (Figure 4.21). Chemokine MCP1 levels increased significantly between early treatment and baseline following TLR4 and TLR7/8 ligand stimulation, with a decline at end of treatment which was not statistically significant (Figure 4.21A and B). IL-8 levels increased following TLR4 and 7/8 with a significant change occurring at end of treatment compared to baseline (Figure 4.21C and D). The numbers in the LTBI cohort were small however, with only eight results at the final timepoint.

It is interesting that no TLR2 changes were found through LTBI treatment in this study. A study reported changes in TLR2 and 9 in patients with LTBI and filariasis co-infection, where ex vivo samples were stimulated with PPD and CFP, then TLR expression and function evaluated.283 The authors found lower TLR2 and TLR9 expression and cytokines production following ligand stimulation which was restored following anti-filarial treatment. These changes were not seen when samples were stimulated with tetanus toxoid as a control. To my knowledge, no studies have reported on TLR studies in LTBI therapy. It is also noteworthy that there was no further follow-up of these patients once
LTBI treatment was completed (from a clinical perspective). Despite the small numbers in this cohort, these observations support the hypothesis that innate immune markers are important in LTBI, although recent reports have argued against an innate immune response in LTBI.284

4.4.3 Challenges and limitations of the present study

There are several limitations in the current study. The study was designed as an observational cohort and challenges in patient recruitment and retention were encountered, particularly in the LTBI cohort. Practical resource limitations, in particular in processing of samples, lead to the restricted patient numbers which limits the power of the statistical analysis.

The potential recruitment bias and similarly, voluntary nature of the study could impact on findings. In the LTBI cohort 38 participants were recruited, of whom 22 samples had baseline laboratory work completed and only 14 had final end of treatment samples collected (63%). The poor compliance with medication adherence / clinic review is well described in the literature and comparable to previous reports in this group of patients.71 Another potential source of selection bias is the recruitment of patients from a single health service. However, Monash Health is the largest health service in Victoria, with an extensive catchment including large migrant and refugee communities. As such those recruited into the MYC active TB cohort are considered to be representative of the distribution of TB notifications in Australia.37 This also results in a heterogeneous study population.

In Chapter 4, the active TB and LTBI cohorts are described separately to enable data analysis of each cohort and therefore the effect of treatment within the group. This prospective study did not have a control group who were uninfected, healthy volunteers, a comparative group with pulmonary (non-tuberculous) infections, or multi-system inflammatory disorders. In the following chapter, the active TB and LTBI groups are compared, in effect using the LTBI cohort as a surrogate ‘control’ group. This is what many research groups utilise when investigating TLR expression and function, often not indicating TST and/or IGRA status, but defining ‘healthy controls’ as those who are asymptomatic with normal chest radiography. Given most studies in TB are undertaken
in medium to high endemicity regions, there is a high likelihood that a significant proportion of the ‘healthy controls’ in such studies are latently infected with Mtb and my comparative study avoids this potential confounder by design.\textsuperscript{146-148,150,169,171}

The laboratory work, in particular TLR staining and flow cytometry, can raise issues of consistency relating to gating, cell culture and cell loss from washes. More extensive gene expression studies would be important to consider in future research to provide reproducible results. Very low / undetectable levels of several cytokines may have been due to laboratory error or specific kit issues and these were excluded. These laboratory issues have been described relating to ELISA, CBA and ES-CBA assays and do not affect the final conclusions (see Section 4.2).

### 4.4.4 TLR studies: effect of treatment, understanding immunopathogenesis and implications for further research

The results shown in this chapter confirm the hypotheses that treatment of Mtb infections, both active and latent, affect innate immune markers as shown by evaluating TLR expression and function. The results discussed thus far highlight previous research in this area of altered TLR2 and 4 expression, as well as cytokine and chemokine release following TLR ligand stimulation. The current work also sheds light on TLR7 expression and function in both active and LTBI, which have not been described in detail previously, despite genetic association studies suggesting strong links to increased susceptibility in certain forms of TB disease.\textsuperscript{7,10} TLR7 expression changes were seen on CD14+ monocytes and NK cells in active TB and on NKT cells in LTBI through treatment. Strong functional changes were seen following TLR ligand stimulation with TLR2,4,7/8 and 9 affecting protein measurements in active TB. The changes in cytokine and chemokines were evident to a lesser extent in LTBI.

Whilst changes occurred through antibiotic treatment in active TB, few markers evaluated (i.e. TLR expression nor cytokine release post TLR ligand stimulation) resulted in return to ‘normal’ at the end of therapy, which is deemed ‘clinical cure’. No relapses were observed during subsequent clinical observation of the patients, indicating cure (personal communication, Dr Grant Jenkin, Head of Mycobacterial Services, Monash Health). It may be prudent to be comparing end of treatment results to healthy,
uninfected controls or those with other, non-tuberculous infections. Innate immune responses are important in sepsis and previous reports have identified TLR2 and 4 regulation in monocytes and more recently on NKT and NK cells (including subsets) of TLR4 expression.

Further evaluation of cell counts and percentages may contribute to better understanding of innate cellular responses in these cohorts, namely differential white cell counts and ratios in comparison between the groups and through treatment. Such descriptions have reported distinct patterns evaluating cell counts. By analysing such results in these cohorts, it may provide further insight into the specific cell-led activity and effects found on TLR studies. Another aspect of TLR expression which was not evaluated are the heterodimers of TLR2:P3C is a potent stimulant of TLR2/1 and whilst results discussed here presume TLR2 activation, it may in fact incorporate TLR2/6 heterodimer effects as well. The significance of differentiation is unclear based on available literature on TLR function and expression, but once again information based on genetic association studies suggest it may have clinical implications.

Whilst the TLR studies undertaken and reported in this chapter have provided important information, the direct cellular mechanisms were not evaluated; that is, I did not investigate which cells with altered TLR expression resulted in altered TLR function downstream. Furthermore, certain TLR expression and function were not evaluated: at the time of commencement of the doctoral studies, few human studies had reported TLR3 or 5 in TB pathogenesis. Similarly, TLR9 intracellular expression has since also been described, but not routinely undertaken. Given the significant changes I found post TLR9 ligand stimulation, it would be prudent that future studies of TLRs in TB incorporate TLR9 expression as well. Also, further evaluation of results to include different monocyte subgroups would be worthwhile (such as CD14\textsuperscript{int}), whereas only strongly CD14+ gating was undertaken for the study.

Another aspect which was not investigated but is important to consider is the innate immune response at the site of disease in patients with active TB, most of which are cases of reactivation disease. Previous studies have shown that TLR responses can in fact be attenuated at the site of disease compared to peripheral blood, seen in TB as well as non-infectious pulmonary illnesses as examples. Future investigation could include
focussed evaluation of TLR responses at the site of disease in comparison to PBMCs. The latter was not undertaken as part of this pilot study in part because of the paucity of data in the current literature through drug therapy evaluating TLR responses and the ease of acquisition of samples.

The combination of higher cytokine/chemokine levels following TLR ligand stimulation with antibiotic treatment, whilst paradoxically reduced levels of concurrent plasma cytokine measurements was intriguing, and this is investigated and detailed in Chapter 6 as to whether an inhibitor was present in plasma of Mtb infected individuals. In Chapter 5 analysis of the variations comparing the two MYC cohorts are discussed. The link(s) with vitamin D, clinical data and innate immune markers investigated in the current study are discussed in detail in Chapter 7.

4.5 Conclusions

The findings of this chapter confirm the hypotheses that innate immune function assessed through TLR expression and function is measurable in PBMCs in patients with both active TB and LTBI. The findings also confirm that changes occur through treatment of both forms of TB infection.

Significant differences were found on NK and NKT cells of TLR4 and 7 expression through the treatment of LTBI. On stimulation of these respective TLR ligands, functional changes cytokine and chemokine levels were also found. No significant changes were found on normalised data with respect to TLR2 expression or functional evaluation in LTBI.

In the MYC active TB cohort, TLR function varied during antibiotic treatment in active TB, demonstrated by altered cytokine measurements in stimulated PBMC supernatants, often reflective of respective TLR expression. Unstimulated plasma cytokines and chemokines varied significantly following anti-tuberculous therapy, with most being lower at the end of treatment. It is challenging to ascertain how much TLR induction leads to production of downstream cytokines measured in plasma in this study.

The combination of patterns of TLR2, 4, 7 expression and TLR2, 4, 7/8 and 9 function allude to complex changes which occur through treatment of both active and LTBI and reinforce the importance of innate immune responses in reactivation TB. It is indeed
interesting that whilst generally plasma pro-inflammatory cytokines and chemokines declined through treatment (with the exception of MCP1, where increases were observed), these cytokines were generally higher when measured following TLR ligand stimulation whilst patients were on antibiotics, and although declined during treatment, never returned to baseline. These observations imply that the pro-inflammatory state in active TB, in part occurring through TLR activation, is altered and antibiotic therapy affects this further. The significant differences found in active and LTBI cohorts also allude to a varied ‘TLR activation or signatory profile’.

TLR markers alone are probably inadequate to enable differentiation of treatment effectiveness or innate immune responses in active TB and LTBI infection. However, further concerted efforts investigating these findings will contribute to better understanding of their role in the immunological spectrum of TB and determining whether patients with active TB and LTBI have a specific ‘TLR profile’. This, in turn, may contribute towards a ‘biomarker platform’ to enable differentiation and treatment efficacy determination.
Chapter 5: Differentiating active from latent tuberculosis infections based on innate immune markers: is it possible?

The role of TLRs in the initial immune response to Mtb infection is becoming better defined. However, despite recent human research and genetic association studies, the cellular and functional effects are still inadequately described and understood. Furthermore, there is a paucity of understanding of the effect of antibiotic treatment on these responses. Understanding these effects are important and could potentially contribute to formation of a biomarker(s) and/or adjuvant therapies.\textsuperscript{23,24,87,88}

It is hypothesised that the innate immune responses vary between LTBI and active TB with respect to TLR expression and consequent function. It is also postulated that these changes will be measurable \textit{ex vivo} in PBMCs of patients diagnosed with the two distinct clinical forms of Mtb infection and these will change progressively during antibiotic treatment. It is also postulated that TLR expression following treatment of these infections will be similar between the two cohorts.

This chapter focuses on the comparison of innate immune responses between active TB and LTBI patients. Surface and intracellular TLR expression was evaluated between the two groups. Evaluations between baseline and end of treatment for both active and LTBI cohorts were also undertaken. TLR function in the form of cytokine and chemokine measurements in supernatants following TLR ligand stimulation of PBMCs was also evaluated. Along with plasma cytokines in the two MYC cohorts, these innate immune markers were examined to determine if there were distinct TLR patterns which may differentiate between the two forms of infection. The pre-treatment LTBI cohort is used as a ‘control’ group in analyses in this chapter.\textsuperscript{146-148,150,169}

Investigative methodology and statistical analyses are described in Chapter 3. The analyses were undertaken using Mann–Whitney U tests between the different timepoints (p values \(\leq 0.05\) are shown; column/bar graphs with lines at mean with SD are shown where possible).
5.1 TLR expression comparison between active and LTBI

Geometric mean fluorescence of TLR expression varied between LTBI and active TB on different cell types evaluated. Key findings are shown in Figure 5.1 and 5.2.

5.1.1 Comparison of active TB through treatment to LTBI baseline

TLR2, 4 and 7 expression were significantly different between baseline active TB and/or end of treatment in active TB and baseline LTBI. The LTBI baseline TLR2 expression was lower than all timepoints in participants through active TB treatment on CD14+ monocytes (Figure 5.1A). TLR2 and 7 expression were lower in LTBI compared to active TB at all timepoints (some were of significance), whilst TLR4 expression was significantly higher in LTBI compared to active TB (at end of treatment) on CD14+ monocytes. TLR7 expression in NK and NKT cells was significantly higher between end of active TB treatment and LTBI baseline (Figure 5.2A and C), active TB base and LTBI base (Figure 5.2B and 5.2C), but not TLR2 or 4 surface expression on NK (including subsets) and NKT cells (data not shown).
Figure 5.1: Comparison of TLR expression on CD14+ monocytes between baseline LTBI and through treatment of active TB

ATB: active tuberculosis; LTBI: latent tuberculosis infection. Each data point represents one participant. p ≤ 0.05 are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.
Figure 5.2: Comparison of TLR7 expression on NKT and NK cells between baseline LTBI and through treatment of active TB

ATB: active tuberculosis; LTBI: latent tuberculosis infection. Each data point represents one participant. p ≤ 0.05 are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.
5.1.2 Comparison of TLR expression between active TB and LTBI at end of treatment and baseline in both cohorts

Interestingly, further significant changes were found when LTBI end of treatment was compared to active TB at end of treatment on CD14+ monocytes (Figure 5.3), overall showing that LTBI baseline and end of treatment TLR2 and TLR7 expression were lower compared to active TB baseline and end of treatment.

The significant differences of TLR expression between LTBI and active TB at end of treatment timepoints and baseline found on NKT and NK cells are shown in Figure 5.4: whilst TLR7 expression was lower in LTBI baseline than active TB (baseline and end of treatment) on NKT and NK CD56\textsuperscript{dim} cells, end of treatment differences were seen between LTBI and active TB on NKT and NK cells. TLR2 expression was commonly significantly lower between end of treatment LTBI and active TB (baseline and end of treatment) on NKT and NK cell subsets (Figure 5.4A, D, G and J). TLR4 expression was also significantly lower in these cells between end of LTBI treatment and baseline active TB (p = 0.0465, p = 0.0118 and p = 0.0396, respectively, on Figure 5.4B, E and H). Interestingly, there were no significant changes of LTBI baseline TLR4 expression and active TB on NKT or NK subsets evaluated (Figure 5.4B, E and H).
Figure 5.3: Comparison of TLR 2, 4 and 7 expression between LTBI and active TB between start and end of treatment on CD14+ monocytes

Each data point represents one participant. \( p \leq 0.05 \) are shown, based on Mann–Whitney two-sided \( t \) tests. Lines represent mean ± SD. Active TB base and final versus LTBI baseline: straight lines, dotted lines represent significant differences between LTBI end of treatment and active TB.
Figure 5.4: Comparison of TLR 2, 4 and 7 expression between LTBI and active TB between start and end of treatment, on NKT, NK cells and NK cell subsets

Each data point represents one participant. $p \leq 0.05$ are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD. Active TB base and final versus LTBI baseline: straight lines, dotted lines represent significant differences between LTBI end of treatment and active TB.
5.2  TLR function comparison between active and LTBI

There were significant differences between the active and LTBI cohorts with respect to their TLR function, measured through cytokine/chemokine measurements of the supernatant of stimulated PBMCs. The numbers of participants analysed varied depending on different proteins measured, due to laboratory limitations discussed previously.

5.2.1  Comparison of active TB cohort through treatment to LTBI baseline TLR function

TNF levels were significantly lower in active TB throughout the treatment course compared to LTBI baseline, and these findings were reflected in TLR9 ligand stimulation evaluations (Figure 5.5A and C, respectively). Following TLR2 ligand stimulation, TNF levels were significantly lower in active TB at baseline and one month into treatment compared to LTBI baseline (Figure 5.5B).

IL-8 levels were generally lower in LTBI baseline than active TB when stimulated with TLR4 and TLR 7/8 ligands. End of treatment and TP2 (two months into treatment) in active TB IL-8 levels were higher than baseline LTBI cohort after TLR4 ligand stimulation, whilst all active TB treatment timepoints when stimulated with TLR7/8 ligand were higher than LTBI (Figure 5.5D and E, respectively).

IL-6 levels were significantly lower throughout active TB treatment compared to LTBI baseline in unstimulated supernatants and following TLR9 ligand stimulation (p = 0.0002–0.0032, Figure 5.6A and D, respectively). In the same patients, early timepoints and baseline active TB levels were lower than baseline LTBI after TLR2 ligand stimulation (p = 0.0457, 0.0275 and 0.0248), whilst there was no significant difference between end of active TB treatment and baseline LTBI (Figure 5.6B). Following TLR4 ligand stimulation, the only significant difference between the two cohorts of IL-6 levels was between baseline active TB and LTBI, where active baseline levels were lower than LTBI baseline p = 0.0394 (Figure 5.6C).
Figure 5.5: TNF and IL-8 levels in supernatants following TLR ligand stimulation in baseline LTBI and active TB through treatment

Each data point represents one participant. p ≤ 0.05 are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.

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There were many significant differences between active TB cohort and LTBI baseline with MCP1 measurements in supernatants after all TLR ligand stimulations, generally with active TB levels being lower than LTBI baseline (Figure 5.7). MCP1 levels were significantly lower in active TB through treatment compared to LTBI baseline in unstimulated and TLR9 ligand stimulated samples, \( p = 0.0006–0.0397 \) (Figure 5.7A and E, respectively). Following TLR2 stimulation, MCP1 levels were lower in active TB at end of treatment, timepoint T1 and baseline, compared to LTBI baseline (\( p = 0.0440, 0.0430 \) and 0.0203, respectively) (Figure 5.7B). TLR7/8 ligand stimulation resulted in active TB baseline levels of MCP1 being less than LTBI baseline (\( p = 0.0190 \), Figure 5.7D). Similarly, following TLR4 ligand stimulation, with MCP1 levels being higher in LTBI baseline than active TB baseline (\( p = 0.0442 \)) (Figure 5.7C).
Figure 5.6: IL-6 levels in supernatants following TLR ligand stimulation of PBMCs in baseline LTBI and active TB through treatment

Each data point represents one participant. \( p \leq 0.05 \) are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.
Figure 5.7: MCP1 levels in supernatants following TLR ligand stimulation in baseline LTBI and active TB through treatment

Each data point represents one participant. p ≤ 0.05 are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.
**5.2.2 Comparing cytokine/chemokine patterns in LTBI and active TB before and after treatment of the infections**

The following comparisons were undertaken to determine if the treatment of LTBI and active TB resulted in similar patterns of innate immune function, as the clinical aim is to ‘cure’ the patient of the infection; therefore, it was hypothesised that the differences at the end of treatment between the two groups would not be significant. Mann–Whitney U tests were applied between end and start of treatment for each of the cytokines/chemokines measured (where at least six samples were available in each cohort).

