

Characterization of *Mycobacterium tuberculosis*-specific Th22 cells in HIV-TB co-infection

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Abstract

Tuberculosis (TB) remains the infectious disease causing the greatest global mortality, with an estimated 10 million incident cases of TB and 1.45 million deaths in 2018. Although there is a cure for TB, the success of the treatment is hampered by multidrug resistant TB and HIV infection. There is an urgent need for an effective TB vaccine to prevent ongoing transmission. The development of a new and efficacious TB vaccine will likely be dependent on our understanding of protective immunity to TB. Although it is well established that Th1 cells are crucial in the response against *Mycobacterium tuberculosis* (Mtb), Th1 cytokines may not be sufficient to control Mtb infection. A major focus of this thesis is the contribution of an understudied Th subset in Mtb immunity, namely Th22 cells, producing the cytokine IL-22. IL-22 functions to preserve mucosal barriers and induce antimicrobial peptides, contributing to protective immunity to a range of extracellular and intracellular bacteria. A recent study in IL-22-deficient mice described a protective role for IL-22 during the development of TB. In humans, soluble IL-22 has been detected at sites of extra-pulmonary tuberculosis (TB), and a polymorphism in the IL-22 promoter has been linked to TB susceptibility. However, much remains to be understood about Th22 cells and their role in protective immunity to Mtb.

In this study, we investigated the contribution of Th22 cells to TB immune responses by providing a detailed characterisation of *Mycobacterium tuberculosis*-specific Th22 cells in latent TB infection (LTBI), TB disease and HIV co-infection, using flow

cytometric techniques. In Chapter 2, we optimised detection of IL-22 and determined the factors that contribute to Mtb-specific IL-22 production by CD4⁺ T cells, as well as characterising some aspects of Th22 cell biology. In Chapter 3, we examined the impact of TB disease and HIV infection on Th22 cells, compared to Th1 and Th17 cells. Finally, in Chapter 4, we explored Mtb-specific cytokine production by CD8⁺ T cells and CD4⁺ T cells following Mtb peptide stimulation, and the effect of TB disease and HIV infection.

We detected significant IL-22 production from CD4⁺ T cells in healthy individuals following whole blood stimulation with Mtb whole cell lysate (MtbL). However, IL-22 responses were poorly detectable when peripheral blood mononuclear cells (PBMC) were stimulated with MtbL. Therefore, we sought to investigate conditions that influence IL-22 detection in whole blood and PBMC, and characterise Th22 cells further. We found that PBMC are able to produce IL-22 in response to Mtb but appear to lack the physiological environment for optimal induction of IL-22. We also discovered that TCR blocking inhibited Mtb-specific IL-22 production, suggesting that responses are stimulated through recognition of Mtb antigen by the TCR, rather than through bystander activation. IL-22 is produced by CD4⁺ T cells that appear to be conventional, rather than MAIT, $\gamma\delta$, NKT or iNKT cells. Indeed, analysis of the TCR clonality using $v\beta$ repertoire typing revealed similar repertoire usage between IL-22, IFN- γ -producing CD4⁺ T cells, and total CD4⁺ T cells. Overall, these data shed more light on the biology of IL-22-producing CD4⁺ T cells.

Next, we examined the effects of HIV infection and TB disease on the magnitude, memory profile and activation phenotype of Mtb-specific Th22 cells, compared them to Th1 and Th17 cells. Blood samples were collected from 72 individuals classified into four groups based on their HIV-1 and TB status, namely HIV-/LTBI, HIV+/LTBI HIV-/active TB and HIV+/active TB. Blood was stimulated with MtbL and analysed for cytokine production using multiparameter flow cytometry. We observed similar frequencies of IL-22 to IFN- γ -producing CD4⁺ T cells in LTBI. Mtb-specific Th22 cells were reduced to a greater extent than Th1 cells by a combination of HIV infection and TB disease. Th22 cells demonstrated differences in their memory and activation phenotype compared to Th1 and Th17 cells. In the context of active TB, Th1 cells were characterised by a high expression of the activation marker HLA-DR. In contrast, Th22 cells did not demonstrate activation using this marker during TB disease. Similarly, Th1 cells were more differentiated in TB disease irrespective of HIV status, while there was no difference in the memory phenotype of Th22 cells during different disease states.

Finally, we characterised Mtb peptide-specific CD4⁺ and CD8⁺ T cell responses in LTBI, active TB and HIV infection. CD4⁺ T cells did not produce detectable IL-22 when blood was stimulated with Mtb peptides, and there was also no IL-22 response from CD8⁺ T cells. Th1 cytokines IFN- γ and TNF- α were detectable from CD4⁺ and CD8⁺ T cells in response to Mtb peptides. Consistent with previous studies, there was a higher proportion of individuals with detectable CD8⁺ responses during active TB and HIV co-infection compared to HIV-infected LTBI individuals, but no difference in the magnitude of response was observed. Interestingly, HIV infection and TB disease

induced similar levels of activation in Mtb-specific CD8⁺ compared to CD4⁺ T cells. Moreover, active TB and HIV co-infection impaired memory differentiation of Mtb-specific CD8⁺ T cells towards a less differentiated profile, compared to LTBI. These results confirm that both CD4⁺ and CD8⁺ T cells contribute to TB immune responses.

In summary, we confirm that Th22 cells constitutes a substantially portion of CD4⁺ T cell response to Mtb. IL-22 appears to be produced by conventional CD4⁺ T cells but may require specific antigen presentation requirements to optimally induce its production. Interestingly, HIV infection during TB disease led to a near absence of Th22 cells in blood. Our results warrant further study of the role of Th22 cells in TB immunity, which may lead to insights that could assist the development of an effective vaccine against TB.