IL-6 measurement comparisons between end and start of treatment of the two TB cohorts showed that following TLR2 and TLR9 ligand stimulation (and without), there were significant changes with lower levels in active TB compared to LTBI (Figure 5.8A, B and D). No significant changes were found between active TB and end of LTBI treatment after TLR4 ligand stimulation in terms of IL-6 production (Figure 5.8C).

TNF levels were also significantly different when pre and post treatment of active TB was compared to LTBI: without stimulation, levels in supernatants were lower in active TB (pre and post treatment) compared to LTBI baseline (p < 0.0001, p < 0.0001; Figure 5.8E). Following TLR2 and TLR9 ligand stimulation, there were additional significant differences, with lower levels in active TB (pre and post treatment) compared to LTBI post treatment (Figure 5.8F and G, respectively). Changes found with the additional comparisons in MCP1 and IL-8 between active TB and LTBI are shown in Figure 5.9A-E and F-J, respectively. MCP1 levels were only significantly lower in active TB pre and post treatment compared to LTBI post treatment after TLR2 ligand stimulation (p = 0.0159 and p = 0.0468, respectively; Figure 5.9B). IL-8 levels following stimulation with TLR2 and TLR9 were significantly different with active TB pre and post treatment compared to LTBI post treatment (Figure 5.9G and J, respectively), whilst there were no significant differences in pre-LTBI treatment with these ligands. IL-8 levels were lower in active TB following TLR2 ligand stimulation, whilst higher after TLR9 ligand stimulation compared to LTBI post treatment (Figure 5.9G and J, respectively). IL-8 levels in baseline active TB was also significantly higher than end of treatment LTBI in supernatants (unstimulated) (p = 0.0185; Figure 5.9F).
Figure 5.8: IL-6 and TNF levels in supernatants following TLR ligand stimulation between LTBI and active TB between start and end of treatment

Each data point represents one participant. $p \leq 0.05$ are shown, based on Mann–Whitney two-sided $t$ tests. Lines represent mean ± SD.
Figure 5.9: MCP1 and IL-8 levels in supernatants following TLR ligand stimulation between LTBI and active TB between start and end of treatment

Each data point represents one participant. p ≤ 0.05 are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.
5.3 Comparing plasma cytokine levels in active and LTBI

Plasma cytokine/chemokine levels were measured through treatment in the active and latent TB infection cohorts. Reported in the chapter are the baseline results for both cohorts and the end of treatment for active TB. The following cytokines were measured: TNF, IL-6, IL-8, MCP1, IL-10, IP-10, IFNγ, IL-17A and IL-12p70, but as discussed in Chapter 4 (Section 4.2.4), not all the data is presented. Presented here are comparisons between active TB (pre and post treatment) and baseline LTBI plasma levels, measured through Magnetic Luminex® Performance Assays.

IL-6, IL-8, IL-10, IP-10, IFNγ, TNF and MCP1 levels were measured and compared using Mann–Whitney two-sided t tests. Figure 5.10 highlights the results which were significant. TNF levels were significantly lower post active TB treatment compared to baseline LTBI, p = 0.0.194 (Figure 5.10A). IP-10 levels were higher at baseline in active TB than LTBI, p = 0.0283, but were not significantly different post active TB treatment (Figure 5.10B). Post treatment IL-8 levels were lower in active TB, whilst post-treatment IFNγ levels were higher, compared to baseline LTBI, p = 0.0198 and p = 0.0028 respectively (Figure 5.10C and D, respectively).
Figure 5.10: Plasma cytokine levels in LTBI and active TB

Each data point represents one participant. $p \leq 0.05$ are shown, based on Mann–Whitney two-sided t tests. Lines represent mean ± SD.
5.4 Discussion

5.4.1 TLR expression with latent and active forms of Mtb infection

TLR2, 4, and 7 expression were significantly different between active TB through treatment and LTBI (pre-treatment) on CD14+ monocytes and similar changes were found with TLR7 expression in lymphocyte subgroups. Following LTBI treatment, further TLR expression changes were observed between the two cohorts.

Similar to findings in evaluation of TLRs in the MYC active TB cohort (Chapter 4), TLR2 and 7 expression had contrasting patterns in comparison to TLR4. TLR2 and 7 GMF were generally higher in active TB, whilst TLR4 expression was lower in this cohort on CD14+ monocytes compared to those with LTBI at baseline (Figure 5.1). On NK (including subsets) and NKT cells, there were no significant differences found between active TB and LTBI baseline with TLR2 or 4 expression, which is in contrast to evaluation within these cohorts explored previously in Chapter 4.

TLR expression differences between persons with LTBI and active TB have been evaluated previously. de Oliveira et al. (2014) reported higher levels of TLR2 and TLR4 mRNA expression in patients through active TB treatment compared to infected controls. They also reported higher percentage of TLR2 and TLR4 positive CD3+ cells in patients with active TB compared to LTBI and TLR2 on CD14+ monocytes.

Another study conducted in Mexico City compared 44 healthy controls (Mtb infection state unknown) to 54 patients with active TB and found increased percentage and expression of TLR2 on CD14+ monocytes, but no difference in the percentage of TLR4 positive monocytes in those with active TB. However, the authors of this study found that the mean fluorescence index of TLR4 on CD14+ monocytes was significantly higher in those with active TB compared to the control group. Whilst the findings in the current study reflect the TLR changes noted in the Mexican study, the TLR4 changes were not replicated. It is important to note, however, that I did find differences following treatment between the MYC cohorts of TLR4 expression on CD14+ monocytes. The other key differences between the two studies is that the ‘control’ group in the MYC cohort has
documented LTBI, whereas the control group in the Mexican study did not report on latent TB infection status.\textsuperscript{148} Another study evaluating TLR4 markers in the serum of patients with confirmed LTBI and active TB infection found significantly lower levels in those with LTBI, once again contrasting the observations of the current study where levels were higher in patients with LTBI.\textsuperscript{149}

A study conducted in Egypt evaluated TLR2 surface expression in patients with active TB and healthy controls (Mtb infection status unknown) and found significantly higher levels of TLR2 expression in the active TB groups on CD14+ monocytes. These authors also evaluated TLR2 polymorphisms in their study groups.\textsuperscript{151} The higher levels of TLR2 expression was similar to that reported by Chang et al. (2006), who also found higher levels of mRNA expression of TLR1 and TLR6 (but not TLR7 and 9) in PBMCs of patients with active TB compared to uninfected controls.\textsuperscript{145} A comparable report of higher TLR2 expression in patients with active TB compared to healthy controls was associated with higher IL-10 levels (unstimulated).\textsuperscript{146} The results of the MYC cohort observations thus concur with these studies.

In contrast, a study conducted in Greece reported no difference between patients with Mtb infection (active and LTBI) and uninfected controls when evaluating TLR1 and 2, whilst reporting a significant difference in TLR4 and TLR6 mRNA levels between the groups, but not between those with active and LTBI.\textsuperscript{152}

Another study reported lower absolute lymphocyte and higher monocyte counts in active TB patients than healthy, infected controls (i.e. LTBI), with a specific, low CD3\textsuperscript{dim} CD56+ NKT profile being found in those with active TB.\textsuperscript{169} Barcelos et al. (2008) reported similar findings of lower absolute levels of NK cells in TST+ compared to those with active TB, as well as a different NK subset profile in those who were latently infected.\textsuperscript{170} Whilst in the current study, specific counts of lymphocytes and monocytes were not undertaken, TLR expression on the cell groups and subsets certainly show differing patterns between those with active TB and LTBI.

An important and previously unreported evaluation in the current study is that of LTBI patients and their TLR responses following therapy. Interestingly, when active TB baseline and final timepoints were compared to LTBI end of treatment, TLR2 and 7
expression were still significantly higher in active TB, but no differences were found on TLR4 expression on CD14+ monocytes (Figure 5.3). Furthermore, analysis of active TB end of treatment to end of LTBI treatment revealed significant differences on NKT and NK cells (including CD56\textsuperscript{dim} and bright subsets) of TLR2 surface expression, and TLR4 expression between active TB baseline to LTBI end of treatment (Figure 5.4). Where significant differences were found, generally, active TB expression of TLR2 and 4 were higher than end of LTBI treatment expression (Figure 5.4). It is important to recall that no significant TLR2 expression changes were found within the LTBI cohort on lymphocytes (Chapter 4).

These findings suggest that the TLR profiles on CD14+ monocytes are significantly different following treatment for active TB and LTBI, contrasting the study hypothesis that bacterial eradication would result in the similar innate immune responses in these two clinical cohorts. Also, to the author’s knowledge, these changes along with those found between active TB and LTBI at start and end of treatment on NKT and NK cells of TLR expression have not been described previously. TLR4 expression was significantly different on NK, NK CD56\textsuperscript{dim} and NKT between baseline active TB and end of LTBI treatment, potentially highlighting the differences in cellular function, as well as the specific TLR.\textsuperscript{171,173,175,172} These findings further allude to the possibility of a specific ‘TLR signatory profile’ in active and LTBI, with the possibility that antibiotic treatment further alters such a profile. It may also further validate the concept of ‘putative resistance’ in those with LTBI that has potentially prevented the development of symptomatic active disease previously.\textsuperscript{170,284}
5.4.2 TLR function in different forms of Mtb infection

TLR function evaluated through cytokine and chemokine measurements following TLR ligand stimulation showed differences between the study cohorts through antibiotic treatment. These changes were distinct from those found assessing the cohorts alone described in Chapter 4 of this dissertation. Variations in cytokine measurements were also evident between end of treatment between the two cohorts, as well as between pre-treatment timepoints. Overall, there was a trend towards lower TNF, IL-6 and MCP1 levels in active TB compared to the LTBI cohort, whilst IL-8 levels were the opposite. Importantly, the changes of TLR function did not necessarily reflect the patterns of TLR expression.

The key observations here show that TLR function varies between active and LTBI, with interesting changes that have not been described previously following LTBI treatment compared to those with active TB infection. The results discussed here confirm that TLR function does not always directly infer TLR expression changes in Mtb infection.\textsuperscript{277} The present findings both reflect and contrast previous reports when evaluating changes in active TB or LTBI cohorts in Chapter 4.\textsuperscript{148,147,149,145,150,153}

A recent study of TLR function (mRNA expression of innate immune markers and cytokines via PCR following TLR2, 4, 5 and 9 stimulation) and IL-8 measurement in supernatants was conducted in healthy controls (clinically well, IGRA/TST status unknown, n = 17), previously treated TB patients (i.e. cured) (n = 11) and 19 patients with untreated pulmonary TB.\textsuperscript{153} The authors of the study evaluated over 40 genes through PCR. Overall, they report a significantly higher level of IL-8 following TLR ligand stimulation in those previously treated for TB and healthy controls compared to those with active TB (TLR2, 4 and 5). Interestingly, they also found that TLR9 agonist stimulation was associated with minimal IL-8 responses in both treated and active TB patients. The reported study did not, however, involve measurement of other cytokines following TLR ligand stimulation. These TLR9 changes are in contrast to findings in the MYC cohorts. It is important to recognise, however, that the current study did not have ‘healthy controls’, rather confirmed latently infected (but otherwise well) participants. It is also of interest that the authors did not find any changes in mRNA expression of over 40
pro-inflammatory genes and receptors, attributing this to changes occurring after transcription.

TLR4 functional changes were generally associated with higher levels of cytokine and chemokine production in LTBI (where significant) compared to the active TB cohort, except for IL-8 (Figure 5.9H). Whilst these changes reflect the patterns of TLR4 expression measured in the current research project, it contrasts a previous report by Feruglio et al. (2013), where the authors reported significantly lower levels of TLR4 pathway serum markers in patients with LTBI compared to those with active TB at baseline and through treatment.\textsuperscript{149}

A group found higher levels of TNF, IL-8 and MCP1 following TLR2, TLR4, TLR6 stimulation (\textit{ex vivo} of neutrophils) in those with active to ‘healthy controls’ (BCG vaccinated, TST or IGRA state unknown), once again similar to several aspects of MYC study results, whilst contrasting with others.\textsuperscript{150} It is important to recognise the aforementioned study evaluated neutrophils, whilst the MYC study relates to findings on PBMCs.

### 5.4.3 Plasma cytokine and chemokine comparison between active and LTBI

Several changes in plasma cytokines were found to be significant following comparison of the LTBI and active TB cohorts. TNF and IL-8 levels were significantly lower at end of active TB treatment compared to baseline in LTBI cohort, whilst IFN\textgreek{g} was the opposite. IP-10 levels were found to be significantly higher in those with active TB prior to treatment compared to baseline LTBI.

Despite the limitation of plasma cytokines/chemokines measured and analysed, the changes observed in the current study are similar to those reported by de Oliveira et al. (2014), with TNF and IFN\textgreek{g} levels being lower in those with LTBI compared to active TB.\textsuperscript{147} Higher levels of IP-10 in patients with active TB compared to household contacts were described in a study evaluating plasma cytokines/chemokines through a 17-plex-cytokine assay, which is similar to the current study, but their findings of higher levels of IFN\textgreek{g} in LTBI contrasts the MYC cohorts.\textsuperscript{198} The authors did not find any significant
changes between their study groups of IL-10, MCP1 nor TNF, the latter once again contrasts the current study results. The authors concluded that combination of five measurements (EGF, fractalkine, IFNγ, IL-4 and MCP3) may be able to differentiate patients with active TB from LTBI. Another study evaluated cytokines in plasma from patients taken from QFT (i.e. both unstimulated and Mtb antigen stimulated) and found that IP-10 and IFNγ was significantly lower in healthy household contacts compared to patients with active TB (where 38% of household contacts had positive QFT results and 42% had positive TST results). Once again, it is difficult to directly liken the MYC cohorts to the latter study, but the results are comparable. A systematic review of studies assessing the role of stimulated and unstimulated cytokines/chemokines in plasma concluded that whilst potential biomarkers might exist, at present the results of many, small and heterogeneous studies are inconclusive.

5.4.4 Limitations of the current study

The main limitations encountered in the MYC study have already been outlined in Chapter 4 (Section 4.4.3). The key limitations are the lack of a comparative Mtb uninfected group. Another cohort of participants who had non-tuberculous pulmonary disease (of both infectious and non-infectious aetiologies) would have been ideal. However, the addition of more sub-groups in an observational study has implications. Given this was a pilot, observational study, no formal power assessment was done prior to study commencement. The heterogeneous aspect to both the MYC study cohorts is also an important consideration, although generally reflective of the Australian active TB epidemiology.

5.4.5 Summary: key investigational findings and contribution to the understanding of the role of TLRs in Mtb infections

The findings described highlight clearly that latent and active TB treatment impacts on TLR responses. Whilst several hypotheses have been confirmed, others have been disputed by the observations made in the current study. It was also postulated that at the end of active TB treatment, the TLR profile would be similar to that of patients with LTBI and this has been challenged by the findings reported in this chapter. The possibilities for this are multifactorial, including small numbers in the LTBI cohort, especially at the end
of therapy. An important consideration is the current appreciation of the spectrum of LTBI on cellular and immune response modulation.\textsuperscript{3,4,93,153,170,6,92} It is also important to consider that 15% of participants in the active TB cohort (Table 4.2) were asymptomatic, despite being diagnosed with active TB disease. As a contrast, whilst all the MYC LTBI cohort were well and asymptomatic, the lack of a gold standard diagnostic test for LTBI has to be taken into consideration, given previous reports of viable Mtb bacteria being found at autopsy in tissues of people (death from unrelated causes).\textsuperscript{51} These factors indicate the need to account for the spectrum of Mtb infection in humans, as it is possible that a patient who is asymptomatic with no clinical evidence to suggest active TB and has cellular responses of Mtb sensitisation (i.e. IGRA or TST positive), may not simply have classic LTBI.\textsuperscript{3,4,203}

The key findings in this chapter confirm that TLR2, 4 and 7 expression are altered between LTBI and active TB, with further significant changes evident when post-LTBI treatment is compared to the active TB cohort on CD14+ monocytes, NK, NKCD56+ subsets and NKT cells. Previous studies have not reported TLR4 changes on NKT cells and NK subsets that occur post LTBI therapy to active TB. These are important changes, as they contribute further to the possibility that TLR4 activation is a ‘protective’ immune response, possibly via its induction through MyD88 independent pathways.\textsuperscript{123,122,137,119,124,135} Another key finding in this study was that TLR2 and 7 expression generally followed a similar pattern. Whilst the TLR2 expression changes found in this study are reflective of others, TLR7 expression has only minimally been previously investigated, especially in human studies.\textsuperscript{18,20,145,287} Whilst TLR8 expression was not evaluated, functional aspects of TLR7/8 stimulation showed that this is an important TLR induced during Mtb infection.\textsuperscript{18,101,140} Despite the stimulant being imiquimod-based and known to induce small viral particles (ssRNA), it is also involved in mycobacterial TLR activation.\textsuperscript{141,280,288} Current observations are supported by genetic association studies which have recently found increased susceptibility to TB infections in those with TLR7/8 polymorphisms.\textsuperscript{7,9,140,156}

In addition, the findings that TLR9 ligand stimulation affects the cytokine responses between active TB and LTBI highlight it is also important in TLR responses to Mtb infection. Previous animal studies have suggested the involvement of TLR9, but results
have been inconsistent.\textsuperscript{101,142-144} Recent human and genetic association studies support the observations made in the current study.\textsuperscript{145,7,8,10,119,153,166,12}

An important limitation is the lack of evaluation of site (or tissue) specific changes of innate immune markers in the MYC cohorts. It has been documented in previous reports that TLR expression and function can be different in PBMCs compared to innate immune cells at the site of disease.\textsuperscript{173,277,118} Of particular interest would be to determine if patients diagnosed with LTBI and confirmed active TB had contrasting changes in tissue samples. However, those diagnosed with LTBI by definition are well and therefore would not have a clinical indication for routine tissue sampling. Such results would however contribute to better understanding of the spectrum of these immune responses and potentially foster the theory that ‘LTBI does not equate to healthy controls’. It may also help researchers appreciate why treated or clinically cured patients do not display similar innate immune markers to healthy controls.\textsuperscript{153,170,172,203}

The results discussed in this chapter highlight several receptors, cellular and functional changes from TLR activation in the two MYC cohorts through therapy. The present results confirm that increased TLR expression does not always equate to increased functional consequences, suggested in previous reports.\textsuperscript{170} Increased or chronic TLR2 signalling for example, has been suggested to contribute to downregulation of antigen presentation to MHCII and continued survival of Mtb in macrophages.\textsuperscript{172,97} Barcelos et al. (2008) have suggested that altered innate cellular activity may in part be responsible for some people being resistant to developing active TB or acquiring Mtb infection.\textsuperscript{170}

It is important to recognise that whilst TLR expression was evaluated on different cell types, TLR function was not in the MYC cohort, and this is a pathway for further investigation. It is also likely that despite specifically not investigating adaptive mechanisms and markers of Mtb control in the current study, the strong link between TLR signalling (and possible direct signalling) between adaptive immune responses, cytokine measurements evaluated may represent the latter. Another key future investigative avenue is to delineate cellular mechanisms involved in these changes, such as the role of regulatory T cells (Tregs) and Th17 effects from TLR signalling.\textsuperscript{6,203}
Enrolment and investigation of more participants, particularly with LTBI, evaluation of other TLRs (such as TLR3 and 5), as well as mRNA expression studies would help evaluate the role of TLRs in human Mtb infections further.

5.5 Conclusions

There are clear differences in TLR expression and function which occur through treatment of latent and active TB infections. The comparative observations made in this study shed light on changes which occur following treatment for LTBI, which are poorly described previously.

The findings of the current study highlight that the differences between the two cohorts occur with respect to TLR2, 4 and 7 expression on different innate immune cells and their functional effect can reflect these changes. The findings of this study also show changes of TLR7 expression and function which have not previously been reported, as well as TLR9 function in the two clinical cohorts. The contrasting findings of TLR2 and 7 versus TLR4 expression and downstream functional changes in measurements of pro-inflammatory cytokines suggest suppression or inhibition of these, which alter post treatment. Importantly, it is plausible that patients with LTBI and active TB have different ‘TLR signatory’ profiles, which alter following antibiotic treatment, but are not equal.

The changes reported here contribute to better understanding of TLR responses in patients with active and latent tuberculosis and have the potential to contribute towards a signatory profile enabling differentiation between the two classic clinical forms of TB, as well as monitoring treatment responses.
Chapter 6: Is there an inhibitor present in patients with active TB preventing normal innate immune responses in reactivation disease?

The initial results from TLR ligand stimulation of PBMCs in a small number of patients in the active TB MYC cohort suggested an unexpected increase in TNF levels in patients with active TB following treatment. A similar trend was seen in IL-6 responses in the same group of patients (n = 6). Based on these preliminary results, and pending further laboratory work, I aimed to determine if there was an inhibitory effect from TB infection that prevented ‘normal’ host innate immune responses that may contribute to reactivation disease.

Also described in this chapter is an experiment conducted to determine which specific cells TNF was released from and how much intracellular TNF was being produced.

6.1 Intracellular TNF measurements post TLR ligand stimulation of PBMCs before and after treatment of active TB

This single experiment involved stimulation of baseline and end of treatment PBMCs from one participant, measuring TNF following TLR ligand stimulation and surface and intracellular staining through flow cytometry to determine which cells are involved (see Section 3.7). Figures 6.1 and 6.2 show a symbol and line plot of the TNF GMF (geometric mean fluorescence) on CD14+ monocytes and NKT cells respectively. Other cells, including NK cells (and subsets) and myeloid dendritic cells were also evaluated (data not shown).
Figure 6.1: Intracellular TNF responses on CD14+ monocyte cells following TLR ligand stimulation

Baseline and end of treatment PBMCs from one participant. Two lines per ligand refer to GMF of TNF at time 0 and 2 hours of Golgi plug addition. There were no significant differences following addition of Golgi plugs between baseline and final (p = NS, Wilcoxon signed-rank test).

Overall, intracellular TNF levels were lower at the end of active TB treatment in this participant, in the cell lines evaluated following TLR ligand stimulation. The exception to this were TNF GMF after TLR4 stimulation, which can be seen most clearly on NKT cells (Figure 6.2). Whilst details are not shown here, there was no definite pattern to TNF levels following TLR ligand stimulation with different times of Golgi plug addition. There was no significant difference between the TNF levels measured at baseline and end of treatment on CD14+monocytes with the Golgi plugs at 0 and two hours (p = 0.6250 and 0.125 respectively). Overall, levels of TNF released were higher on CD14+ monocytes and the least from NKT cells. Similar to results of cytokine measurements in supernatants following TLR ligand stimulation, higher levels were seen after TLR7/8 stimulation. There were no significant differences on NKT cell TNF responses with Golgi plug addition either (G0 p = 0.6250, G2 p = 0.0625) (Wilcoxon signed-rank test).
Intracellular TNF responses on NKT cells following TLR ligand stimulation

Baseline and end of treatment PBMCs from one participant. Two lines per ligand refer to GMF of TNF at time 0 and 2 hours of Golgi plug addition. There were no significant differences following addition of Golgi plugs between baseline and final (p = NS, Wilcoxon signed-rank test).

6.2 Stimulation of healthy control PBMCs with TB infected plasma: is there an inhibitory effect?

The following experiments were conducted using methodology described in Section 3.8. In summary, healthy control PBMCs were stimulated with TLR ligands, and simultaneously incubated with plasma from different patients with active TB (before antibiotic treatment). Cytokines and chemokine levels in the supernatants were subsequently measured using BD™ Cytometric Bead Array Human Protein Flex Sets. These experiments were undertaken with single replicates only.

6.2.1 Healthy control inhibitor effect: preliminary experiment

In the first experiment, one healthy control (C1), had plasma separated and PBMCs harvested from whole blood. PBMCs were then incubated with TLR ligands, and Mtb, LTBI plasma or control plasma. Plasma and media were also evaluated for levels of cytokines. For this experiment, plasma:cell ratio of 50:50 was used (see Section 3.8.1). The preliminary results from this experiment suggested that the levels of cytokines were reduced following addition of TB infected plasma, but inconsistently (data not shown). Therefore, the experiment was repeated using three healthy controls (C1-C3), addition of plasma from three patients with active TB and using higher plasma:cell ratio of 80:20 (see Section 3.8.2). The results of TNF, IL-6 and MCP1 measurements are shown in Figures 6.3.
to 6.5 below. The graphs show each healthy control with addition of TB infected plasma and their own plasma in sections. For each Mtb patient plasma addition, stimulation with TLR2, 4, 7/8 and 9 and unstimulated cytokine/chemokine measurements are shown.

Figure 6.3 shows TNF levels following stimulation with TB infected plasma in three healthy controls. The results shown have had TNF levels measured in neat TB infected plasma / control plasma and media accounted for (0.0 pg/mL in all). Compared to addition of healthy control plasma, overall there is less TNF in supernatants following addition of TB infected plasma, and this is particularly evident following TLR7/8 and TLR2 ligand stimulation. The greatest reduction in TNF levels were seen in healthy control C3. Whilst addition of TB infected plasma also affected TNF levels post TLR9- and TLR4 ligand stimulation, a consistent pattern wasn’t found. The least reduction was also seen with addition Mtb2 plasma addition (detailed evaluations not shown). Overall, however, a significant difference between groups was found with application of a two-way Anova, with both plasma addition and TLR ligand stimulation (p = 0.0011 and 0.0004 respectively).

Figure 6.3: TNF in healthy control supernatants

C1–C3 refers to healthy controls 1–3. Mtb1–3 indicates the plasma from three different patients with active TB whose baseline plasma was added prior to incubation. C1 + C1 plasma refers to healthy control plasma with their respective plasma being added at time of incubation. Absolute amounts of cytokine measured are represented by different coloured column sections in the stacked bar graph. Two-way Anova results shown.
IL-6 levels following stimulation of healthy control PBMCs with TB infected plasma is presented in Figure 6.4. The addition of Mtb plasma resulted in a reduction in IL-6 production with most TLR ligands, but not to the extent of TNF reduction. However, this trend did not apply to the addition of Mtb2 plasma to any of the healthy controls and was generally associated with an increase in IL-6 levels measured. Whilst the reasons for this are unclear from the experiment conducted, a two-way Anova found the effect of plasma addition and TLR ligand stimulation to be significantly different between the groups (p < 0.0001 and p < 0.0001 respectively).

**Figure 6.4: IL-6 in healthy control PBMCs stimulated with TLR ligands and Mtb or control plasma**

C1–C3 refers to healthy controls 1–3. Mtb1–3 indicates the plasma from three different patients with active TB whose baseline plasma was added prior to incubation. C1 + C1 plasma refers to healthy control plasma with their respective plasma being added at time of incubation. Absolute amounts of cytokine measured are represented by different coloured column sections in the stacked bar graph. Two-way Anova results shown.

MCP1 levels measured following stimulation of healthy controls with TB infected plasma is shown in Figure 6.5. The trends seen with this chemokine are different to those of TNF and IL-6. Most MCP1 levels increased following addition of Mtb2 plasma or Mtb3 plasma, compared to addition of respective healthy control plasma. The addition of Mtb1 plasma was associated with a decrease in levels of MCP1 measured post TLR ligand stimulation (with all three healthy controls C1-C3). Two-way Anova evaluation showed significant
differences with both plasma addition and TLR ligand stimulation (p = 0.0010 and p = 0.0022 respectively).

Figure 6.5: MCP1 in healthy control supernatants

C1–C3 refers to healthy controls 1–3. Mtb1–3 indicates the plasma from three different patients with active TB whose baseline plasma was added prior to incubation. C1 + C1 plasma refers to healthy control plasma with their respective plasma being added at time of incubation. Absolute amounts of cytokine measured are represented by different coloured column sections in the stacked bar graph. Two-way Anova results shown.

6.2.2 Healthy control inhibitor effect: variable plasma ratios

To assess the results above further, altered proportions of TB infected plasma was added in the next experiment to determine if plasma did contain inhibitory effects on cytokine responses, on the presumption that a true inhibitory affect may be dose-related. All TB infected plasma was taken from baseline active TB patients and pooled together for this experiment. Plasma:cell ratios of 80:20, 50:50 and 20:80 were used (details of the plasma volumes used are in Section 3.8.2). The number of PBMCs in each tube remained the same (approximately 250,000 cells). Cytokines/chemokines measured included TNF, IL-6 and MCP1. MCP1 levels were beyond the range of the CBA kit (even with dilutions) and are not discussed. Unstimulated, TLR2 and TLR9 ligand stimulations were undertaken.
TNF levels measured via CBA without TLR ligand stimulation and addition of TB infected plasma or healthy control plasma can be seen in Figure 6.6. Columns represent different plasma:cell ratios and scatter lines represent the respective measurements of TNF with control plasma added to control PBMCs at time of incubation. No clear pattern with reduction of TB plasma ratios were found. Following TLR2 ligand stimulation and addition of TB infected (or healthy control plasma), a reduction in TNF levels was evident (except in C4, where there was higher level of TNF measured with plasma:cell 20:80 compared to 50:50 ratio). The majority of TNF measurements at the respective ratios with control plasma were much lower than following TB infected plasma addition. These findings contrast those reported above in Section 6.2.1, where addition of Mtb plasma appeared to be associated with a reduction in TNF levels, suggesting the possibility of an inhibitory effect on TLR function contained in TB infected plasma.

Figure 6.6: TNF in supernatants of healthy control PBMCs stimulated with TB infected plasma

Figure 6.7: TNF in supernatants of healthy control PBMCs stimulated with TB infected plasma and TLR2 ligand (P3C)


TNF measurements following TLR9 ligand stimulation in this experiment are shown in Figure 6.8. With addition of plasma:cell 50:50 of TB infected plasma, the levels of TNF measured were less than when plasma:cell 80:20 was added, the trend did not continue with the lowest plasma:cell TB infected plasma addition. Also, no pattern was evident comparing addition of control plasma to TB infected plasma. The measurements TNF in patients with active TB where supernatants of stimulated PBMCs are shown in Figure 6.9 for comparison (of note these are absolute values, rather than normalised or fold changes, which were discussed in Chapter 4). It highlights that the levels of TNF measured post stimulation of healthy control (with varying TB plasma concentrations) were usually much higher than those in patients in the study with active TB (without addition of their own plasma during the experiments).
Figure 6.8: TNF in supernatants of healthy control PBMCs stimulated with TB infected plasma and TLR9 ligand (CpG)


Figure 6.9: TNF levels in supernatants measured following TLR ligand stimulation of PBMCs in patients with active TB
IL-6 post TLR2 ligand stimulation results of healthy control PBMCs is shown in Figure 6.10. Unstimulated and TLR9 stimulated results are not shown as the results were inconsistent, without a pattern, similar to that of TNF measurements following TLR9 ligand stimulation and without stimulation discussed above. IL-6 levels with aTLR2 ligand stimulation appear to reduce with lower levels of TB infected plasma, with the exception of C4 (where the lowest ratio of TB infected plasma 20:80 was associated with higher levels of IL-6 than 50:50 ratio). IL-6 levels with control plasma addition did not appear to have a pattern with the altered plasma ratios. Figure 6.11 shows IL-6 measurements in active TB supernatants following TLR2 ligand stimulation for comparison.

![Graph showing IL-6 in healthy controls with TLR2 stimulation, with/out MTB plasma](image)

**Figure 6.10: IL-6 in supernatants of healthy control PBMCs stimulated with TB infected plasma and TLR2 ligand**

6.3 Discussion and summary

6.3.1 Intracellular TNF measurements

The intracellular TNF measurements revealed that there was a change following antibiotic treatment in active TB. However, the results were not consistent between the unstimulated and TLR ligand stimulated samples. Similarly, no clear pattern was seen with respect to the increase or decrease following treatment, except that it mostly reduced after antibiotic therapy. Given the nature of this experiment compared to those undertaken looking at TNF levels in supernatants following TLR ligand stimulation (and therefore direct comparison is inappropriate), the fold changes seen in supernatants that TLR4 ligand stimulation was lower at the end of treatment compared to early, whilst unstimulated was higher at end of treatment compared to baseline and early in unstimulated, TLR7/8 and TLR9 ligand stimulation (Figure 4.10G, A, J and M, respectively).
It has supported studies showing that CD14+ monocytes are an important site of innate immune responses in TB, with higher GMF of TNF in these cells noted in this experiment.\textsuperscript{148,146,151} It is important, however, to note that only one patient’s blood samples were used in this experiment.

This experiment does highlight that while intracellular TNF may contribute to overall levels measured following stimulation, significant conclusions cannot be made. Further studies using participants pre and post treatment, as well as healthy controls, will help towards better understanding of responses, evaluate whether the results can be replicated and determine whether a more formative pattern can be found. The evaluation of determining which cells and how much intracellular TNF is being produced is challenging and this experiment further reinforces this notion.\textsuperscript{150,177,188}

6.3.2 Investigating presence of an inhibitor in plasma of patients with active TB disease

The cytokine responses following healthy control evaluation of innate TLR function with TB infected plasma addition provides interesting results: preliminary data discussed above showed a distinct pattern of lower cytokine measurements following addition of TB plasma prior to cell culture, potentially suggesting the presence of an inhibitor that was part of the hypothesis. An overall similar pattern of TNF and IL-6 responses were seen, which in general is considered in line with work discussed in Chapter 4. MCP1 responses appear to be different to the latter pro-inflammatory/ immunoregulatory cytokines, but interestingly similar to TLR4 functional responses. Once again, these are in line with the results from Chapter 4 of TLR functional responses in active TB. Importantly, whilst there was significant reduction of cytokines measured following the addition of Mtb infected plasma, the extent of reduction was not uniform.

Following the use of variable TB infected plasma ratios, generally a ‘dose effect’ was seen with TLR2 functional assays but this was inconsistent. However, the patterns were opposite to the previous experiment and the reasons for this are unclear. Possible factors include the use of pooled plasma from patients with active TB, or a ceiling effect being reached by the addition of plasma with the number of cells remaining the same.
In summary, TB infected plasma does appear to have an effect on TLR function. The lack of dose-effect described in Section 6.2.2, as well as contrasting results in Section 6.2.1, highlights the problems experienced with these experiments. Further work has to be done to determine its direction. In addition to conducting the experiments with a greater number of healthy controls, as well as multiple replicates, it would be important to consider evaluating patients with active TB in parallel. To further clarify whether the effects measured are a result at a transcriptional level, messenger RNA studies could also be conducted.
Chapter 7: Vitamin D and clinical correlates of toll-like receptor markers in active tuberculosis infection

The classical role of vitamin D in bone and calcium homeostasis is well recognised. Its non-classical roles in immune responses, including infections such as tuberculosis are also becoming better understood. Historically, in the pre-antibiotic era, exposure to sun was used as a treatment for those diagnosed with tuberculosis, including lupus vulgaris. More recently, vitamin D deficiency has been associated with the development of active TB.

Humans obtain vitamin D mainly from sunlight activity inducing 7-dehydrocholesterol conversion in the skin to produce vitamin D3 (colecalciferol) and to a lesser extent through dietary sources. The importance of sunlight in vitamin D production alludes to noted seasonality in Mtb infections in humans. Multiple immunomodulatory effects of vitamin D have been described, including effects on both innate and adaptive immunity. It is likely that the immune modulation is not simply from vitamin D alone, but also possibly from the effects of ultra-violet radiation on skin.

TLR2 and 4 expression variations and cytokine changes have been shown to occur in healthy adults (ex vivo) following vitamin D supplementation. Vitamin D deficiency has been shown to be associated with impaired mycobacterial killing in monocyte-macrophage cells (ex vivo) and reversed with vitamin supplementation. Vitamin D is also required for TLR-stimulated production of cathelicidin. Genetic association studies have shown both protective and increased susceptibility to Mtb infections with TLR2, 4 and vitamin D receptor (VDR) polymorphisms. It also plays an important role in autophagy.