List of abbreviations

AFB	Acid fast bacilli
Ad85A	Adenovirus expressing antigen 85A
Ag	Antigen
Ag85A	Antigen 85A
AhR	Aryl hydrocarbon receptor
AIDS	Acquired Immunodeficiency Syndrome
ALS	Alternate lysing solution
AMs	Alveolar macrophages
APC	Allophycocyanin
APC	Antigen presenting cells
APC-H7	Allophycocyanin-H7
ART	Antiretroviral therapy
ARV	Antiretroviral drugs
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCG	Bacille Calmette Guérin
BEI	Biodefense and emerging infections research resources repository
BFA	Brefeldin-A
BV	Brilliant violet
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
CLA	Cutaneous lymphocyte antigen
CMC	Chronic mucocutaneous candidiasis
CMV	Cytomegalovirus
CO₂	Carbon dioxide

COPD	Chronic obstructive pulmonary disorder
CTL	Cytolytic CD8+ T cells
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3 grabbing non-integrin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
ECD	Electron coupled dye
ED	Early differentiated
DOTS	Directly observed treatment, short course
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	6kDa Early Secretory Antigen
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
Foxp3	Forkhead box p3
FSC	Forward scatter
GALT	Gut associated lymphoid tissue
GEM	Germline-encoded mycolyl lipid reactive cells
GIT	Gastrointestinal tract
GM-CSF	Granulocyte macrophage colony stimulating factor
gp120	Glycoprotein 120 from HIV
HAART	Highly active antiretroviral treatment
HIV	Human Immunodeficiency virus
HLA	Human leukocyte antigen
HREC	Human Research Ethics Committee
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule-1

ICS	Intracellular cytokine staining
IDM	Institute of Infectious Disease and Molecular Medicine
IDO	Indoleamine 2,3-dioxygenase 1
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IGRA	Interferon gamma release assays
IL	Interleukin
ILC	Innate lymphoid cells
iNKT	Invariant natural killer T cells
IP-10	IFN- γ inducible protein 10
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
IVE-TB	In vivo expressed M.tb antigens
LAM	Lipoarabinomannan
LD	Late differentiated
LLTI	Lectin-like transcript 1
LTBI	Latent tuberculosis infection
LTI	Lymphoid tissue inducer cells
Mtb	<i>Mycobacterium tuberculosis</i>
MAIT	Mucosal associated invariant T cells
MCP-1	Monocyte chemoattractant protein 1
MCP-3	Monocyte chemoattractant protein 3
MDM	Monocyte-derived macrophages
MDR-TB	Multi-drug resistant tuberculosis
MFI	Median Fluorescent Intensity
MHC	Major histocompatibility complex
min	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
mM	Millimolar

MMP	Matrix metalloproteinases
MOI	Multiplicity of infection
MR	Mannose receptor
MR1	Major histocompatibility complex, class I-related
mRNA	Messenger ribonucleic acid
MST	Median survival time
MVA85A	Modified vaccinia ankara expression Adenovirus expressing antigen 85A
ng	Nanogram
NHP	Non-human primate
NIH	National Institutes of Health
NK	Natural killer
nM	Nanomolar
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
NOS	Nitric Oxide Synthase
OI	Opportunistic infections
p24	HIV protein 24
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cy5
PE-Cy5.5	Phycoerythrin-Cy5.5
PE-Cy7	Phycoerythrin-Cy7
PET-CT	¹⁸ F-fluorodeoxyglucose positron emission tomography and computed tomography
PILAR	Proliferation-induced lymphocyte-associated receptor
PMA	Phorbol 12-myristate 13-acetate
PMT	Photomultiplier tube

PPD	Purified protein derivative
pVL	Plasma viral load
QFT	Quantiferon
RA	Rheumatoid arthritis
RBC	Red Blood Cell
RD-1	Region of difference 1
Reg	Regenerating proteins
RANTES	Regulated on activation, normal T expressed and secreted
RNA	Ribonucleic acid
RORC	RAR-related orphan receptor gamma
RORγt	RAR-related orphan receptor gamma t
ROS	Reactive Oxygen species
Rpf	Resuscitation promoting factors
RPMI	Roswell Park Memorial Institute cell culture medium
RR-TB	Rifampicin-resistant tuberculosis
RT	Room temperature
SNP	Single nucleotide polymorphisms
SIV	Simian immunodeficiency virus
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TCM	Central memory T cell
TCR	T cell receptor
TD	Terminally differentiated
TE	Effector T cell
TEM	Effector memory T cell
Tfh	T follicular helper cells
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor

TNF-α	Tumor necrosis factor alpha
TPA	12-O-Tetradecanoylphorbol-13-acetate
Treg	Regulatory T cell
TST	Tuberculin skin test
VCC	Virus containing compartment
ViViD	LIVE/DEAD Fixable Violet Dead Cell Stain
VL	Viral load
WBA	Whole blood assay
WCL	Whole cell lysate
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis
μl	Microliter
μM	Micromolar

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CHAPTER 1

Literature Review

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1.1 Introduction

Tuberculosis (TB) remains on the leading cause of death from an infectious disease globally (WHO, 2019). In 2018, approximately 10 million people developed TB, and 1.45 million died from the disease (WHO, 2019). HIV is the greatest risk factor for TB illness and death, with over 250 000 of the people who died from TB in 2018 being HIV-infected.

The World Health Organisation (WHO) has established the “End TB strategy” that aims to reduce TB deaths by 90% and TB incidence by 80% by the year 2030, compared to 2015 cases. The priorities for the “End TB Strategy” include development of a vaccine to lower the risk of infection, and a vaccine or a new drug treatment to cut the risk of TB disease in individuals with latent tuberculosis infection (LTBI; WHO, 2019). The only licensed TB vaccine, Bacille Calmette-Guérin (BCG), is universally administered at birth in TB endemic countries. However, BCG provides only partial protection against severe TB in children but does not provide consistent or durable protection against adult disease (Mangtani *et al.*, 2014). Therefore, there is a need for a safe and more effective TB vaccine to reduce the burden of TB morbidity and mortality and to reduce *Mycobacterium tuberculosis* (Mtb) transmission in both HIV-infected and HIV-uninfected populations (Knight *et al.* 2014). In order to develop a more effective TB vaccine, we need to understand the type of immunity that confers protection against TB.

In this thesis, I explore features of adaptive immunity to TB in latent and active TB in persons with and without HIV infection. My main focus is investigating the contribution of a less well-studied subset of T helper cells, Th22 cells, in TB immunity and how HIV infection impacts Th22 immune responses. In this introductory chapter, the current knowledge on TB immunity, defects caused by

HIV that may lead to increased risk of developing TB, advances in vaccine development and the potential role of Th22 cells in Mtb control are reviewed.

1.2 Epidemiology of TB

1.2.1 Burden of TB disease

Approximately 1.7 billion people are latently infected with TB and are at risk of developing active TB during their lifetime. A global estimate of people with TB disease has been stable over the last 10 years (range: 9.0 – 11.1 million). The burden of disease varies depending on geographical location, and eight countries account for two thirds of TB prevalence: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) (WHO, 2019). **Figure 1.1** shows global incidence of TB in the year 2018.