Vitamin D deficiency has been shown to be associated with acquisition of Mtb infection, as well as development of active disease. High dose vitamin D supplementation resulted in faster sputum clearance in patients with certain VDR polymorphisms, whilst other studies have not shown a clear effect. Vitamin D supplementation is seen as an adjunct in TB treatment and its role as a host directed therapy continues to be further
investigated. Current guidelines of vitamin D deficiency and supplementation generally relate to its classical role(s), and these definitions of 'replete' and 'deficient' may not apply to patients with and at risk of Mtb infections. The current Therapeutic Drug Guidelines in Australia define vitamin D deficiency as levels < 50 nmol/L, with mild deficiency being 30–49 nmol/L, moderate 12.5–29 nmol/L and severe < 12.5 nmol/L.

In this chapter, qualitative analyses of vitamin D levels in participants in the active TB MYC cohort are undertaken, as a continuous and categorical variable, comparing it to TLR expression. Serum vitamin D levels (25-hydroxy vitamin D (25(OH)D) nmol/mL) were measured through Monash Pathology when participants were having blood samples collected at baseline, one and two months into treatment and end of treatment. In addition, general clinical correlates are also described.

7.1 Epidemiological and clinical associations of TLR markers in active tuberculosis treatment

The following variables were evaluated to determine if there were any associations with TLR expression through treatment: age, gender, region of birth (according to WHO classification1), duration of time lived in Australia, disease burden, comorbidities, presence of diabetes, previous treatment for active TB and drug sensitivity profile. These were not able to be evaluated through a multivariate analysis because of the large number of laboratory variables and the small numbers in each group to be analysed. Clinical data was evaluated as categorical or continuous data.

7.1.1 Demographics and influence on TLR expression

The following variables were assessed using GEEs. The latter were utilised to determine the effect of the following variables on TLR expression, as well as adjusting for changes of these variables over time, using the natural logarithm of TLR expression. Each variable was assessed individually for TLR2, 4 and 7 expression in different cell types (CD14+ monocytes, NKT cells and NK cells).
**Age and gender**

There was no effect of gender on TLR expression on any cell types evaluated. There was a trend to an association of reduced TLR2 expression on NKT cells for every year of age in this cohort (coefficient $-0.01$, 95% CI $-0.02$ to $0.00$, $p = 0.05$) and a similar 1% reduction in TLR2 expression on NK cells (coefficient $-0.01$, 95% CI $-0.02$ to $0.00$, $p = 0.06$).

**Region of birth**

Participants in the active TB cohort were born in different countries representing each of the five regions of birth (see Table 4.1). Due to the small numbers in all but two regions, TLR expression was only evaluated in Southeast Asian and Western Pacific regions (accounting for 81% of study participants). There was a trend to lower TLR4 expression on NKT and NK cells between these two regions. The coefficient for NK cells of TLR4 expression was $-0.13$ (95% CI $-0.27$ to $0.01$), $p = 0.07$, suggesting that those born in the Western Pacific region have 12.2% lower NK cell TLR4 expression compared to those from the Southeast Asia region. NKT TLR4 expression also had a trend to be lower in those from the Western Pacific region with a coefficient of $-0.12$ (95% CI $-0.26$ to $0.02$), $p = 0.09$, suggesting an 11.3% lower TLR4 expression.

There was no significant effect of region of birth with respect to TLR2 and 7 on different cell lines using GEE.

**Duration of time living in Australia**

There was no effect of length of stay in Australia on TLR expression in the active TB cohort. Evaluation was undertaken as less than five years in Australia, more than five years in Australia, born in Australia or unknown.
7.1.2 Clinical associations with TLR markers over time

GEE were applied to the following clinical parameters: previous treatment for active TB, presence of diabetes or other comorbidities. The effect of disease burden, culture sensitivity is discussed below. The effect of vitamin D is discussed separately in Section 7.2.

Disease burden

Disease burden was defined as mild/moderate or severe: those with more than one site of disease, cavitary pulmonary TB, CNS disease, miliary TB or involvement of multiple lobes on chest radiography were deemed to have a ‘severe’ burden of disease. It is important to recognise that this may not be classified as ‘severe’ in high endemicity nations.

There was a trend of 16% lower TLR4 expression on CD14+ monocytes in those severe burden of disease (coefficient −0.17, 95% CI −0.34 to 0.01, p = 0.07). Disease burden did not appear to significantly affect TLR2 or 7 expression.

Signed rank-sum tests were applied to determine if there was an effect of disease burden on cytokine measurements following TLR ligand stimulation. The only significant findings were at one month with IL-6 levels following TLR9 ligand stimulation, where the median IL-6 level was lower in those with severe disease 30.79 pg/mL (22.00, 55.00 IQR, n = 6), compared to those with mild/moderate disease 202.50 pg/mL (97.00, 240.00 IQR, n = 26, p = 0.002) (Figure 7.1). At the same timepoint, TNF levels following TLR9 ligand stimulation were also significantly lower 1.00 pg/mL (1.00, 4.00 IQR, n = 6) in those with severe disease compared to those without 9.00 pg/mL (1.00–16.00, n = 26, p = 0.025) (Figure 7.2).
Figure 7.1: Differences in IL-6 levels following TLR ligand stimulation based upon burden of disease classification

Cytokine measurements in pg/mL. Box plots show line at median and IQR.

Figure 7.2: Differences in TNF levels following TLR ligand stimulation based upon burden of disease classification

Cytokine measurements in pg/mL. Box plots show line at median and IQR.
Univariate analyses (using rank sum) was used to evaluate the differences in plasma cytokine levels with different variables. There were minimal significant differences found with the cytokines and the above mentioned variables, with the exception of disease burden. Several cytokines (IL-6, IL-8, IL-10, IP-10, TNF and IFNγ) varied through treatment with respect to disease burden classification. These differences were particularly marked for IL-10 and IP-10 and found to be higher with more severe disease throughout treatment than in participants with mild/moderate disease burden (Table 7.1).

Table 7.1: Association of plasma cytokine levels and disease burden in MYC active cohort

<table>
<thead>
<tr>
<th>Plasma cytokine</th>
<th>Mild/moderate disease (n = 37) (median, IQR)</th>
<th>Severe disease (n = 16) (median, IQR)</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>38.79 (26.91, 59.44)</td>
<td>65.36 (37.73, 239.03)</td>
<td>0.029</td>
</tr>
<tr>
<td>Early TP*</td>
<td>37.73 (24.91, 77.01)</td>
<td>82.80 (61.62, 119.24)</td>
<td>0.004</td>
</tr>
<tr>
<td>Final TP</td>
<td>38.79 (24.91, 55.07)</td>
<td>43.30 (29.20, 69.42)</td>
<td>0.605</td>
</tr>
<tr>
<td>IP-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>140.21 (101.17, 239.43)</td>
<td>325.80 (186.23, 1009.85)</td>
<td>0.001</td>
</tr>
<tr>
<td>Early TP</td>
<td>141.47 (91.26, 211.36)</td>
<td>272.40 (214.81, 492.53)</td>
<td>0.007</td>
</tr>
<tr>
<td>Final TP</td>
<td>105.06 (76.96, 121.94)</td>
<td>139.95 (100.90, 273.68)</td>
<td>0.017</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16.99 (3.75, 64.91)</td>
<td>38.35 (6.27, 104.51)</td>
<td>0.301</td>
</tr>
<tr>
<td>Early TP</td>
<td>6.34 (3.36, 45.44)</td>
<td>147.54 (9.53, 469.88)</td>
<td>0.002</td>
</tr>
<tr>
<td>Final TP</td>
<td>6.32 (3.29, 17.46)</td>
<td>11.29 (6.34, 83.59)</td>
<td>0.096</td>
</tr>
<tr>
<td>TNF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>6.44 (4.91, 10.55)</td>
<td>11.39 (5.95, 25.80)</td>
<td>0.096</td>
</tr>
<tr>
<td>Early TP</td>
<td>7.25 (4.85, 10.55)</td>
<td>13.08 (8.04, 18.50)</td>
<td>0.015</td>
</tr>
<tr>
<td>Final TP</td>
<td>7.25 (4.07, 10.55)</td>
<td>8.89 (3.87, 10.65)</td>
<td>0.798</td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.45 (0.58, 5.71)</td>
<td>4.47 (1.98, 18.46)</td>
<td>0.056</td>
</tr>
<tr>
<td>Early TP</td>
<td>2.17 (0.88, 6.86)</td>
<td>2.32 (1.62, 14.79)</td>
<td>0.411</td>
</tr>
<tr>
<td>Final TP</td>
<td>3.37 (1.27, 4.57)</td>
<td>5.56 (2.04, 21.29)</td>
<td>0.109</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.48 (3.65, 8.44)</td>
<td>17.06 (9.53, 24.22)</td>
<td>0.004</td>
</tr>
<tr>
<td>Early TP</td>
<td>4.58 (3.38, 10.12)</td>
<td>5.48 (0.20, 13.62)</td>
<td>0.945</td>
</tr>
<tr>
<td>Final TP</td>
<td>3.38 (2.34, 5.00)</td>
<td>3.91 (2.67, 12.84)</td>
<td>0.613</td>
</tr>
</tbody>
</table>

* TP: timepoint. ‘Early TP’ refers to either one or two month timepoint plasma cytokine measurement. IQR: interquartile range.
**Culture and sensitivity results**

Using GEE to evaluate the effect of culture sensitivity results revealed interesting findings; whilst there was no effect seen between fully sensitive, isoniazid resistant and/or MDR-TB (multi-drug resistant TB), there was an effect seen between culture negative TB and fully sensitive isolates (9/53 and 38/44 respectively; see Table 4.2).

TLR2 expression was significantly reduced on NK, NKT and CD14+ monocytes in participants with culture negative active TB, compared to those with fully sensitive disease (Table 7.2). There was reduced expression of 35%, 33.6% and 23.7%, respectively. There appeared to be a significant effect of TLR4 expression on NK and NKT as well, with a reduction of 17.3% and 18.1%, respectfully in culture negative TB compared to fully sensitive disease (Table 7.2). There was no effect on TLR7 expression in cells evaluated with culture and sensitivity testing.

**Table 7.2: Effect of culture and drug sensitivity testing on TLR expression on different cell types compared to fully sensitive TB infection**

<table>
<thead>
<tr>
<th>Cell type and TLR</th>
<th>Culture negative</th>
<th>Isoniazid resistant</th>
<th>Multi-drug resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient (95% CI)</td>
<td>p value</td>
<td>Effect</td>
</tr>
<tr>
<td>CD14+ monocytes</td>
<td>-0.27 (0.54, -0.01)</td>
<td>0.042</td>
<td>23.7% reduction</td>
</tr>
<tr>
<td>TLR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK cells TLR2</td>
<td>-0.43 (-0.79, -0.08)</td>
<td>0.017</td>
<td>35% reduction</td>
</tr>
<tr>
<td>NKT TLR2</td>
<td>-0.41 (0.77, -0.06)</td>
<td>0.021</td>
<td>33.6% reduction</td>
</tr>
<tr>
<td>NK cells TLR4</td>
<td>-0.19 (0.34, -0.03)</td>
<td>0.017</td>
<td>17.3% reduction</td>
</tr>
<tr>
<td>NKT TLR4</td>
<td>-0.2 (-0.35, 0.05)</td>
<td>0.010</td>
<td>18.1% reduction</td>
</tr>
</tbody>
</table>

* GEE were used with natural logarithm of TLR expression, hence coefficients are exponential. Significant p values < 0.05 are in bold. There was no effect on TLR7 expression on cell types evaluated using this statistical method (data not shown).
7.2 Vitamin D and TLR expression associations

Vitamin D levels were recorded pre-antibiotics and at the time of one, two months and final timepoint blood sampling (where available). Vitamin D supplementation was guided by local recommendations and individual patient treatment decided by the clinician. Spearman’s coefficient was used to determine if associations existed between two variables, at each timepoint of TLR expression between baseline vitamin D levels. Correlations of vitamin D levels at different timepoints and TLR expression were also evaluated. GEE of vitamin D levels (as a continuous variable) were evaluated to determine the effect on TLR expression over the treatment period.

7.2.1 Baseline (or pre-treatment) vitamin D level and TLR expression correlation

There were several weak associations between vitamin D levels and (the natural logarithm of) TLR expression. These were mainly of TLR4 and TLR7 expression on different cell types.

**CD14+ monocytes**

On CD14+ monocytes, TLR7 expression with baseline vitamin D level was associated with a weak positive correlation at one month (correlation coefficient 0.360, p = 0.013), and two months timepoints (correlation coefficient 0.295, p = 0.044) (Figure 7.3). At baseline, this did not reach significance (p = 0.056, coefficient 0.265).

There were no significant changes found between TLR2 and TLR4 using this analysis method.
Figure 7.3: Baseline vitamin D and TLR7 expression on CD14+ monocytes through treatment

Natural logarithm of TLR expression shown with significant Spearman’s correlation coefficients $p < 0.05$.

**NK cells**

TLR4 expression had a weak negative association with baseline vitamin D levels at one month (correlation coefficient $-0.337$, $p = 0.021$), whilst at baseline it did not reach significance (correlation coefficient $-0.249$, $p = 0.072$) (Figure 7.4).

There was a weak association with TLR7 expression at the end of treatment (correlation coefficient 0.287, $p = 0.041$), whist at two months, significance was not reached (correlation coefficient 0.271, $p = 0.062$) (Figure 7.5).

There were no significant changes found between TLR2 and baseline vitamin D.
**Figure 7.4: Baseline vitamin D and TLR4 expression on NK cells through treatment**

Natural logarithm of TLR expression shown with significant Spearman's correlation coefficients $p < 0.05$.

**Figure 7.5: Baseline vitamin D and TLR7 expression on NK cells through treatment**

Natural logarithm of TLR expression shown with significant Spearman's correlation coefficients $p < 0.05$. 
**NKT cells**

Similar to NK cells, there were negative weak associations with TLR4 and positive correlations with TLR7 expression. Baseline vitamin D was associated with lower TLR4 expression at baseline (correlation coefficient $-0.322$, $p = 0.019$), one month (correlation coefficient $-0.366$, $p = 0.011$) and end of treatment (correlation coefficient $-0.277$, $p = 0.046$) (Figure 7.6). The slightly higher TLR7 expression was found at two months (correlation coefficient 0.290, $p = 0.048$; see Figure 7.7).

There were no significant changes found between TLR2 and baseline vitamin D.

**Figure 7.6: Baseline vitamin D and TLR4 expression on NKT cells through treatment**

Natural logarithm of TLR expression shown with significant Spearman’s correlation coefficients $p < 0.05$. 
7.2.2 Vitamin D levels through treatment and association with TLR expression through treatment timepoints

Vitamin D level changes between timepoints were compared to TLR expression at different timepoints using Spearman’s coefficient (of the natural logarithm of TLR expression). This was undertaken to see if there was a correlation between vitamin D supplementation (therefore consequent increase in serum 25(OH)D levels), and TLR expression.

The effect of vitamin D levels and TLR expression through treatment longitudinally was assessed with GEE. This was undertaken with vitamin D as a continuous variable as well as categorically based on deficiency and extent of deficiency where applicable. Given the small numbers in subgroups based on the latter, presented results relate only to vitamin D levels as a continuous variable.

Overall, the mean serum 25(OH)D levels were 36 nmol/L, 76 nmol/L and 66 nmol/L at baseline, early in treatment (one or two months) and end of treatment, respectively.
7.2.2.1 Changes in vitamin D and correlation with TLR expression at varying timepoints

On CD14+ monocytes, the change in vitamin D levels from baseline to end of treatment was associated with higher end of treatment TLR2 expression (correlation coefficient 0.305, p = 0.039). A trend towards lower TLR7 expression at one month was found when comparing vitamin D change from baseline to one month (coefficient –0.287, p = 0.073) on CD14+ monocytes.

Vitamin D changes from base to end of treatment was associated with an increase in end of treatment TLR4 expression on NKT cells (correlation coefficient 0.315, p = 0.033) and a similar association on NK cells for the same evaluation (correlation coefficient 0.313, p = 0.034). A trend towards lower end of treatment TLR7 expression was found when comparing vitamin D change from baseline to one month (coefficient –0.278, p = 0.067) on NK cells.

7.2.2.2 Vitamin D effect on TLR expression through treatment using GEE

The use of GEE was to ascertain the effect of vitamin D in a similar way as other variables in Section 7.1.1. Baseline vitamin D was associated with TLR7 expression on CD14+ monocytes, where an increase of 1 nmol/L was associated with a 1% increase in TLR7 expression (95% CI 0.00, 0.01) (p = 0.04). When evaluating TLR4 expression on NK cells, an increase in vitamin D of 1 nmol/L was associated with a 0.5% decrease in expression (95% CI –0.01, 0.00) (p = 0.02). A similar effect was seen NKT cells and TLR4 expression (95% CI –0.01, 0.00) (p = 0.01).

When vitamin D was evaluated as a categorical variable, there were significant associations only when levels were replete (≥ 70 nmol/L). On CD14+ monocytes, TLR7 expression was 85% greater when vitamin D levels were ≥ 70 nmol/L (95% CI 0.03, 1.22) (p = 0.039). A trend towards 33% lower TLR2 expression with replete vitamin D levels was found on CD14+ monocytes (95% CI –0.82, 0.02) (p = 0.064). The implications of the latter results are unclear as they involve small numbers.
7.3 Discussion

The predominantly qualitative analyses in this chapter highlight several clinical and epidemiological factors that were important in this cohort of patients with active TB infection, through an association with or having an effect upon TLR expression.

7.3.1 Epidemiological and clinical factors affecting TLR expression in active TB

7.3.1.1 Trends suggesting an influence of region of birth and age on TLR expression

TLR4 expression appeared to be influenced by region of birth in the MYC active TB cohort on NKT and NK cells. Whilst there were only small numbers in each group (i.e. Southeast Asia or Western Pacific regions), it is possible these trends represent underlying genetic polymorphisms which have been well described to be associated with tuberculosis susceptibility.\(^7\)\(^-\)\(^9\)\(^,\)\(^12\)\(^,\)\(^155\)\(^,\)\(^162\) It is important to recognise, however, that no genetic association studies were undertaken in the current study but would be an important avenue to consider investigating in the future especially if these trends are confirmed.

There were trends to suggest TLR2 expression was reduced with advancing age in this cohort. A previous study reported positive correlation with age of TLR2 expression on monocytes, whilst finding reduced TLR4 and 8 function increasing age, in a cohort of healthy participants with no significant chronic infections, malignancies or autoimmune disease.\(^2\)\(^16\)

7.3.1.2 TLR2 and 4 expression were significantly lower in those with culture negative TB

Other interesting findings relate to altered TLR expression in those with culture negative TB diagnosis compared to participants with fully sensitive disease. There were marked differences in TLR2 and 4 expression on different cell types (described in Table 7.2), with 23–35% reduction in TLR2 expression and 17–18% reduction in TLR4 expression in this group of patients. Whilst only nine participants had culture negative disease, it is plausible that theirs was a ‘paucibacillary’ form of active disease and therefore resulting in altered immune responses.\(^2\)\(^0\)\(^6\)\(^4\)\(^,\)\(^2\)\(^9\)\(^1\)\(^,\)\(^2\)\(^9\)\(^2\) It is also possible that the lack of any significant
findings between those with isoniazid resistant or MDR-TB was due to small numbers in the latter two groups (n = 3 each). There is a paucity of information available in the literature regarding TLR expression in those with culture negative disease, mainly because most translational studies involve patients in high-endemicity countries where those with symptomatic disease and positive culture results are generally recruited. It is plausible then that patients with paucibacillary disease may in fact have a different ‘immune profile’ or signature and these altered innate immune responses may be an important way of differentiating or identifying patients along the TB spectrum (Figure 2.4).

7.3.1.3 TLR markers in active TB vary with disease burden

IL-6 and TNF levels post TLR9 ligand stimulation was lower at one month in those with severe disease compared to those classified as having mild/moderate disease. Once again, the numbers being compared are small in each group but these findings suggest the importance of TLR9 in active TB, where there is a paucity of translational research data available.

Plasma cytokines measurements varied with disease burden in the active TB cohort and importantly these significant changes were found at baseline or early during the intensive phase of treatment. IL-10, IL-8, IP-10, TNF, IL-6 and IFNγ levels were significantly higher in those with severe disease (Table 7.1). Interestingly, the stimulated cytokines were lower (IL-6 and TNF) in severe disease in analyses where it was found to be significant (see above). Though plasma cytokines represent adaptive and innate immune responses, plasma sampling is convenient and easy to access, and therefore any changes found are important to consider as part of a platform in the search for biomarkers.  

7.3.2 Vitamin D and TLR expression: cause or effect?

Baseline vitamin D levels were associated with negative TLR4 expression, whilst associated with weak positive TLR7 through treatment on different cell types.

Whilst the correlations found in this part of the study are weak and did not occur through all timepoints on cell types evaluated, the findings nevertheless provide insight into the relationship between vitamin D and TLRs in active TB infection. The trends found with
vitamin D and TLR4 and 7 expression are similar to trends of TLR expression on these cell lines described in Chapter 4, with TLR2 and 7 generally increasing through treatment, whilst TLR4 decreased on NK cells and CD14+ monocytes. Supplementation of vitamin D has been shown to affect TLR expression in other translational studies, including TLR2 expression and TLR4 function. Whilst there was no significant effect seen on TLR2 expression using Spearman’s correlation in baseline vitamin D evaluations, several trends and effects were found using GEE and changes in vitamin D through treatment. Further analyses need to be undertaken to evaluate whether there are correlations with TLR function and vitamin D in the MYC active TB cohort. The findings reported in this chapter do not concur with a report by Sadeghi et al. (2006), where the authors found that TLR2 and 4 expression were downregulated by incremental vitamin D supplementation (ex vivo) of human monocytes. However, the human samples were not Mtb infected in the latter study.

To the author’s knowledge, there have been no translational reports of TLR7 associations with vitamin D in Mtb infections. Vitamin D modulation of TLR7 expression in tumour pathogenesis has been described, and decreased TLR7 expression in immune cells (such as monocytes and B and T lymphocytes) reported with increased serum vitamin D levels. My findings have implications for the role of vitamin D and TLR7, especially given genetic association studies in recent years suggesting a role in disease susceptibility and severity.

It is reported that anti-tuberculous therapy is associated with lowering of vitamin D levels. Whilst this pattern was also seen in the MYC cohort, the reasons for the decline cannot be attributed to TB therapy in the current study, as vitamin D supplementation often occurred (and at the treating clinician’s discretion). Baseline vitamin D levels in those born in Australia in this cohort were replete according to Australian Guidelines; however, it is possible that the recommended levels in Australia are inadequate in the management of active TB (rather the definitions and replacement guidelines are for bone and calcium homeostasis management). It is likely that patients with active TB require higher levels of vitamin D for adequate innate and adaptive responses which are vitamin D dependent. The majority of participants in the cohort were vitamin D deplete. However, the majority were also born in high TB endemicity countries and therefore it is
not possible to associate vitamin D deficiency alone as a risk factor in these participants. Importantly, it can be acknowledged that the single most represented country in this cohort was India, and it is established that persons with dark skin do not produce vitamin D as efficiently and have lower vitamin D levels with similar sun exposure to those with fair skin.\textsuperscript{30} It has also been shown that migrants have lower vitamin D levels post resettlement compared to those living in the country of origin, suggesting that this may be a risk factor for TB reactivation soon after migration.\textsuperscript{30,295} However, the role of low vitamin D levels in TB reactivation and infection is essentially undisputed based on observational studies over many decades, showing that those who are fair-skinned are also at higher risk with low vitamin D levels.\textsuperscript{30,296,15} The latter also raises an important risk of a group in Australia with potential for reactivation TB that is often under-recognised: the elderly.\textsuperscript{37} The MYC cohort highlights this with respect to vitamin D and age, as well as TLR expression, but also with respect to the numbers of participants in the older age groups (Table 4.1). The role of age in TLR expression, serum vitamin D levels and cathelicidin was investigated in healthy patients of different age groups, with authors finding no significant effect of vitamin D on TLR expression with advancing age, but lower cathelicidin levels and negatively correlated TLR expression (with advancing age) and with the cathelicidin levels on different PBMCs cell lines.\textsuperscript{216}

\textbf{7.3.3 Limitations of the present study}

The small numbers of participants in this study, particularly with a heterogenous cohort, is the main limitation. This is particularly so when evaluating multiple clinical and demographic variables in comparison to laboratory data.

The evaluation of the effect of vitamin D is further confounded by the observational nature of the study and therefore no control over management of participants’ vitamin D serum sampling or supplementation. Similarly, whilst the effects were mainly analysed with vitamin D levels as a continuous variable, it would be prudent to evaluate results further based on different categorical definitions; the current guidelines in Australia for vitamin D supplementation was used, although literature pertaining to tuberculosis would suggest alternate classifications.\textsuperscript{290,204,205}
Another limitation of the study is lack of analysis of the LTBI cohort, as well as detailed evaluation of TLR function in relation to the epidemiological and clinical variables. The main reason the latter was not undertaken was due to small numbers in several cytokines measured at different timepoints.

Lastly, further investigational steps as described above and in previous chapters would enable better understanding of the TLR effect at a cellular level, rather than relying on observational results presented here.

### 7.4 Summary and future investigative directions

The role and importance of TLR expression and consequent function is reinforced through the demographic and clinical variable correlates reported in this chapter: age, burden of disease and region of birth appear to have significance in TLR expression, particularly TLR4 and 7 on NK and NKT cells. The findings of culture negative disease having altered TLR expression compared to those with fully sensitive disease is of interest and, to the author's knowledge, not previously described.

The current study results confirm the hypothesis that vitamin D is associated with TLR expression. This translational, observational study also highlights previous reports in the literature that vitamin D has an important role in active TB. It also further establishes a link and potential effect on TLR expression and/or its role in TB immunopathogenesis being affected by TLR expression. The contrasting associations of TLR4 and TLR2/7 are generally consistent with findings described in previous chapters with respect to TLR expression on varied cell lines evaluated. Whilst it is unlikely that the main reason for this is vitamin D levels, it is likely to be a contributing factor.

To the author's knowledge, this is the first report of association of vitamin D levels and TLR7 expression reported in active TB patients. It further illustrates the potential role of TLR7 in innate immune responses to Mtb infection, which have been alluded to in recent years through genetic association studies but have not been reported extensively at a cellular or molecular level in humans.

Evaluation of the LTBI cohort and comparison of vitamin D levels and correlations of TLR expression would potentially shed further light on the role of vitamin D in Mtb infections,
but also of whether it contributes to ‘protection’ or a ‘putative resistant’ (to developing active TB) profile as suggested in previous studies.\textsuperscript{18,147,297,170} Further follow-up of the LTBI in the future to determine their TLR expression/ vitamin D levels and development of active TB would also be enlightening.

Whilst the results discussed show strong correlation with TLR expression on different cell types, further investigation into determining the cellular level of its effects would be prudent, especially with TLR7. Further investigation of TLR and VDR polymorphisms in select groups of these patients with active TB disease may also shed light on their specific role(s).

\textbf{7.5 Conclusions}

Age, disease burden and region of birth are associated with altered TLR signalling in active TB disease. These trends need to be further explored in future studies. Vitamin D plays a key role in immune responses in active TB. The observational data explored here further establishes the role of vitamin D in relation to innate immune responses in the form of TLR expression in participants with active TB disease. Whilst more detailed studies need to be conducted to establish its mechanism of effect (or being affected by TLR activation), the potential for its ongoing use as an adjuvant or host-directed therapy is reinforced by the observations of the present study.
Chapter 8: QuantiFERON-TB Gold Plus: evaluation of a new interferon-gamma release assay in a cohort of patients with active and latent tuberculosis in Melbourne, Victoria

Interferon-gamma release assays (IGRAs) have become common place in diagnosing latent tuberculosis infections (LTBI) in developed countries. The diagnosis of LTBI relies on the exclusion of active tuberculosis by clinicians, as there is no gold standard test currently. The impact of identifying and treating those with LTBI in high risk groups in low-income countries is important and has widespread economic ramifications, as well as being a vital part of the WHO’s Global End TB Strategy. The WHO recently recommended the utilisation of IGRAs in LTBI diagnosis in resource-poor settings. Similarly, IGRA was recommended for LTBI diagnosis instead of TST in recently published IDSA/CDC guidelines for persons over the age of five.

IGRAs and tuberculin skin tests (TST) are surrogate markers of Mtb infection, measuring cellular immune responses to Mtb antigen sensitisation in an individual, whilst not differentiating between remote or recent exposure and infection. Advantages include high sensitivity and specificity to Mtb, and minimal cross reactivity in persons who have had BCG vaccination compared to TST based testing. It also has less cross-reactivity with non-tuberculous mycobacterial infections. However, disadvantages include cost, laboratory requirements for the assays and inability of the tests to differentiate between active and latent TB infections. Other disadvantages include reduced sensitivity in the immunocompromised host and children due to altered immune responses.

Two forms of IGRA tests are currently available commercially: QuantiFERON-TB Gold (QFT) / QuantiFERON-TB Gold Plus (QFT-Plus) (Qiagen, Hilden, Germany) and T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK). They both assess Mtb-specific T cell responses by measuring IFNγ levels through ex vivo stimulation of peripheral blood T cells with Mtb antigens. QFT measures IFNγ using ELISA, whilst T-SPOT.TB uses an...
enzyme-linked immunospot (ELISPOT) technique. QFT uses peptides eliciting responses of CD4+ T cells to ESAT-6, CFP-10 and TB 7.7, whilst T-SPOT uses Mtb antigens representing ESAT-6 and CFP-10. Currently available sensitivity and specificity data regarding these assays are detailed in Chapter 2 (Section 2.6.1).

8.1 QFT-Plus: the main changes from QFT

QFT-Plus measures IFNγ levels using peptides associating with MHC Class I and II molecules. The new test includes altered peptide lengths of Mtb antigens to associate with CD4+/MHC-II in TB antigen tube 1 (TB1), with the addition of shorter length peptides to associate with CD8+/MHC-I T cells in TB antigen tube 1 (TB2). Compared to QFT, the new test uses ESAT-6 and CFP-10 as the Mtb antigens (TB 7.7 was excluded). Hence TB2 measures IFNγ responses of Mtb-specific CD4+ and CD8+ T cells. Petruccioli et al. (2016), confirmed that TB1 elicited CD4+ T cell response, while TB2 induced both CD4 and CD8 responses, through intracellular staining of PBMCs harvested at the time of QFT-Plus sampling. The authors also suggest a stronger TB2 response based on higher CD8 intracellular staining in patients with greater TB disease severity and microbiological burden, as well as an association with active TB and recent LTBI infection.

In studies evaluating the new QFT-Plus, authors report a high agreement between QFT-Plus, QFT and/or T-SPOT.TB. Where applicable, most authors reported similar or improved sensitivity of the newer test in people living with HIV and/or who are immunocompromised, and lower overall IFNγ measurements in the newer QFT-Plus, whilst one study reported higher levels. The only study which assessed QFT-Plus through antibiotic treatment suggested this may be a plausible option for treatment monitoring. Several studies suggested lowering the cut-off for a ‘positive’ result from its current 0.35 IU/mL for QFT-Plus. Published reports of QFT-Plus research are summarised in Chapter 2 (Table 2.3).

Based on the recent literature, I hypothesise that the newer QFT-Plus will have similar sensitivity results to its predecessor, but potentially different IFNγ levels in the new antigen tubes. I also hypothesise that there will be a change, likely a decrease, in absolute IFNγ release at the end of anti-tuberculous treatment. I will also evaluate whether this decline will permit treatment response monitoring. Qiagen has not been involved in any
data analyses. Methodology and statistical analyses are described in Section 3.9 and 3.10, respectively. Qiagen Ltd provided the tubes for the QFT and QFT-Plus tubes (initially F1-F6, consequently TB1 and TB2).

8.2 Results

Forty-two patients with active TB and 21 with LTBI were recruited. Of these, 39 and 21 respectively were planned to have longitudinal blood sampling through their treatment. However, in the active TB cohort, 29 (74%) participants had a minimum of three blood samples collected, whilst in the LTBI cohort only seven (33%) had three blood samples collected, whilst nine (43%) provided end of treatment samples. Poor compliance with attendance for follow-up is well documented in the literature in patients with LTBI.228 None of the participants were HIV positive; several had underlying condition(s) which may have contributed to altered immune responses including diabetes mellitus (n = 3, 4.7%), pregnancy (n = 1, 1.6%) and hepatitis/liver dysfunction or respiratory disease (n = 13, 20.6%). One participant in the active TB group refused end of treatment blood sampling but consented to previous samples being used in the research, whilst one female in the LTBI cohort withdrew (no reason provided). Another participant in the LTBI cohort became pregnant during the study and chose to stop isoniazid therapy, but still consented to having baseline blood sampling to be used in the research study.

There were no significant differences between the active and LTBI cohort with respect to IFNγ responses between the genders (Figure 8.2).
Figure 8.1: Numbers of patients treated for active TB at Monash Health (2011–2015)

Figure 8.2: Age and gender distribution of participants in active TB and LTBI cohorts, QuantiFERON study group (2013–2016)

Only patients whose blood samples were analysed are included.
8.2.1 Demographics of the active TB and LTBI cohorts

Table 8.1 and Table 8.2 summarise the clinical and demographic details of participants in the QFT active TB cohort and Table 8.3 the demographic details of QFT-LTBI cohort.