Rifampicin is the most effective first-line drug against TB. Of the 10 million people who developed TB in 2018, 500 000 were infected by rifampicin resistant Mtb (RR-TB), and of these, 78% had multidrug-resistant TB (MDR-TB). Among cases of MDR-TB in 2018, 6.2% were estimated to have extensively drug-resistant TB (XDR-TB). MDR-TB is defined as resistance to rifampicin and isoniazid, while XDR-TB is defined as MDR-TB plus resistance to at least one drug in the following two classes of medicines used in treatment of MDR-TB: fluoroquinolones and second-line injectable agents (WHO, 2019). It is estimated that 3.5% of new TB cases and 18% of previously treated cases were infected by MDR/RR-TB (WHO, 2019).

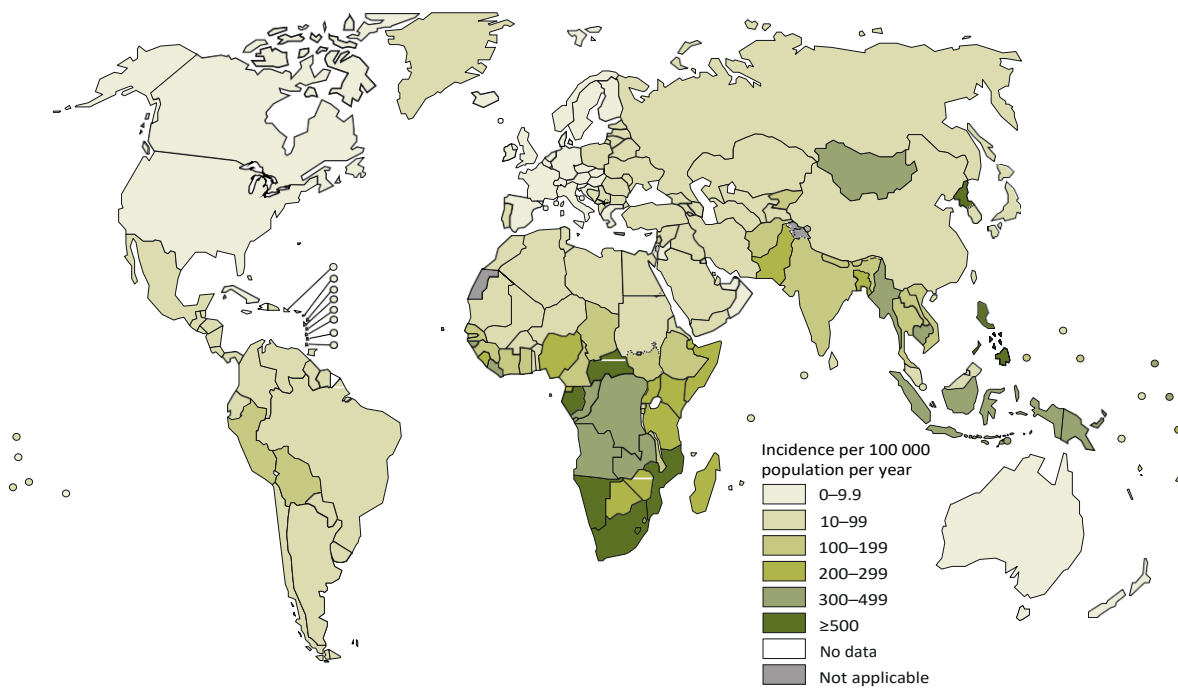


Figure 1.1: Estimated worldwide tuberculosis incidence rate in 2018. Taken from the Global Tuberculosis Report 2019, World Health Organisation.

The current recommended treatment for cases of drug-susceptible TB is a 6-month regimen of four first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide. This regimen is highly effective against TB (at least 82% treatment success rate) in cases of drug-susceptible TB. However, treatment for MDR-TB is longer, more expensive and comprises more toxic drugs. Furthermore, MDR-TB and XDR-TB cases have lower treatment success rate (56% and 34%, respectively) (WHO, 2019). Moreover, successful treatment does not confer protection against relapse or reinfection (Lambert *et al.*, 2003). Recently, the FDA approved a new treatment for drug-resistant forms of TB, pretomanid. This drug is approved to be used in combination with bedaquiline and linezolid, two other recently approved drugs, and this drug regimen has the potential to shorten treatment duration of extensively drug-resistant TB (Silva *et al.*, 2018; WHO, 2019).

The current standard for TB diagnosis is the use of culture-based methods, however, these tests have a long turn-around time (up to 12 weeks) (WHO, 2019). Molecular assays are increasingly used in combination with culture techniques to reduce time to diagnosis. The Xpert® MTB/RIF assay (Cepheid, USA) is currently the only rapid molecular test that is recommended by WHO, although microscopy and culture remain essential for treatment monitoring. The Xpert® MTB/RIF test can simultaneously detect TB and resistance to rifampicin. Other assays are available for testing resistance to rifampicin and isoniazid as well as resistance to fluoroquinolones and injectable anti-TB drugs (e.g. line probe assays (LPAs) (WHO, 2019). These tests require sputum sample; therefore, it is difficult to effectively diagnose individuals with lack of cavitation (Charmie *et al.*, 2010).

Latent TB infection can be diagnosed using IFN- γ release assays (IGRA), such as QuantiFERON®-TB Gold In-Tube. These assays measure the secretion of IFN- γ after stimulation with Mtb peptide antigens. WHO recommends IGRA tests for diagnosis of latent TB infection, although these tests cannot distinguish between LBTI and active TB disease (Menzies *et al.*, 2008).

1.2.2 HIV and TB co-infection

HIV infection is the greatest known risk factor for TB, being associated with increased risk of active TB development, disseminated TB and high TB mortality (Walker *et al.*, 2013). Globally, high HIV prevalence and high TB incidence rates are significantly correlated (WHO, 2019). This deadly synergy of HIV and TB is highest in Africa, where 25% of TB patients in 2018 had HIV-associated TB (WHO, 2019). The risk of TB is increased even within a year of HIV infection, at any CD4 count. Factors that may be responsible for the high mortality rate of TB in HIV-infected

individuals include: (i) compromised immune responses by HIV leading to rapid TB disease progression, and (ii) complication of TB diagnosis as a result of HIV infection, resulting in delayed TB treatment (Kwan and Ernst, 2011). Concerning the latter, HIV-associated TB is more likely have a false negative result from a TB sputum smear test (WHO, 2019), which could result from lack of cavitation in HIV-infected individuals.

HIV co-infection also causes complications for TB treatment. The success rate of TB treatment in HIV co-infection patients is lower compared to HIV-uninfected individuals (77% vs 82% success rate, respectively) (WHO, 2019). It is thought that antiretrovirals (ARVs), taken for control of HIV, interact with anti-TB drugs that could lead to patients having adverse effects such as peripheral neuropathy and rash (Dean *et al.*, 2002), and also result in poor ARV retention and faster elimination of drugs (Kwan and Ernst, 2011). Studies show that HIV infection increases the chances of recurrent TB, especially when CD4 counts are low (Perriens *et al.*, 1991; Pulido *et al.*, 1997; Mallory *et al.*, 2000).

1.3 TB pathogenesis

1.3.1 Early events in Mtb infection

Tuberculosis is an infectious bacterial disease caused by the pathogen *Mycobacterium tuberculosis* (Mtb) (Wolf *et al.*, 2007). Mtb is primarily transmitted by the respiratory route, and typically causes disease in the lungs (pulmonary TB), although it can cause disease in most organs (extrapulmonary TB). The primary target for Mtb infection is alveolar macrophages (AM) in the lungs; the infection results in the engulfment of the bacilli by resident phagocytic cells, alveolar macrophages, neutrophils or dendritic cells (DC) (**Figure 1.2**) (Giacomini *et al.*, 2001). The

bacteria then induce the recruitment of macrophages and may trigger granuloma formation (Ndlovu and Marakalala, 2016). Upon engulfment of the bacilli, the adaptive immune response is then activated by DC trafficking to draining lymph nodes and presenting antigens to T cells (Giacomini *et al.*, 2001). B cells, T cells and other cells are then recruited to restrain Mtb bacilli at the centre of the granuloma. However, as the disease progresses, necrotic breakdown of granuloma cells may result in cavitation of granulomas which could lead the lung to collapse and Mtb bacilli to be released into the airway (Philips and Ernst, 2012).

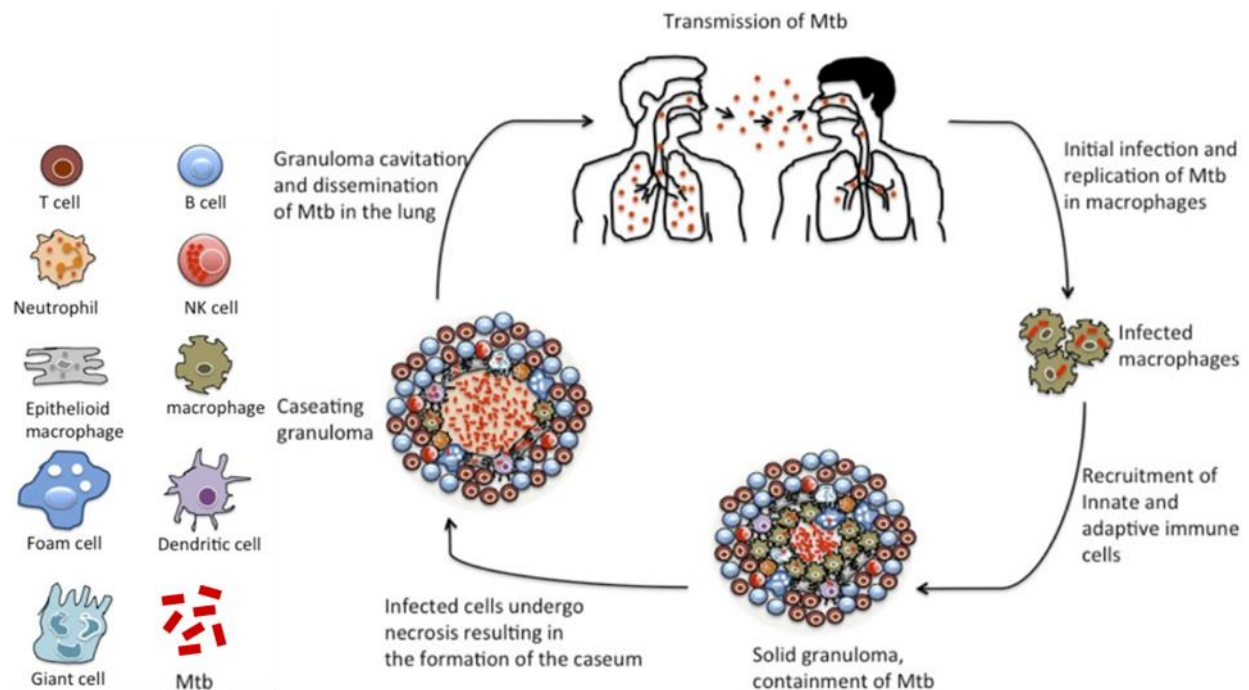


Figure 1.2: Early events leading to Mtb infection. Mtb bacilli are inhaled into the lungs, leading to a local inflammatory response resulting in the recruitment of monocytes and macrophages and other innate immune cells to the site of infection. Adapted from Ndlovu and Marakalala, 2016.

It is estimated that at least one third of the world's population is infected with Mtb, but infection does not usually lead to active disease. Infection is controlled in 90% of immunocompetent individuals establishing latent infection (LTBI), while progressive primary disease occurs at a rate of 2-10% in a lifetime (O'Garra *et al.*, 2013). Given the observed complexity between the host and bacterial interaction and outcome, more recent trends in understanding Mtb infection and TB disease have moved away from the binary distinction and view LTBI and active TB as stages on a spectrum, which include clearance, resistance, subclinical, mild and severe manifestations of TB (Barry *et al.*, 2009).

1.3.2 Granuloma formation

A granuloma is characterized by an organized aggregate of epithelioid macrophages, foamy macrophages, and multinucleated giant cells (MGCs), which form by fusion of the plasma membranes of multiple macrophages at the centre, surrounded by B and T lymphocytes and other cell types including neutrophils, dendritic cells, natural killer (NK) cells, and fibroblasts (**Figure 1.2**) (Ndlovu and Marakalala, 2016).