Table 8.1: Baseline demographics of QuantiFERON active TB cohort

<table>
<thead>
<tr>
<th>Demographic characteristics (total 42)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>24 (57)</td>
</tr>
<tr>
<td>Female</td>
<td>18 (43)</td>
</tr>
<tr>
<td>Mean age (years ± SD)</td>
<td>32.5 (SD 17.5 years)</td>
</tr>
<tr>
<td>Region of birth*</td>
<td></td>
</tr>
<tr>
<td>SE Asia</td>
<td>16 (38)</td>
</tr>
<tr>
<td>Subcontinent</td>
<td>16 (38)</td>
</tr>
<tr>
<td>Middle East</td>
<td>4 (9)</td>
</tr>
<tr>
<td>Africa</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Europe</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Australia</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Previous TB treatment</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>36 (85)</td>
</tr>
<tr>
<td>Yes</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Data not available</td>
<td>2 (5)</td>
</tr>
<tr>
<td>BCG vaccination</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20 (48)</td>
</tr>
<tr>
<td>Childhood vaccination</td>
<td>19 (95)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (5)</td>
</tr>
<tr>
<td>No</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Data not available</td>
<td>19 (45)</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>26 (62)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Hepatitis / liver dysfunction</td>
<td>6 (14)</td>
</tr>
<tr>
<td>COPD / asthma</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Cardiac / vascular disease</td>
<td>7 (17)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>0</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>0</td>
</tr>
</tbody>
</table>

* Region of birth according to WHO grouping: Australia listed separately; would otherwise be included in Western Pacific region.¹
Table 8.2: Clinical disease and microbiological findings in active TB cohort

<table>
<thead>
<tr>
<th>Clinical and microbiological characteristics (total 42)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms (present)</td>
<td>35/42</td>
</tr>
<tr>
<td>TB disease</td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>28 (67)</td>
</tr>
<tr>
<td>non-cavitary</td>
<td>18 (64)</td>
</tr>
<tr>
<td>cavitary</td>
<td>10 (36)</td>
</tr>
<tr>
<td>LN disease</td>
<td>18 (43)</td>
</tr>
<tr>
<td>Pleural</td>
<td>2 (5)</td>
</tr>
<tr>
<td>&gt; one site</td>
<td>6 (14)</td>
</tr>
<tr>
<td>Smear positive (sputum / BAL sampling)*</td>
<td>10/28</td>
</tr>
<tr>
<td>Culture</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33 (79)</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (21)</td>
</tr>
<tr>
<td>MTB PCR and/or histology consistent with TB*</td>
<td>7/9 (78)</td>
</tr>
<tr>
<td>Drug sensitivity</td>
<td></td>
</tr>
<tr>
<td>Pan-sensitive</td>
<td>29/33</td>
</tr>
<tr>
<td>Isoniazid resistance only</td>
<td>2/33</td>
</tr>
<tr>
<td>MDR-TB*</td>
<td>1/33</td>
</tr>
</tbody>
</table>

# TB disease according to site.
* Respiratory samples only tested for acid fast bacilli (AFB) included. BAL: bronchoalveolar lavage.
∞ MTB PCR IS6110 results where applicable; anatomical pathology results showing necrotising granulomatous changes / caseating granulomas or non-necrotising granulomas.
Φ MDR-TB defined as per WHO Guidelines.¹
Table 8.3: Clinical and demographics of participants with LTBI in QFT cohort

<table>
<thead>
<tr>
<th>Demographic and clinical characteristics (total 21)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>11 (52)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (48)</td>
</tr>
<tr>
<td>Mean age (years ± SD)</td>
<td>33.5 (± 9)</td>
</tr>
<tr>
<td>Region of birth*</td>
<td></td>
</tr>
<tr>
<td>SE Asia</td>
<td>10 (47)</td>
</tr>
<tr>
<td>Subcontinent</td>
<td>2 (9)</td>
</tr>
<tr>
<td>Middle East</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Africa</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Australia</td>
<td>4 (20)</td>
</tr>
<tr>
<td>South America</td>
<td>1 (5)</td>
</tr>
<tr>
<td>BCG vaccination</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (33)</td>
</tr>
<tr>
<td>Childhood vaccination</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Data not available</td>
<td>10 (47)</td>
</tr>
<tr>
<td>CXR</td>
<td></td>
</tr>
<tr>
<td>Clear/normal</td>
<td>15 (71)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>6 (29)</td>
</tr>
</tbody>
</table>

* Region of birth according to WHO grouping: Australia listed separately; would otherwise be included in Western Pacific region.¹

8.2.2 Active TB results

As mentioned previously, a total of 42 participants were recruited for the active TB cohort, of whom 39 were eligible to provide blood samples through their treatment course. Early treatment samples were available (TP1 or TP2) for 26/39 (66.7%) and 26/39 (66.7%), respectively. End of treatment blood sampling occurred for 30/39 (76.9%) (Table 8.4).

Table 8.4: Blood sample collection success through treatment in active TB cohort

<table>
<thead>
<tr>
<th>Baseline recruited n</th>
<th>Timepoint 1 (denominator n = 39)</th>
<th>Timepoint 2 (denominator n = 39)</th>
<th>Timepoint final (denominator n = 39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collected samples n</td>
<td>42 (100)</td>
<td>26 (66.7)</td>
<td>26 (66.7)</td>
</tr>
</tbody>
</table>
Overall, there were no significant differences (p > 0.05) noted in sensitivity or agreement between QFT and QFT-Plus in the active TB cohort. There were three discordant results and two indeterminate results (occurred in the same samples in both QFT and QFT-Plus) (Table 8.5). In the discordant results, two samples were QFT positive and QFT-Plus negative and one was QFT-Plus positive and QFT negative.

Table 8.5: Discordant results in active TB cohort between QFT and QFT-Plus

<table>
<thead>
<tr>
<th></th>
<th>QFT negative</th>
<th>QFT positive</th>
<th>QFT indeterminate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>QFT-Plus negative</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>QFT-Plus positive</td>
<td>1</td>
<td>110</td>
<td>0</td>
<td>111</td>
</tr>
<tr>
<td>QFT-Plus indeterminate</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>9</td>
<td>112</td>
<td>2</td>
<td>123</td>
</tr>
</tbody>
</table>

Discordant results are in bold.

Table 8.6 highlights the details of the discordant samples in active TB. There were no significant clinical differences of note amongst these individuals compared to the rest of the cohort. Participant A111 was a 68-year-old male with smear negative pulmonary TB and diabetes, participant A114 was a 68-year-old with pleural TB and diabetes, whilst participant A120 was a 66-year-old male with smear positive pulmonary TB who was a heavy smoker and had a significant alcohol use history. It is interesting to note that the rest of each individual’s samples were concordant between QFT and QFT-Plus, but not necessarily both QFT-Plus antigen tubes. Similarly, the absolute IFNγ measurements were borderline in the QFT-Plus antigen tubes in those who were QFT positive/ QFT-Plus negative.
Table 8.6: Details of IFNγ measurements in participants where discordant QFT/QFT-Plus results occurred in the active TB cohort

<table>
<thead>
<tr>
<th>Study ID</th>
<th>TP (months)</th>
<th>Nil control</th>
<th>QFT-nil</th>
<th>TB1-nil</th>
<th>TB2-nil</th>
<th>Mitogen-nil</th>
</tr>
</thead>
<tbody>
<tr>
<td>A111</td>
<td>0</td>
<td>0.22</td>
<td>2.19</td>
<td>2.2</td>
<td>2.27</td>
<td>8.26</td>
</tr>
<tr>
<td>A111</td>
<td>1</td>
<td>0.14</td>
<td>0.52</td>
<td>0.57</td>
<td>0.72</td>
<td>4.35</td>
</tr>
<tr>
<td>A111</td>
<td>2</td>
<td>0.21</td>
<td>0.44</td>
<td>0.54</td>
<td>0.79</td>
<td>3.51</td>
</tr>
<tr>
<td>A111</td>
<td>6</td>
<td>0.08</td>
<td>0.35</td>
<td>0.18</td>
<td>0.21</td>
<td>16.12</td>
</tr>
<tr>
<td>A114</td>
<td>0</td>
<td>0.17</td>
<td>0.01</td>
<td>0.02</td>
<td>0.43</td>
<td>18.65</td>
</tr>
<tr>
<td>A114</td>
<td>1</td>
<td>0.17</td>
<td>–0.03</td>
<td>–0.08</td>
<td>0.03</td>
<td>20.08</td>
</tr>
<tr>
<td>A114</td>
<td>3</td>
<td>0.02</td>
<td>0.04</td>
<td>0.02</td>
<td>0.24</td>
<td>10.5</td>
</tr>
<tr>
<td>A114</td>
<td>6</td>
<td>0.04</td>
<td>–0.02</td>
<td>0.05</td>
<td>0.16</td>
<td>17.23</td>
</tr>
<tr>
<td>A120</td>
<td>0</td>
<td>0.23</td>
<td>0.72</td>
<td>0.34</td>
<td>0.15</td>
<td>13.68</td>
</tr>
<tr>
<td>A120</td>
<td>1</td>
<td>0.01</td>
<td>0.13</td>
<td>0.19</td>
<td>0.13</td>
<td>8.26</td>
</tr>
<tr>
<td>A120</td>
<td>2</td>
<td>0.03</td>
<td>0.69</td>
<td>0.45</td>
<td>0.39</td>
<td>14.41</td>
</tr>
<tr>
<td>A120</td>
<td>9</td>
<td>0.03</td>
<td>0.37</td>
<td>0.56</td>
<td>0.35</td>
<td>28.84</td>
</tr>
</tbody>
</table>

All results for each individual shown. Discordant results are in bold.

There was no difference between the sensitivity results of QFT and QFT-Plus overall when compared to the currently available gold standard of a positive culture result. When various diagnostic criteria were included, there were no significant differences between the two QuantiFERON-TB tests, although overall sensitivity was reduced (see Table 8.7).
Table 8.7: Comparison of QFT and QFT-Plus with varied microbiological and pathological diagnostic confirmation

<table>
<thead>
<tr>
<th>Diagnostic basis (prevalence %)</th>
<th>Test</th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture positive (78)</td>
<td>QFT</td>
<td>96.9 (83–99.9)</td>
<td>11 (0.3–48)</td>
<td>79.5 (63–90)</td>
<td>50 (1–98)</td>
</tr>
<tr>
<td></td>
<td>QFT-Plus</td>
<td>96.9 (83–99.9)</td>
<td>11 (0.3–48)</td>
<td>79.5 (63–90)</td>
<td>50 (1–98)</td>
</tr>
<tr>
<td>Culture or histology positive (90)</td>
<td>QFT</td>
<td>94.6 (81.8–99)</td>
<td>0 (0–60.2)</td>
<td>89.7 (75–97)</td>
<td>0 (0–84)</td>
</tr>
<tr>
<td></td>
<td>QFT-Plus</td>
<td>94.6 (81.8–99)</td>
<td>0 (0–60.2)</td>
<td>89.7 (75–97)</td>
<td>0 (0–84)</td>
</tr>
<tr>
<td>Culture or MTB PCR or histology positive (93)</td>
<td>QFT</td>
<td>94.7 (82–99.4)</td>
<td>0 (0–70)</td>
<td>92.3 (79–98)</td>
<td>0 (0–84)</td>
</tr>
<tr>
<td></td>
<td>QFT-Plus</td>
<td>94.7 (82–99.4)</td>
<td>0 (0–70)</td>
<td>92.3 (79–98)</td>
<td>0 (0–84)</td>
</tr>
</tbody>
</table>

Calculations determined on prevalence of cumulative diagnostic basis.

8.2.2.1 Agreement and comparison of QFT test performance through antibiotic treatment in active TB

Overall, the agreement between the two tests is high: 97.56% with Cohen’s κ of 0.86, varying between 0.83 and 1.00 when the tests were compared at each timepoint (see Table 8.8).

There was a strong correlation among QFT, TB1 and TB2 (ranging from 0.91 to 0.99) (Figure 8.3B, D and F). The smallest difference was observed between TB1 and TB2 with an average difference of 2.3 with limits of agreement (LOA) between −25.1 and 29.7. The difference between the QFT antigen tube and TB1 was 9.8 (LOA −53.7 to 73.4), while the mean difference between QFT and TB2 was 7.1 (LOA −51.5 to 65.7) (Figure 8.3). The differences between all tests increase as the value of the test increases. The latter findings are similar to those reported in recent QFT-Plus research, but the reports are inconsistent.267,261,262,270,271
Table 8.8: Overall agreement in active TB and LTBI cohorts through treatment*

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>Number</th>
<th>Agreement %</th>
<th>Expected agreement %</th>
<th>Cohen’s κ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>63</td>
<td>96.8</td>
<td>79.7</td>
<td>0.84</td>
</tr>
<tr>
<td>TP 1</td>
<td>38</td>
<td>97.4</td>
<td>74.6</td>
<td>0.89</td>
</tr>
<tr>
<td>TP 2</td>
<td>31</td>
<td>100</td>
<td>87.9</td>
<td>1.00</td>
</tr>
<tr>
<td>TP final</td>
<td>42</td>
<td>95.0</td>
<td>70.4</td>
<td>0.83</td>
</tr>
<tr>
<td>Overall</td>
<td>125</td>
<td>97.6</td>
<td>82.8</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* Includes all sampling of active and LTBI cohorts through treatment.
TP1: timepoint 1 or one month into treatment; TP: timepoint 2 or two months into treatment; TP final: final timepoint / end of treatment.
Figure 8.3: Bland-Altman (left) and regression (right) plots

A & B: QFT and TB1 of absolute IFNγ levels. Average difference of 9.8 (Limits of agreement −53.7 to 73.4).
C & D: QFT and TB2 of absolute IFNγ levels. Average difference of 7.1 (Limits of agreement −51.5 to 65.7). The limits of agreement suggest a difference in mean between QFT and TB2.
E & F: Comparing TB1 and TB2 absolute IFNγ levels. Average difference of 2.3 (Limits of agreement −25.1 to 29.7).
Figures 8.4A and 8.4B show the trends in IFNγ through antibiotic treatment over time. The figures highlight the large variation in IFNγ responses between individuals and also through antibiotic treatment.

**Figure 8.4:** QFT and QFT-Plus IFNγ release according to antigen tubes through treatment in active TB (2013–2016)

A: Absolute IFNγ values (IU/mL). Each data point represents one participant at each timepoint. Lines represent median values. B: Median IFNγ measurements with IQR.

To enable comparison over time (i.e. through treatment) between the two tests’ antigen tubes, GEE were used and the outcomes log transformed. Based on a population-averaged model, there was a significant difference found between baseline and all timepoints in QFT (21%, 26% and 54% reduction, respectively, p < 0.0001 for all three evaluations), whilst there was a significant difference between baseline and end of treatment for TB1 and TB2 (23% reduction p < 0.0001, and 31% reduction p = 0.005 respectively; see Table 8.9). No significant trend was found when the difference or ratios of TB1 and TB2 were evaluated (data not shown). (It is important to recognise that the following evaluations were done using Wilcoxon signed-rank paired tests, which doesn’t allow for missing values and the continuum that is accommodated using GEE as already discussed above.)

Whilst overall there is a decline in the QFT, TB1 and TB2 through antibiotic therapy, there are only significant differences when univariate tests of the median IFNγ is evaluated in QFT and TB2, but not in TB1, TB2-TB1 difference or TB2:TB1 ratios (see Table 8.10). QFT TP2 versus baseline and TP-final versus baseline were significant (p = 0.044 and
p = 0.003) respectively, whilst in TB2 there was a trend to a difference when TP-final was compared to baseline (p = 0.098). Interestingly, when IFNγ measurements were taken as a maximum of 10 (as per manufacturer’s recommendations), the results were altered: QFT TP2 versus baseline p = 0.069, TP-final versus baseline p = 0.001, whilst TB2 TP-final versus baseline p = 0.028. Such differences have been reported previously in QFT, but due to the variability in individual results, QFT has been shown in the past to be inadequate in treatment response monitoring.236,230,237 Reports are conflicting of recent QFT-Plus evaluations (Table 2.3).269

Table 8.9: Comparison of changes through anti-tuberculous drug therapy between QFT and QFT-Plus antigen tubes in active TB using GEE

<table>
<thead>
<tr>
<th>TB antigen tube</th>
<th>Timepoint</th>
<th>Exponentiation coefficient (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>QFT</td>
<td>TP1 vs TP0</td>
<td>0.79 (0.70, 0.88)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>TP2 vs TP0</td>
<td>0.74 (0.66, 0.83)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>TP final vs TP0</td>
<td>0.46 (0.41, 0.51)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>QFT-Plus TB1</td>
<td>TP1 vs TP0</td>
<td>0.99 (0.87, 1.13)</td>
<td>0.926</td>
</tr>
<tr>
<td></td>
<td>TP2 vs TP1</td>
<td>1.04 (0.91, 1.20)</td>
<td>0.492</td>
</tr>
<tr>
<td></td>
<td>TP final vs TP0</td>
<td>0.77 (0.68, 0.87)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>QFT-Plus TB2</td>
<td>TP1 vs TP0</td>
<td>0.97 (0.74, 1.26)</td>
<td>0.816</td>
</tr>
<tr>
<td></td>
<td>TP2 vs TP0</td>
<td>1.09 (0.83–1.43)</td>
<td>0.527</td>
</tr>
<tr>
<td></td>
<td>TP final vs TP0</td>
<td>0.69 (0.54, 0.90)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

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Table 8.10: IFNγ levels through treatment in active TB comparing QFT, QFT-Plus antigen tubes and QFT-Plus antigen tube ratios and differences

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>QFT</th>
<th>TB1 (IQR)</th>
<th>TB2 (IQR)</th>
<th>TB2-TB1 (IQR)</th>
<th>TB1/TB2 ratio (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATB TP0</td>
<td>7.73 (2.19, 25.17)</td>
<td>2.65 (1.20, 13.54)</td>
<td>6.49 (1.40, 14.58)</td>
<td>0.10 (-0.05, 1.35)</td>
<td>1.06 (0.95, 1.30)</td>
</tr>
<tr>
<td>ATB TP1</td>
<td>9.24 (2.65, 40.94)</td>
<td>4.38 (1.20, 24.62)</td>
<td>5.61 (1.83, 25.92)</td>
<td>0.23 (-0.19, 2.15)</td>
<td>1.06 (0.91, 1.36)</td>
</tr>
<tr>
<td>ATB TP2</td>
<td>6.19 (1.92, 22.51)</td>
<td>3.56 (0.80, 17.84)</td>
<td>4.53 (1.17, 19.98)</td>
<td>0.27 (-0.11, 2.78)</td>
<td>1.13 (0.96–1.40)</td>
</tr>
<tr>
<td>ATB TF</td>
<td>3.21 (0.49–25.03)</td>
<td>5.22 (0.40–12.52)</td>
<td>1.65 (0.57–21.96)</td>
<td>0.17 (-0.40–0.51)</td>
<td>1.20 (0.97–1.90)</td>
</tr>
</tbody>
</table>

IU/mL, median with IQR shown. ATB: active TB.

8.2.2.2 QuantiFERON responses in active TB disease severity

One of the possible advantages of the newer QuantiFERON test is suggested to be the differentiation between more severe active TB disease.\(^{247,248,249,252,255}\) In the cohort of patients with active TB, only few fall into a classic ‘severe’ disease with 3+ smear positivity, multiple cavities on CXR, multiple lobe involvement, or multiple sites of disease. In fact, seven of the 42 (16%) patients enrolled did not have any symptoms at the time of diagnosis, which confirms that the current study cohort has proportionately less severe disease than those in high endemic countries.

I assessed the following criteria individually and together, to determine if there were any trends in QFT or QFT-Plus responses. ‘Severe disease’ was classified as more than one of:

- presence of a pulmonary cavity
- involvement of more than one lobe
- smear positivity (sputum or bronchoalveolar lavage sampling)
- greater than one site of disease
- a serum albumin level of 25 g/L or less.