The granuloma was initially believed to contain Mtb bacilli and enable mycobacterial killing. However, some studies suggest that granuloma formation creates an environment that may result in mycobacterial persistence and dissemination (Ramakrishnan, 2012; Ehlers and Schaible, 2013). The heterogeneity of lung granulomas has been demonstrated during both active TB and latent tuberculosis infection (LTBI), where some granulomas were sterile, while others contained vastly different numbers of bacteria in cynomolgus macaques (Lin *et al.*, 2014; Gideon *et al.*, 2015). Sterile granulomas were characterized by a balance of proinflammatory and regulatory cytokines

(Gideon *et al.*, 2015), indicating the importance of cytokine responses in the fate of mycobacteria in granulomas.

1.4 Immune response to TB

1.4.1 Innate Immunity

Cells of the innate system are responsible for initiating the immune response to pathogens. Macrophages, dendritic cells (DC) and neutrophils are the primary phagocytes involved in the uptake of *Mycobacterium tuberculosis* in the lung (Walker *et al.*, 2013).

Macrophages

Following pulmonary Mtb infection, AM are the first cells to encounter the inhaled Mtb bacilli. Macrophages can then present Mtb antigens to CD4⁺ T cells via major histocompatibility complex (MHC) II enabling CD4⁺ T cells to activate AM through cytokines and inhibit bacterial growth (Chan *et al.*, 1992; Flynn *et al.*, 1995). Macrophages can also secrete proinflammatory cytokines (IFN- γ and TNF- α) and induce a granulomatous inflammatory response (Giacomini *et al.*, 2001). Moreover, macrophages can kill Mtb directly via apoptosis (Behar *et al.*, 2011) and autophagy (Gutierrez *et al.*, 2004). However, Mtb has developed multiple strategies to escape macrophage killing such as inhibiting phagosomal maturation (Sturgill-Koszycki *et al.*, 1994; Toossi *et al.*, 2012), inhibiting antigen presentation/macrophage activation (Baena and Porcelli, 2009; Grace and Ernst, 2016) and inhibiting autophagy (Shin *et al.*, 2010). It is important to note that different forms of macrophages exist in the lung and they play different roles in advancing or controlling infection (Tripathi *et al.*, 2014). In mice, AM have been shown to be more susceptible to Mtb growth compared to interstitial macrophages (IM) (Huang *et al.*, 2018; Cohen *et al.*, 2018). It is

important to note that different forms of macrophages exist in the lung and they play different roles in advancing or controlling infection (Tripathi *et al.*, 2014). In mice, AM have been shown to be more susceptible to Mtb growth compared to interstitial macrophages (IM) (Huang *et al.*, 2018; Cohen *et al.*, 2018).

Dendritic cells

The primary function of DC is the induction of the antimycobacterial T cell immune response (Banchereau and Steinman, 1998; Giacomini *et al.*, 2001). After phagocytosis of Mtb, DCs become activated and upregulate co-stimulatory and MHC molecules for enhanced antigen presentation and the capacity to stimulate T cells (Henderson *et al.*, 1997; Giacomini *et al.*, 2001). DCs then traffic to draining lymph nodes and present antigens to T cells to activate the adaptive immune response. However, Mtb can delay activation of the adaptive responses by preventing migration of DCs to lymph nodes (Marino and Kirschner, 2004; Wolf *et al.*, 2007; Reiley *et al.*, 2008).

Neutrophils

The importance of neutrophils in controlling Mtb infection has been reported. Studies suggest that neutrophils play a protective role against Mtb during the early phase of infection (Martineau *et al.*, 2007; Lowe *et al.*, 2012). Upon Mtb infection, neutrophils rapidly migrate to the lung, phagocytose bacilli, and contribute to the early clearance of infection through their antimicrobial peptides (Eruslanov *et al.*, 2005; Martineau *et al.*, 2007; Lowe *et al.*, 2012). Indeed, granzyme B expression by neutrophils was found in the lung granulomas of Mtb-infected cynomolgus macaques (Mattila

et al., 2015). However, neutrophils have also been associated with pathological effects in TB by disseminating viable bacteria (Eruslanov *et al.*, 2005; Lowe *et al.*, 2012).

Innate Lymphoid cells (ILCs)

The involvement of ILCs in the control of Mtb has been investigated. Recently, group 3 ILCs (ILC3s) were shown to mediate protective immunity during the acute stage of Mtb infection by orchestrating the recruitment of alveolar macrophages in mice (Ardain *et al.*, 2019). Furthermore, RNA sequencing of ILC2s and ILC3s from resected human lungs revealed that ILCs are activated and recruited to the lung during Mtb infection (Ardain *et al.*, 2019). In addition, ILCs share similar characteristics with CD4⁺ Th cells (Spits *et al.*, 2013) and are able to produce cytokines (IFN- γ , IL-17 and IL-22), which are involved in protective TB immunity (Cupedo *et al.*, 2009; Ardain *et al.*, 2019; Cella *et al.*, 2019).

1.4.2 Adaptive Immunity

CD4⁺ T cells

It is well established that CD4⁺ T cells are critical for immunity against Mtb (Jasenosky *et al.*, 2015). CD4-deficient mice had higher bacterial burden in the lungs, spleen and liver and increases Mtb dissemination (Caruso *et al.*, 1999). Moreover, using MHC class II chimeric mice, it was demonstrated that CD4⁺ T cells must directly detect Mtb infected lung myeloid cells for optimal control of intracellular Mtb (Srivastava and Ernst, 2013). In humans, the importance of CD4⁺ T cells is illustrated in the case of HIV infection, which causes progressive CD4⁺ T cell depletion that strongly correlates with increasing risk of TB (Selwyn *et al.*, 1989; Lawn *et al.*, 2002). However, TB susceptibility due to HIV infection is likely not limited to CD4⁺ T cells, with other

immune mechanism involving cells of the innate immune system (inhibition of phagocytosis and autophagy in macrophages) having been documented (Bell *et al.*, 2017).

CD4⁺ T cells differentiate into a number of T helper (Th) subsets that produce a range of cytokines (Kara *et al.*, 2014). Differentiation of naïve CD4⁺ T helper cells is induced by the presence of various cytokines and is dependent on the expression of transcription factors, effector cytokines, and chemokine receptors (**Figure 1.3**) (Kara *et al.*, 2014; Knochelmann *et al.*, 2018).