All individual univariate analyses (Mann–Whitney U test) are shown in Table 8.11 and 8.12, and the combined evaluation in Table 8.12. Results contrast those of recent studies (see Table 2.3). Only one patient had an albumin \(\leq 25\) g/L at time of TB diagnosis, whilst six had more than one site of disease. Individually, the involvement of more than one lobe, smear positivity and low albumin levels were not associated with significantly different
IFNγ responses in either of the QuantiFERON tests, nor the differences between the QFT-Plus antigen tubes. Interestingly, when more than site of disease was involved, both QFT-Plus TB1 and TB2 had lower IFNγ responses which were significant (p = 0.048 and 0.042, respectively). Importantly, it was found that the presence of a cavity and severe disease (by above definition) were associated with lower surrogate CD8+ T cell responses (i.e. TB2-TB1; p = 0.018 and 0.034, respectively). This is in contrast with the findings of Barcellini et al. (2016), who described severe disease (smear positivity) as being associated with a greater difference compared to those who were smear negative.265

### Table 8.11: Clinical factors associated with disease severity

<table>
<thead>
<tr>
<th>Cavity present (n)</th>
<th>&gt; 1 lobe (n)</th>
<th>&gt; 1 site of disease (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (32)</td>
<td>Yes (10)</td>
</tr>
<tr>
<td>QFT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.45 (2.62, 25.7)</td>
<td>5.26 (1.12, 20.2)</td>
</tr>
<tr>
<td>TB1</td>
<td>5.3 (1.19, 13.9)</td>
<td>1.52 (1.2, 10.6)</td>
</tr>
<tr>
<td>TB2</td>
<td>8.01 (1.4, 14.8)</td>
<td>1.54 (1.2, 9.85)</td>
</tr>
<tr>
<td>TB2-TB1</td>
<td><strong>0.41 (0.02, 2.83)</strong></td>
<td><strong>-0.035 (-0.7, 0.1)</strong></td>
</tr>
</tbody>
</table>

Discordant results are in bold.

Median IFNγ responses (IU/mL) with IQR shown. Univariate analyses using Mann–Whitney U test.
Table 8.12: QuantiFERON antigen tube responses with severe disease; univariate analyses using Mann–Whitney U test

<table>
<thead>
<tr>
<th>Smear positive (n)</th>
<th>Albumin low (n)*</th>
<th>Severe disease (n)#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (31)</td>
<td>Yes (10)</td>
</tr>
<tr>
<td></td>
<td>No (41)</td>
<td>Yes (1)</td>
</tr>
<tr>
<td></td>
<td>No (31)</td>
<td>Yes (11)</td>
</tr>
<tr>
<td>QFT</td>
<td>8.7 (2.19, 26.2)</td>
<td>5.26 (1.95, 19.2)</td>
</tr>
<tr>
<td></td>
<td>7.67 (2.19, 23.1)</td>
<td>178 (178, 178)</td>
</tr>
<tr>
<td></td>
<td>10.2 (3.05, 32)</td>
<td>4.14 (1.12, 20.2)</td>
</tr>
<tr>
<td>TB1</td>
<td>6.91 (1.08, 14.2)</td>
<td>1.52 (1.2, 4.19)</td>
</tr>
<tr>
<td></td>
<td>2.53 (1.2, 11.9)</td>
<td>120 (120, 120)</td>
</tr>
<tr>
<td></td>
<td>6.41 (1.29, 13.5)</td>
<td>1.49 (0.47, 16.2)</td>
</tr>
<tr>
<td>TB2</td>
<td>9.9 (1.27, 14.8)</td>
<td>1.61 (1.47, 6.49)</td>
</tr>
<tr>
<td></td>
<td>5 (1.33, 14.4)</td>
<td>116 (116, 116)</td>
</tr>
<tr>
<td></td>
<td>8.93 (1.4, 14.8)</td>
<td>1.55 (1.2, 14.2)</td>
</tr>
<tr>
<td>TB2-TB1</td>
<td>0.15 (-0.05, 1.3)</td>
<td>0.08 (-0.07, 1.35)</td>
</tr>
<tr>
<td></td>
<td>0.15 (-0.035, 1.48)</td>
<td>-3.6 (-3.6, -3.6)</td>
</tr>
<tr>
<td></td>
<td>0.41 (0.02, 2.83)</td>
<td>0 (-1.81, 0.42)</td>
</tr>
</tbody>
</table>

Discordant results are in bold.

* Low albumin defined as albumin ≤ 25 g/L.

# Severe disease defined as more than one of presence of a cavity on CXR / > one lobe involvement / > one site of clinical disease / albumin ≤25 g/L.

There is a trend to lower TB2 responses in older patients in the active TB cohort in the present study. With advancing age, TB2 responses were lower (correlation coefficient −0.279, p = 0.077 using Pearson’s r). A similar response was reported by Yi et al. (2016). This trend was not found in TB1 or QFT responses in with age, nor in the LTBI group. This may be due to impaired IFNγ responses in older patients, not necessarily related to responses to Mtb infection per se.

8.2.3 Latent TB results

Overall, there was a high agreement with LTBI cohort QFT and QFT-Plus qualitative and quantitative results. There were two discordant results amongst 49 samples collected through the course of enrolment in this cohort; one was indeterminate with both QFT-Plus and QFT. The two discordant results were negative with QFT and positive with QFT-Plus (Table 8.13). The agreement was 95.83% (expected 66.15%) with Cohen’s κ of 0.88. Details of all the results of the two participants with discordant test are detailed in Table 8.14. Participant L121 was a 28-year-old man who was referred as part of migrant screening, with presumed remote infection, whilst participant L122 was a 21-year-old female referred as part of a contact tracing program in Victoria and had a TST conversion.
Table 8.13: Discordant results in LTBI cohort between QFT and QFT-Plus

<table>
<thead>
<tr>
<th></th>
<th>QFT negative</th>
<th>QFT positive</th>
<th>QFT indeterminate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>QFT-Plus negative</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>QFT-Plus positive</td>
<td>2</td>
<td>37</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>QFT-Plus indeterminate</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11</td>
<td>37</td>
<td>1</td>
<td>49</td>
</tr>
</tbody>
</table>

Discordant results are in bold.

* Results that were invalid/inconclusive based on the manufacturer's recommendations.27,28

There were no significant differences between QFT and QFT-Plus when absolute IFNγ release was evaluated (see Table 8.15 and Figure 8.5). On evaluation with GEE, there were no significant differences noted through treatment in the LTBI cohort, with respect to QFT, TB1, TB2, or QFT-Plus antigen tube differences or ratios (data not shown).
Table 8.15: IFNγ levels through treatment in LTBI TB comparing QFT, QFT-Plus antigen tubes and QFT-Plus antigen tube ratios and differences

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>QFT IU/ml median (IQR)</th>
<th>TB1 IU/ml median (IQR)</th>
<th>TB2 IU/ml median (IQR)</th>
<th>TB2-TB1 IU/ml median (IQR)</th>
<th>TB1/TB2 ratio median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTBI TP0</td>
<td>3.05 (0.39, 12.80)</td>
<td>2.08 (0.30, 12.37)</td>
<td>2.63 (0.64, 14.89)</td>
<td>0.09 (-0.03, 0.59)</td>
<td>1.04 (1.00, 1.35)</td>
</tr>
<tr>
<td>LTBI TP1</td>
<td>3.38 (0.64, 33.88)</td>
<td>2.25 (0.97, 18.48)</td>
<td>2.21 (1.04, 16.14)</td>
<td>-0.26 (-0.90, 0.17)</td>
<td>1.06 (0.91, 1.36)</td>
</tr>
<tr>
<td>LTBI TP2</td>
<td>0.52 (0.23, 4.03)</td>
<td>0.82 (0.15, 3.34)</td>
<td>1.92 (0.04, 13.99)</td>
<td>0.22 (-0.04, 3.41)</td>
<td>1.06 (0.73, 1.24)</td>
</tr>
<tr>
<td>LTBI TPF</td>
<td>1.31 (0.36, 3.00)</td>
<td>1.49 (0.87, 3.57)</td>
<td>1.50 (1.03, 3.23)</td>
<td>0.02 (0.01, 0.36)</td>
<td>1.09 (1.01, 1.13)</td>
</tr>
</tbody>
</table>

IU/mL. Median with IQR shown.

There was no trend or significant difference in absolute IFNγ measurements between QFT and QFT-Plus TB antigen tubes (TB1 and TB2) when those who had recent contact with a person(s) with pulmonary TB were compared to those who did not. These results are different to that of recent studies suggesting a stronger association with TB2 IFNγ responses in those who have had recent TB exposure²⁵⁸,²⁶². Similarly, I did not find any significance in the above when the mean difference in TB2-TB1 was calculated, using this
value as a surrogate for CD8+ T cell responses, which have been reported as being associated with recent TB exposure (Table 8.16), but inconsistently.\textsuperscript{249,255,261,258}

Interestingly, there was a significant difference ($p < 0.05$) between those who had been in Australia for less than five years, compared with those who had been here longer when evaluating QFT-Plus antigen tube IFN\textgamma{} response difference (i.e. TB2-TB1): for those who had been in Australia for less time TB2-TB1 was 0.585 IU/mL (0.24, 1.3) (median with IQR) ($n = 6$), those living here for greater than five years 0 IU/mL (−0.18, 0.21) ($n = 8$) $p = 0.015$. The numbers being evaluated are small, and it is an important consideration, however it may represent surrogate CD8+ T cell responses associated with mycobacterial load; it is well described in the literature that those who are recent settlers from a high TB endemic country are more likely to develop reactivation disease and whilst clinically all the participants in this cohort were deemed to have LTBI, there is no way to determine their true risk of reactivation (or mycobacterial load) in the spectrum of disease that Mtb infection represents.\textsuperscript{4,303}

There were no significant differences found between those who had recent exposure to a person with active pulmonary TB and those who did not (Table 8.16). Similarly, there were no differences found between participants with LTBI who had an abnormal CXR (calcified nodules or inactive changes) and those with normal CXRs (data not shown).

Table 8.16: Comparison of IFN\textgamma{} levels in the QFT and QFT-Plus antigen tubes in participants in the LTBI cohort with and without a history of recent pulmonary tuberculosis exposure history, of baseline timepoint blood sampling

<table>
<thead>
<tr>
<th></th>
<th>Recent PTB exposure</th>
<th>No recent PTB exposure</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB1</td>
<td>1.34 (0.165, 46.9)</td>
<td>6.44 (1.09, 11.5)</td>
<td>0.787</td>
</tr>
<tr>
<td>TB2</td>
<td>1.96 (0.42, 55.1)</td>
<td>6.18 (1.24, 12.7)</td>
<td>1.000</td>
</tr>
<tr>
<td>QFT</td>
<td>1.97 (0.285, 49.3)</td>
<td>5.95 (0.925, 11.8)</td>
<td>0.939</td>
</tr>
<tr>
<td>TB2-TB1</td>
<td>0.12 (−0.06, 1.2)</td>
<td>0.14 (−0.09, 0.455)</td>
<td>0.710</td>
</tr>
<tr>
<td>TB2/TB1</td>
<td>1.1 (1, 1.4)</td>
<td>1.05 (0.993, 1.18)</td>
<td>0.689</td>
</tr>
</tbody>
</table>

Median IFN\textgamma{} responses. IU/mL, IQR shown.
8.2.4 Comparison of latent to active TB cohorts

There were no significant differences between the active and LTBI cohorts when comparing QFT and QFT-Plus results. This applied to qualitative and quantitative comparisons, including when the differences and ratios of the QFT-Plus antigen tubes were analysed (see Figure 8.6B). Figure 8.6A shows that while there seems to graphically be a trend to a lower IFNγ level in those with LTBI compared to those with active TB, when formal univariate analyses are applied, there is no significant difference between the cohorts. This general trend is seen throughout treatment in both cohorts (Figure 8.7A-C).

Similarly, there was no significant difference in TB2 and TB1 when comparing active and LTBI cohorts. The lack of any difference between the cohorts both contrasts and agrees with findings from recent studies. The overall findings comparing active and LTBI cohorts were unchanged when the maximum IFNγ responses equalling 10 were applied as per manufacturer's recommendations (data not shown).
<table>
<thead>
<tr>
<th></th>
<th>Latent</th>
<th>Active</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>21</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>TB1</td>
<td>2.1 (0.3, 12)</td>
<td>2.6 (1.2, 14)</td>
<td>0.414</td>
</tr>
<tr>
<td>TB2</td>
<td>2.6 (0.64, 15)</td>
<td>6.5 (1.4, 15)</td>
<td>0.504</td>
</tr>
<tr>
<td>QFT</td>
<td>3 (0.39, 13)</td>
<td>7.7 (2.2, 25)</td>
<td>0.131</td>
</tr>
</tbody>
</table>

Figure 8.6A: Univariate analyses of TB antigen tubes in QFT-Plus (TB1 and TB2) and QFT (Wilcoxon signed-rank test) and graphical representation of the range of TB1, TB2 and QFT antigen tube IFNγ levels IU/mL. All results from baseline sampling.
<table>
<thead>
<tr>
<th></th>
<th>Latent</th>
<th>Active</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>21</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>TB2-TB1</td>
<td>0.095 (-0.03, 0.585)</td>
<td>0.1 (-0.05, 1.35)</td>
<td>0.690</td>
</tr>
<tr>
<td>TB2/TB1</td>
<td>1.04 (1, 1.35)</td>
<td>1.06 (0.955, 1.3)</td>
<td>0.868</td>
</tr>
</tbody>
</table>

Figure 8.6B: Differences and ratios in QFT-Plus antigen tubes between active TB and LTBI cohorts

Median IU/mL. IQR shown. All results from baseline sampling.
Figure 8.7: Comparison of active TB to LTBI cohort through antibiotic treatment of QFT-Plus and QFT antigen tubes responses

A: IFNγ responses to TB1. B: IFNγ responses to TB2. C: IFNγ responses to QFT.

Lines represent median values, with IQR.
8.3 Discussion

The observations from this study confirm the high sensitivity of the newer QFT-Plus tests in both active TB and LTBI. There was a high level of agreement between the older and newer generation tests, as well as between the two TB tubes in QFT-Plus. The findings of this study also show that changes in IFNγ responses occur through antibiotic therapy in these participants, confirming the hypotheses.

8.3.1 Comparison of concordance and sensitivity profiles between QFT and QFT-Plus

The results of this observational study reinforce recent publications that QFT-Plus is comparable to QFT with an agreement of 97.10% overall (Cohen’s κ 0.869), whilst for the active TB cohort it was 97.56% (Cohen’s κ 0.86) and LTBI cohort 95.83% (Cohen’s κ 0.88).258,259,260,261,262 This is an important finding as the study cohorts were heterogeneous in nature, whilst most of the previously reported studies are not.

The sensitivity in the active TB cohort was the same for both QFT and QFT-Plus, with the highest occurring with positive culture only (sensitivity 96.9, 95% CI 83–99.9), whilst the lowest occurred when positive culture or histology were included (94.6, 95% CI 81.8–99) (see Table 8.7). Not surprisingly, the positive predictive value (PPV) was best when all diagnostic methods were included 92.35 (95% CI 79–98). The specificity is inaccurate, as there was no control/uninfected group. These results are comparable to recent reports where sensitivity was not significantly higher in QFT-Plus compared to QFT (in studies which evaluated them head to head).267,270 Overall, similar sensitivity of QFT-Plus was reported in one study265, whilst it was lower 83% (95% CI 75–90) in another where the majority of participants were HIV positive.264 A study in Japan found lower sensitivity of QFT-Plus based on current manufacturer’s guidelines of 0.35 IU/mL in either TB antigen tubes.271
8.3.2 QFT versus QFT-Plus: general observations

The current study results of lower absolute IFNγ measurements in QFT-Plus compared to QFT reflect those reported by Hoffman et al. (2016), Pieterman et al. (2018), Takasaki et al. (2018), and Yi et al. (2016)\(^{262,267,270,271}\), whilst contrasting the findings of Petriccioli et al. (2017).\(^{261}\) This may be due to the exclusion of TB7.7 from the new antigen profile compared to the QFT. Based on the literature, I would have expected to find a difference in those with active TB in the responses in TB2 but did not.

There were several discordant results based on the current cut-off of 0.35 IU/mL in either TB1 or TB2. Of the three patients with active disease who had discordant results, two had diabetes, whilst the other had a history of alcohol excess. The potential implications of diabetes on immune responses and IGRA results has been established, and also noted in another QFT-Plus study.\(^{245,270}\)

8.3.3 QFT-Plus in disease severity

Several recent studies have found strong associations of higher levels of IFNγ responses in TB2, or as a marker surrogate of CD8+ T cell responses as a sign of more severe TB disease (i.e. using TB2-TB1).\(^{265,271}\) The findings were alluded to in a report from Italy that TB2-TB1 levels were associated with worse disease was confirmed by flow-cytometry eliciting CD4+ and CD8+ responses by the same team.\(^{247,248}\) These findings were not replicated in all studies evaluating QFT-Plus in active TB (Table 2.3). The current study findings in fact conflict with these results: a significant difference in TB1, TB2, as well as TB2-TB1, of more severe disease being associated with a lower IFNγ level (Table 8.11 and 8.12). ‘Severe disease’ was defined as one or more of presence of a cavity, smear positivity or more than one lobe or site of disease. These would not necessarily be classified as ‘severe’ disease in other circumstances, especially high endemicity countries, but in this cohort, where several patients had little if any symptoms, it was important to attempt to differentiate possible subgroups. Studies have shown that Mtb-specific CD8+ T cell responses vary with mycobacterial load, and also correlate with treatment responses, but once again with variable consistency.\(^{252,253,254,255}\) There within lies one possible reason for the findings: patients with severe disease are believed to have impaired IFNγ responses, and this is likely to be multifactorial.\(^{254,271}\)
8.3.4 Treatment monitoring potential of QuantiFERON tests

The present study is the only one that has evaluated QFT-Plus through LTBI treatment and the second in active TB treatment to the authors’ knowledge. There was a significant difference through treatment in QFT and QFT-Plus: there was a reduction in IFNγ levels of 21%, 26% and 54% in QFT between baseline and one month, baseline and two months, and baseline and end of treatment respectively in the active TB cohort (p < 0.001 in all evaluations). There was a reduction of 23% and 31% in TB1 and TB2 between baseline and end of treatment IFNγ, respectively (p < 0.001 for both) (see Table 8.9). In univariate analyses of median IFNγ responses through the treatment course, however, the difference was less pronounced in TB2, not evident in TB1 and most significant for QFT (Table 8.10). These results were not consistent when IFNγ levels were ‘capped’ at 10 IU/mL, as per manufacturer’s recommendations, and underpin an issue with IGRA tests of variability between patients. However, the GEE discussed previously, provide the strongest indication of change through antibiotic therapy in both QFT and QFT-Plus. Kamada and Amashima (2017), found a reduction in both TB1 and TB2 responses at the three-month treatment mark, but TB2-TB1 at the end of treatment was significant. The findings in the current study do not reflect theirs directly but do suggest a reduction through treatment in active TB. There is also a trend to reduction in IFNγ levels through LTBI treatment, although no significant differences were found. The complex interplay between Mtb specific CD8+ and CD4+ T cells is likely responsible for the variation in results.