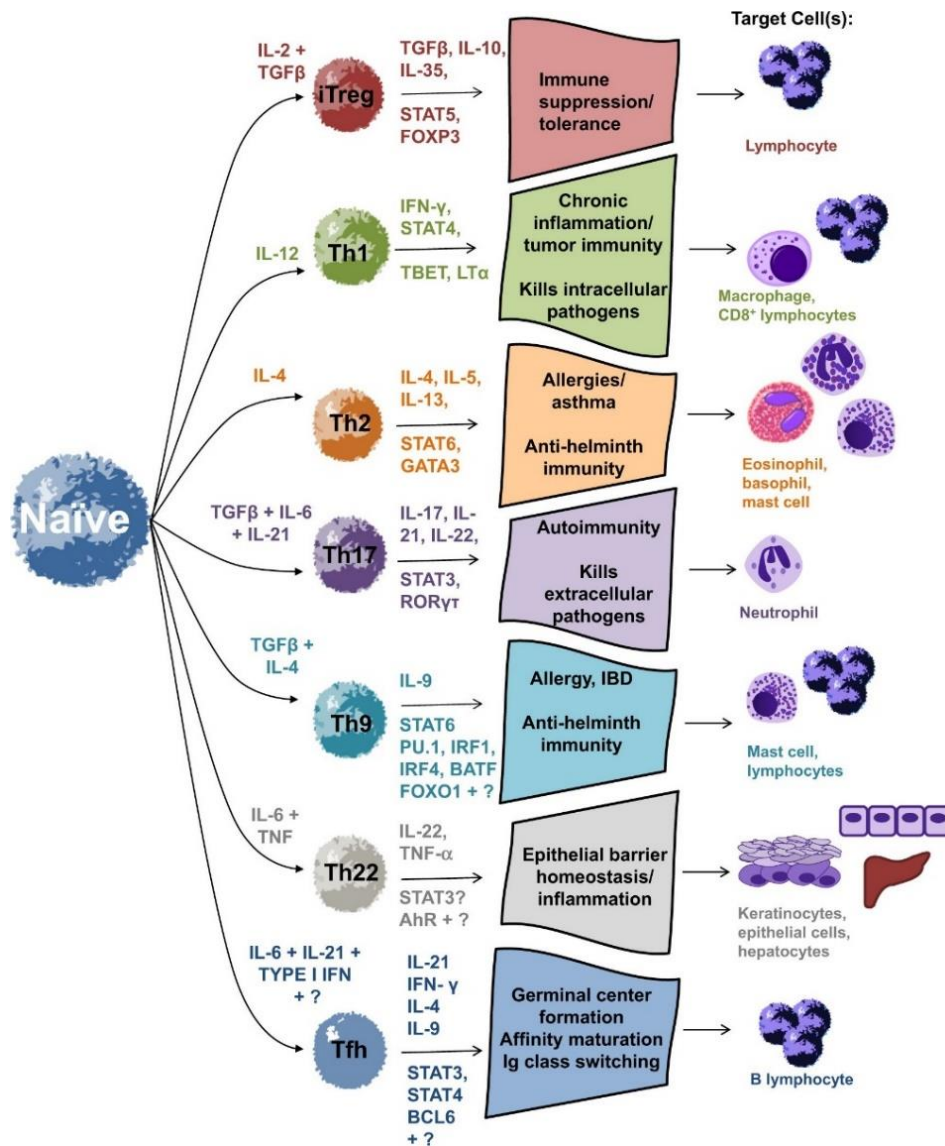


Figure 1.3: Diversity of CD4+ T cells and factors that modulate the differentiation of naïve CD4+T cells. Th1, Th2, Th9, Th17, Th22, Tfh and Treg lineages are defined based on the expression of transcription factors, signature cytokines, and chemokine receptors. Question marks denote properties that are unknown. Taken from Knochelmann *et al.*, 2018.

Th1 cells which produce cytokines IFN-γ and TNF-α have been intensively studied and recognised as vital in protective TB immunity (Jasenosky *et al*, 2015). Challenging mice deficient in IFN-γ or TNF-α with Mtb resulted in high bacterial loads, disseminated disease, necrosis and consequently rapid death after Mtb challenge (Flynn, 1993; Flynn *et al*, 1995). In humans, studies have shown

an increased prevalence of mycobacterial infections in individuals with genetic defects in the Th1 pathway compared to the general population (Ottenhoff *et al.*, 1998). Similarly, individuals with auto-antibodies against IFN- γ were shown to have increased susceptibility to mycobacterial diseases (Kampmann *et al.*, 2005). Furthermore, individuals receiving anti-TNF immunotherapy for rheumatoid arthritis or Crohn's disease had an increased rate of aTB reactivation (Keane *et al.*, 2001; Harris *et al.*, 2010). Although the importance of IFN- γ is clearly established, it is evident that IFN- γ may not be sufficient to protect against TB. This was initially illustrated by the modified Vaccinia Ankara virus expressing antigen 85A (MVA85A) vaccine that was designed to enhance the protective efficacy of BCG (Tameris *et al.*, 2013). This vaccine was shown to induce highly durable (>1000 days) Ag85A-specific CD4+ Th1 responses but was not effective in TB control in phase II clinical trials (efficacy of 17.3%) (Oduola *et al.*, 2012; Tameris *et al.*, 2014). This may indicate that Th1 CD4+ T cells alone are not sufficient for control of Mtb. In fact, evidence shows that overproduction of Mtb-specific Th1 responses resulting from PD-1 blockade may increase the risk or severity of tuberculosis in mice and humans (Sakai *et al.*, 2016; Chu *et al.*, 2017; Picchi *et al.*, 2018; Barber *et al.*, 2019). Therefore, studies have pointed to IFN- γ independent mechanisms of CD4+ T cells protection (Wozniak *et al.*, 2010a; Gallegos *et al.*, 2011; Lu *et al.*, 2019). Additional CD4+ T helper subsets such as Th17 and Th22 cells may thus play a role in protective TB immunity.

Adoptive transfer of mycobacterial-specific Th17 cells in IFN- γ deficient mice provided protection against Mtb challenge (Wozniak *et al.*, 2010a). Furthermore, defective granuloma formation and poor immune control of Mtb has been observed in mice lacking IL-17 (Yoshida *et al.*, 2010; Gopal *et al.*, 2014). Moreover, IL-17 was also shown to be important in vaccine induced protective

immunity in mice (Khader *et al.*, 2007) and NHP (Wareham *et al.*, 2014). In humans, IL-17 CD4⁺ T cells were found to be lower in TB patients compared to Mtb-exposed individuals (Perreau *et al.*, 2013; Heidarneshad *et al.*, 2016) and single nucleotide polymorphisms (SNPs) in IL-17 resulted in reduced risk of TB disease (Milano *et al.*, 2016; Yu *et al.*, 2017). IL-17 inhibits Mtb growth by regulating chemokine receptors (CXCL-1 and CXCL-5) to induce neutrophil-mediated inflammation and promote granuloma maturation (Khader *et al.*, 2007; Umemura *et al.*, 2007; Yoshida *et al.*, 2010). Moreover, IL-17 was also shown to be important in vaccine-induced protective immunity in mice (Khader *et al.*, 2007) and NHP (Wareham *et al.*, 2014). These data indicate that Th17 cells play a role in protective immunity against Mtb. The potential role played by Th22 cells is discussed in detail in Section 1.5 below.

CD8⁺ T cells

There is a growing body of evidence supporting a protective role for CD8⁺ T cells in TB. CD8⁺ major histocompatibility complex (MHC) class 1-restricted T cells specific for secreted immunodominant Mtb antigens have been identified (Lalvani *et al.*, 1998; Lewinsohn *et al.*, 2006). Studies have observed a higher proportion of circulating CD8⁺ T cells responders to ESAT-6/CFP-10 in individuals with active TB compared to LTBI individuals, but the magnitude of responses are similar (Caccamo *et al.*, 2009; Day *et al.*, 2011; Rozot *et al.*, 2013; Amelio *et al.*, 2019). Mtb-specific CD8⁺ T cells display different memory phenotypes between LTBI (T_{EMRA}: CD45RA⁺CCR7⁻) and active TB individuals (T_{EM}: CD45RA⁻CCR7⁻) (Caccamo *et al.*, 2009; Rozot *et al.*, 2013).

In non-human primates, BCG-vaccinated rhesus macaques lacking CD8⁺ T cells had significantly lower vaccine-induced immunity against TB (Chen *et al.*, 2009). In vitro studies have shown that CD8⁺ T cells are able to kill Mtb-infected macrophages or suppress Mtb growth in macrophages through perforin, granulysin and/or Fas-Fas interactions (Stenger *et al.*, 1997; Brookes *et al.*, 2003; Andersson *et al.*, 2007; Canaday *et al.*, 2014) by targeting heavily Mtb-infected cells (Lewinsohn *et al.*, 2003). Similarly, a subset of CD8⁺ T cells expressing granulysin is depleted during anti-TNF therapy, leading to increased risk of reactivating LTBI (Bruns *et al.*, 2009; Miller and Ernst, 2009).

1.4.3 Unconventional T cells

Unconventional T cells denote to cells that don't recognize antigens presented by cell-surface proteins of the MHC such as MHC class I or MHC class II. These cells are also referred to as donor-unrestricted T cells, and include $\gamma\delta$ T cells, mucosal associated invariant T cells (MAIT) and CD1-restricted cells (Beckman *et al.*, 1994; Tilloy *et al.*, 1999; Sieling *et al.*, 2000).

$\gamma\delta$ T cells

$\gamma\delta$ T cells express the $\gamma\delta$ TCR and recognize non-protein antigens such as synthetic alkyl phosphates and isoprenoids produced by bacteria (Tanaka *et al.*, 1995; Morita *et al.*, 2000). Presentation of antigens to $\gamma\delta$ T cells is mediated through extracellular pathway that does not require antigen uptake, antigen processing, or MHC class I or class II expression (Morita *et al.*, 1996). $\gamma\delta$ T cells secrete a range of effector cytokines including IFN- γ and cytotoxic molecules in response to mycobacteria (Barnes *et al.*, 1993; Poggi *et al.*, 2009; Steinbach *et al.*, 2016) and have been reported to expand during active tuberculosis (Ness-Schwickerath and Morita, 2011). A

subset of $\gamma\delta$ T cells (V γ 9V δ 2) that recognizes microbial phosphoantigens has also been described in Mtb infection (Shen *et al.*, 2002). These cells are thought to regulate Mtb infection through the release of IFN- γ and IL-10 (Pinheiro *et al.*, 2012; Chen *et al.*, 2013). Furthermore, administration of phosphoantigen (HMBPP) and IL-2 induced expansion and pulmonary accumulation of V γ 9V δ 2 T cells which resulted in reduced Mtb burden in non-human primates (Chen *et al.*, 2013).

Mucosal associated invariant T cells (MAIT)

Mucosal associated invariant T (MAIT) cells express a semi-invariant TCR (V α 7.2-Ja33/20/12 in humans, paired with a limited number of V β chains and can either be activated through recognition of MHC class I-related molecule, MR1 (Tilloy *et al.*, 1999; Reantragoon *et al.*, 2012) or by cytokines such as IL-12 and IL-18 (Ussher *et al.*, 2014; Suliman *et al.*, 2019). MAIT cells exert their function by producing inflammatory cytokines (IFN- γ , TNF- α , IL-17) which can act directly on infected cells to inhibit Mtb growth (Gold *et al.*, 2010; Le Bourhis *et al.*, 2010). MAIT cells can also mediate perforin-dependent killing of infected cells (Kurioka *et al.*, 2015). Evidence shows that these cells are lower in blood of active TB individuals (Le Bourhis *et al.*, 2010; Kwon *et al.*, 2015; Coulter *et al.*, 2017) and present in the lungs or pleural effusions of patients with active TB (Le Bourhis *et al.*, 2010; Jiang *et al.*, 2016) suggesting that they may be migrating to Mtb-infected tissues.

CD1-restricted cells

There are five types of CD1-restricted T cells divided into three groups: group 1 (CD1a, CD1b and CD1c), group 2 (CD1d) and group three (CD1e) (Calabi and Milstein, 1986; Young and Moody, 2006). All five CD1 types recognize lipid and lipid-associated antigens and present them to T cells.