Petruccioli et al. (2017) reported better concordance (Cohen’s κ 0.7) in a group of patients treated for TB previously, compared to those with active TB (Cohen’s κ 0.5) between the two QFT tests; the authors, however, didn’t evaluate the active TB cohort through the antibiotic treatment course. In contrast, comparison of agreement through treatment was very high in the current study’s active TB cohort (> 0.8 for all timepoints) (see Table 8.8).

Whilst these changes are promising, the potential for variability (established in QFT), but unknown in QFT-Plus, has to be considered and evaluated prior to using QFT-Plus as a treatment response monitoring tool.
8.3.5 Does QFT-Plus differentiate between disease states?

Several recent reports suggest the new TB antigen tube (TB2), measuring Mtb-specific CD8+ T cell responses may help differentiate between active and LTBI, be associated with recent TB exposure, as well as be a marker of more severe disease in those with active TB.\textsuperscript{258,268,261,262} However, these findings are not consistent, and some reports in fact contrast others (see Table 2.3).

The comparisons between baseline active TB and LTBI cohorts agree with the findings of Hofland et al. (2016), that QFT-Plus does not differentiate between these two disease states of Mtb infection.\textsuperscript{268} This observation is based upon univariate analyses of IFNγ levels in TB1, TB2, difference between the two antigen tubes, as well as their ratios (see Figure 8.6A-B). Similarly, whilst there is a trend to a lower level of IFNγ responses in these groups in QFT-Plus compared to QFT, it isn’t significant (see Figure 8.6A and 8.7A-C). Analysis of documented recent contact in the LTBI cohort didn’t show an association with higher IFNγ responses or a greater difference between TB antigen tubes in QFT-Plus, contrasting recent reports.\textsuperscript{258,268,262} The reasons for these observations in the current study are likely to be multifactorial, but possibly mainly due the relatively small size of the LTBI cohort, as well as the impact of the potential selection bias.

8.3.6 Limitations of the study

This was an observational study of adults in a single hospital network in Melbourne. The implications of the latter include small sample size, no control or TB uninfected group and heterogeneity of the cohorts. Similarly, there may be a degree of selection bias in those who were offered to enter the study, because of the plan for longitudinal sampling through treatment. The latter consideration was especially important for the LTBI cohort, but unfortunately retention was still poor in this group.\textsuperscript{228} Most (90%, 38/42) of the study participants with active disease were born in TB-endemic countries. The majority of participants (62% in the active TB cohort) had no comorbidities; none had HIV or significant immunosuppression, whilst 7% in the active TB cohort had diabetes mellitus. These are important considerations as the newer QFT-Plus is advocated to have improved sensitivity and specificity in these subgroups of patients.\textsuperscript{226}
All the ELISA processing was undertaken at a non-diagnostic research laboratory, allowing the evaluation of absolute IFNγ levels accurately across both tests and reducing inter-personnel variability. However, between 2013 and 2015, Qiagen provided tubes F1-F6, of which two tubes were consequently renamed TB1 and TB2 (F3 = TB1, F6 = TB2); hence, for much of the study samples were not processed in standard kits.

### 8.3.7 Implication of the study on future research

The newer QFT-Plus IGRA test is now established in diagnostic laboratories. The benefits over its predecessor, however, are not obvious. The present study both confirms and contrasts findings of other researchers who have evaluated QFT-Plus (either with or without direct comparison to QFT). QFT-Plus is at least as sensitive as QFT in qualitative evaluations, using incremental diagnostic strategies. The concordance between the two tests is also very high.

The extended application of QFT-Plus compared to QFT in special groups such as paediatric patients, those who are immunocompromised or HIV positive was not evaluated in this study and is an important area for further clarification.

The overall reduced levels of IFNγ responses in patients with Mtb infection with QFT-Plus is a very important consideration: further studies are needed to assess both the variability of the newer test and evaluate whether a lower cut-off is warranted, as suggested by several authors recently. The present findings support this notion.

Whilst the potential for QFT-Plus as a treatment monitoring tool is supported by this data, the range and variability of responses need further evaluation before it can be clinically applied. Further studies detailing the *ex vivo* responses of CD4+ and CD8+ cells, alongside the QFT-Plus responses may advance understanding of this newer test and provide more avenues for determining whether it can be used in conjunction with other tests as a biomarker in Mtb infection.
\section*{8.4 Conclusions}

The sensitivity of QFT-Plus compared to QFT in this study was similar. Quantitative pooled data showed no significant difference in the IFNγ levels in LTBI or those with active TB prior to antibiotic therapy commencement. The agreement between QFT and QFT-Plus in this study is high: when all samples of both LTBI and active TB cohorts were combined the agreement was 97.1\% (Cohen's kappa 0.869), which is higher than the expected agreement of 77.63\%.

This study supports a previous report that QuantiFERON test results are affected by antituberculous therapy.\textsuperscript{269} This is only the second reported study looking at QFT-Plus through antibiotic treatment and the first reporting of such in LTBI. GEE showed a significant change in QFT responses through treatment, with a 54\% reduction at end of therapy, whilst similar changes of a lower magnitude were found in TB1 and TB2 (23\% reduction). Overall, there was a greater difference between TB1 and QFT with respect to absolute IFNγ responses, whilst there was a significant but smaller difference between TB2 and QFT.

Given the variability of IFNγ responses, these findings need to be confirmed through larger, more extensive studies to establish their clinical utility in monitoring treatment responses, assessing disease severity and potentially determining whether LTBI acquisition was recent or remote.
Chapter 9: Final summary and conclusions

Tuberculosis is one the greatest global challenges in infectious diseases the world faces currently. This mycobacterial infection alone accounts for an estimated 10 million new active cases annually, with nearly two million deaths.\(^1\) Whilst there have been advances in diagnostic technologies, improved accessibility to treatment and several promising therapeutic options, there are still many impediments to reaching the "End TB Strategy 2035" goal.\(^2\) In addition to the timely diagnosis and commencement of anti-tuberculous therapy, the challenges facing health care practitioners and policy makers include, but not limited to, identifying those at risk of developing reactivation disease, differentiating active TB from LTBI, drug-resistant TB, HIV-TB co-infection, development of an efficacious vaccine and an effective marker of 'cure'.\(^5,3^0^4\) There is thus a desperate need for appropriate biomarkers.\(^2^4,2^7^3,3^1,3^5,2^2\) The complex nature and interplay of human immune responses and the bacterium have meant improved, but still incompletely understood mechanisms of immunopathogenesis. The innate immune responses with no true formation of 'memory', in an infection which is recognised as a spectrum from latent to active disease in humans, are the gatekeepers of the initial reaction to Mtb infection.\(^3,4\) TLRs form an integral component of these initial responses and hence the reason for this observational pilot study to evaluate the differences between active TB and LTBI in patients undergoing treatment for the respective infections. Investigated also was the newer QuantiFERON-TB Gold Plus IGRA through antibiotic treatment.

In this dissertation, the investigative chapters 4 to 8 addressed the original hypotheses that were outlined in Chapter 2. The following summary presents the key findings and synopses derived from the investigative chapters.
Toll-like receptor markers through active and latent TB infection treatment

The observations made in the present study confirm the hypotheses that there were distinct variations in TLR expression and function on PBMCs in those with active TB and LTBI, which also altered through antibiotic therapy.

In active TB, the changes were seen on both surface and intracellular TLR expression, with the key trends of TLR2 and TLR7 being overall increased at the end of treatment compared to TLR4, which declined through treatment. Significant changes of TLR7 expression were seen in CD14+ monocytes and NKT cells, whilst TLR2, 4 and 7 variations through treatment were found on NK cells. Evaluations also showed significant reduction in TLR2 and 4 expression was associated with culture negative active TB on CD14+ monocytes and NK subsets.

Cytokines and chemokines measured in supernatants following TLR ligand stimulation of PBMCs changed through antibiotic treatment, although the variation(s) did not always necessarily mirror the respective TLR expression changes. The key patterns seen suggest a pro-inflammatory response following TLR ligand stimulation, which declines through treatment, whilst remaining higher than pre-treatment levels. The main exception to this was seen with TNF, where levels increased through treatment without stimulation and post TLR2 and 9 ligand stimulation. A similar increase was seen in IL-6 production post TLR2 ligand stimulation. Significant differences were observed through various timepoints through treatment with respect to IL-6, TNF, MCP and IL-8 measurements.

Unstimulated plasma cytokines and chemokines (TNF, IL-6, IL-8, IL-10, IP-10 and MCP1) levels varied significantly following anti-tuberculous therapy, with most being lower at end of treatment. All cytokines except IFNγ and TNF reduced significantly between end of treatment and start of antibiotics. Importantly, absolute measurements of these cytokines/chemokines in plasma were much lower than following TLR ligand stimulation, alluding to a potential inhibitor presence resulting in this paradoxical response. Whilst further laboratory investigations suggested such an effect on healthy PBMCs when cultured with TLR ligands and plasma from TB-infected patients, the overall results were inconclusive.
LTBI TLR expression and function assessment suggested predominantly TLR4 mediated immune changes in this cohort. TLR4 expression was significantly lower following treatment on NK and NKT cells, with similarly reduced expression of TLR7 in NKT cells. These changes were reflected with significant differences observed following TLR4 and TLR7/8 ligand stimulation with MCP1 and IL-8 measurements. Such observations may support previous reports that TLR4 represents a protective role in Mtb immune responses.\textsuperscript{164,149}

**Comparison of TLR markers through treatment between active and latent TB infection**

TLR2, 4 and 7 expression were significantly different between active TB through treatment and LTBI (pre-treatment) on CD14+ monocytes, with TLR2 and 7 expression generally being higher and TLR4 expression being lower in active TB. TLR7 expression was also higher in lymphocyte subgroups through active TB compared to baseline LTBI. Following LTBI treatment, further TLR expression changes were observed between the two cohorts, with changes particularly significant on lymphocyte subsets between active TB and the end of LTBI treatment. To the author’s knowledge, this is the first description of significant changes found of altered TLR markers comparing end of LTBI treatment to active TB.

TLR function evaluated through cytokine and chemokine measurements following TLR ligand stimulation showed differences between the study cohorts through antibiotic treatment. Variations in cytokine measurements were also evident between end of treatment between the two cohorts, as well as between pre-treatment timepoints. Overall TNF, IL-6 and MCP1 levels tended to be lower in the active TB compared to the LTBI cohort, whilst IL-8 levels were the opposite.

Significant changes in plasma cytokines levels were found following comparison of the LTBI and active TB cohorts. TNF, IL-8 and IFN\(\gamma\) were significantly different between end of active TB treatment and baseline the LTBI cohort. IP-10 levels were found to be significantly higher in those with active TB prior to treatment compared to baseline LTBI. TLR responses to Mtb infections varied between active TB and LTBI. The results of TLR4 changes following LTBI treatment compared to active TB shed new light in this area. This
work also contributes to knowledge of TLR7/8 and 9 roles on a functional level in Mtb infections, description of which in the current literature is limited.

Overall, the comparison of TLR markers in Mtb infections allude to a pro-inflammatory state in which cytokines levels often alter following TLR ligand stimulation, whilst generally paradoxical levels of the same cytokines occur in plasma. It also appears that despite antibiotic therapy, the pro-inflammatory state does not return to ‘normal’ after treatment. Importantly, as distinct and measurable differences exist between those with active TB and LTBI, such variations may be utilised in future to create a ‘TLR signatory profile’ enabling differentiation.

**Role of serum vitamin D and TLR expression in active TB**

This study confirms that patients with active TB often have deplete serum vitamin D levels. This study describes weak associations with baseline vitamin D levels with negative TLR4 expression and weak positive TLR7 expression through antibiotic treatment on different cell types evaluated. To the author’s knowledge, there have been no previous translational reports of TLR7 associations with vitamin D in Mtb infections.  

**QFT-Plus IGRA: evaluating an updated IGRA test**

The newer QFT-Plus is a highly sensitive test and the observations from this study show high concordance with the former QFT. The findings of this study also show that changes in IFNγ responses occur through antibiotic therapy in these participants, confirming the hypothesis, but QFT-Plus fails to differentiate between these two disease states of Mtb infection.

**Pertinent investigation findings and potential future investigation directions**

The overall observations from the current study assert that there are potential TLR signatory changes which could contribute to differentiating between the two key forms of Mtb infection in humans. Importantly, whilst several of the hypotheses in this dissertation are confirmed, others are disputed through this work. The current results
confirm that increased TLR expression does not always equate to increased functional consequences, similar to previous reports.\textsuperscript{170}

The findings of varied cytokine responses between the two cohorts following TLR9 ligand stimulation highlight that this TLR is also important in human Mtb immune responses. Whilst animal studies have alluded to this is the past, results have been inconsistent.\textsuperscript{101,142-144} The current results are in conformity with recent human studies of \textit{ex vivo} TLR9 expression and functional assays, as well as genetic association studies.\textsuperscript{145,7,10,119,153,166,12} Similarly, the findings of TLR7/8 in these cohorts are of great interest, and further evaluation is important.

The combination of TLR expression, function and plasma cytokine variations following treatment could help create distinct profiles to distinguish between the two cohorts with further augmentation of the current work. Further evaluation may also potentially contribute to the development of these changes as part of a platform for a biomarker differentiating these two classic forms of Mtb infection.

Another key future investigative avenue is to delineate cellular mechanisms involved in these changes, such as the role of regulatory T cells (Tregs) and Th17 effects from TLR signalling.\textsuperscript{6,203} Enrolment and investigation of more participants, particularly with LTBI, evaluation of other TLRs, such as TLR3 and 5, as well as mRNA expression studies would help evaluate the role of TLRs in human Mtb infections further.

Evaluation of TLR responses at the site of disease (such as broncho-alveolar fluid or lymphoid tissue), with direct comparison to peripheral blood mononuclear cells would provide further information of innate immune effects involved in reactivation disease.

The observational nature and the small numbers included in the heterogeneous cohorts are this study’s main limitations. This study was undertaken in a single tertiary health service in Australia, with a low annual incidence of active TB. Large observational studies are currently underway through international collaborations (the RePORT International Study), in which investigation of TLR markers are included.\textsuperscript{36} Such large studies are bound to shed light on many aspects of Mtb immunopathogenesis. With ongoing worldwide research in animals and translational work such as the current study, it is
hoped that delineating the spectrum of Mtb infection will be made clearer, and its role as the greatest killer of young adults from an infectious disease will become history.
References


SABINE DE SILVA


Appendices

Appendix 1

Buffer / reagent list

Appendix 2

Raw data from TLR stimulation studies which had minimal cytokine levels (IL-10, IFNγ, IL-17A, IL-17F, IL-12p70

Appendix 3

ELISA and CBA cytokine measurements 2017 done simultaneously for correlation studies (IL-6 and TNF)

Appendix 4

Summary of cytokine measurements found to be significant in supernatants through active TB treatment in MYC cohort following TLR ligand stimulation of PBMCs using ELISA and CBA.

Appendix 5

Other published work

Appendix 6

Poster presentations

Appendix 7

Scholarships
Appendix 1: Buffer / reagent list

RPMI-media

RPMI (Sigma Life Sciences, US), supplemented with 1% penicillin, 1% L-glutamine and 5% foetal calf serum

Freezer mix

20% dimethyl sulfoxide (DMSO) and foetal bovine serum

Coating buffer (ELISA TNF and IL-6)

0.1 M sodium carbonate, pH 9.5 7.13 g NaHCO₃, 1.59 g Na₂CO₃; q.s. to 1.0 L; pH to 9.5 with 10 N NaOH
Appendix 2: Raw data from TLR stimulation studies which had minimal cytokine levels

IL-10, IFNγ, IL-17A, IL-17F, IL-12p70 in pg/mL

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### Appendix 4: Summary of cytokine measurements found to be significant in supernatants through active TB treatment in MYC cohort following TLR ligand stimulation of PBMCs using ELISA and CBA

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Wilcoxon signed-rank test between each timepoint. p < 0.05 shown. Results from cytokine bead arrays and ELISA are shown.
Appendix 5: Other published work

**Publications**


Appendix 6: Poster presentations

*Lorne Infection and Immunity 2014*

Marked changes in toll-like receptor expression and function in a cohort of patients with active tuberculosis in Melbourne, Australia

S. De Silva, G. Jenkin and K. Visvanathan

*Australasian Society for Antimicrobials: AGM 2016*

Evaluating the next generation of QuantiFERON assay in tuberculosis: results of a pilot study at Monash Health, Melbourne

S. De Silva, G. Jenkin, S. Vogrin, V. Sundararajan, J. Yu and K. Visvanathan

Potential use of toll-like receptor expression and function as markers of differentiating active from latent TB infection: results from a translational study in Melbourne, Australia

S. De Silva, G. Jenkin and K. Visvanathan
Appendix 7: Scholarships

*NHMRC: Dental and Medical Postgraduate Scholarship 2011–2015*