However, these CD1-restricted T cells may serve different functions, as the CD1 receptors are expressed differentially on Langerhans cells and skin resident DCs (mostly CD1a), myeloid DCs (CD1b), monocyte-derived DCs, B cells, and Langerhans cells (CD1c) and CD1d on many cell types, including macrophages, and epithelial cells (Angénioux *et al.*, 2000; De La Salle *et al.*, 2005; Roura-Mir *et al.*, 2005; Young and Moody, 2006; Barral and Brenner, 2007; Dougan *et al.*, 2007). CD1e is the only member of the CD1 group that is not expressed on the surface of APCs (Angénioux *et al.*, 2000). The role of CD1-restricted cells in TB control is not clear. Mtb infection or immunization with Mtb lipids has been shown to elicit group 1 CD1-restricted T cell responses in mice (Felio *et al.*, 2009). Furthermore, group 1 CD1-restricted T cells are cytotoxic and produce IFN- γ and TNF- α in response to mycobacterial antigens (Ulrichs *et al.*, 2003; Gilleron *et al.*, 2004; Layre *et al.*, 2009). In addition, CD1-restricted cells producing IFN- γ have been found in higher frequencies in Mtb-infected individuals in comparison to healthy controls (Ulrichs *et al.*, 2003). Polymorphisms in CD1a and CD1d have been shown to lead to greater susceptibility to TB in human cohorts (Seshadri *et al.*, 2014; Taheri *et al.*, 2019).

B cells

Unlike T cells, the role of B cells in controlling Mtb was traditionally thought to be minimal, although antibody responses have been detected in both LTBI and TB disease (Wilkinson *et al.*, 1997, 1998; Achkar *et al.*, 2015). Indeed, B cell-deficient mice presented with higher bacterial burdens when challenged with Mtb (Vordermeier *et al.*, 1996). Moreover, passive transfer of monoclonal antibodies against Mtb components have been shown to be effective in reducing bacterial load in spleens and lungs, reduced weight loss and resulted in long-term survival of mice after Mtb infection (Teitelbaum *et al.*, 1998; Hamasur *et al.*, 2004; López *et al.*, 2009; Balu *et al.*,

2011). Recently, IgM and class-switched IgG and IgA antibody responses were detected in a cohort of individuals who appeared to be resistant to TB infection ('resistors'), namely household contacts who were exposed to Mtb yet remained persistently IGRA and TST negative (Lu *et al.*, 2019). These studies highlight B cells as underestimated players in Mtb control.

1.5 Characteristics of IL-22 and its potential role in TB immunity

1.5.1 Biology of IL-22

Interleukin (IL)-22 is a member of the IL-10 family of cytokines, and it is produced by a variety of cells, including T cells (Th17, Th22, Th1 and $\gamma\delta$ T cells) and a range of innate cells (innate lymphoid cells (ILCs) and NK cells) (Rohan Dhiman *et al.*, 2009; Witte *et al.*, 2010; Cella *et al.*, 2019). IL-22 signals through a type 2 receptor (IL-22R), which is composed of two heterodimeric subunits, IL-22R1 and IL-10R2 (Logsdon *et al.*, 2004). IL-22R is expressed on non-hematopoietic cells, particularly epithelial cells (Yoon *et al.*, 2010), with a few reports of upregulation on macrophages (Rohan Dhiman *et al.*, 2009; Treerat *et al.*, 2017). The transcription factor aryl hydrocarbon receptor (AHR) has been shown to be involved in the production of IL-22 in both mice and human (Trifari *et al.*, 2009; Basu *et al.*, 2012). Exogenous cytokines IL-6, IL-23, TNF and IL-1 β have also been shown to promote IL-22 production (Duhon *et al.*, 2009; Trifari *et al.*, 2009; Basu *et al.*, 2012; Plank *et al.*, 2017).

IL-22 signalling is mainly mediated by signal transducer and activator of transcription (STAT)-3, STAT-1 and STAT-5 phosphorylation (**Figure 1.4**) (Dudakov *et al.*, 2015). Activation of receptor-associated Jak1/Tyk2 kinases activation is induced by ligation of the IL-22-IL-22R1-IL-10R2 complex, Jak1/Tyk2 kinases then phosphorylates IL-22 receptors and STAT proteins.

Evidence suggests that IL-22 can also signal through p38 and the mitogen-activated protein (MAP) kinase pathways (Dudakov *et al.*,2015).

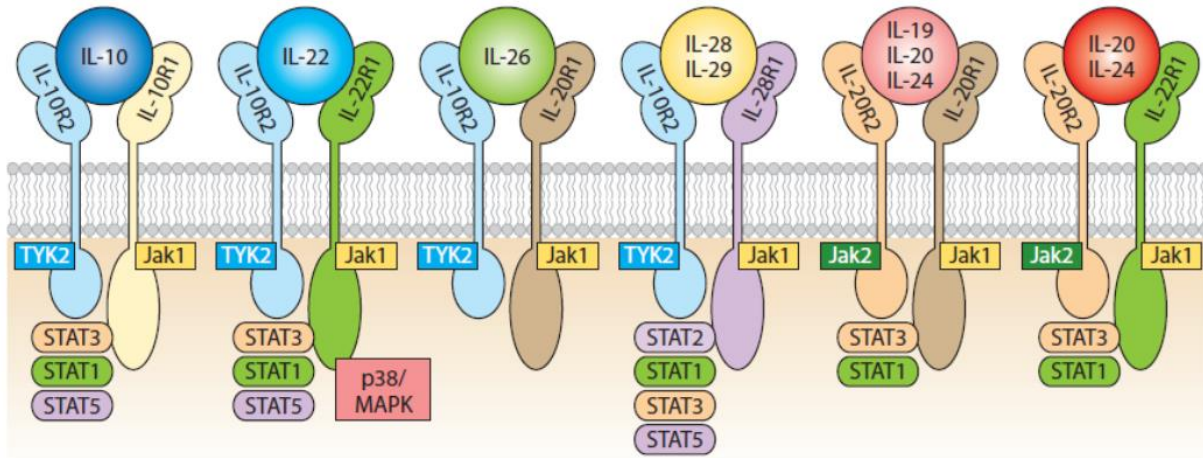


Figure 1.4: Receptors and Jak-STAT molecules of the IL-10 family of cytokines. IL-22 receptor is composed of two subunits: the IL-10R2 subunit and the IL-22R1 subunit. The IL-10R2 is shared with the receptors for IL-10, IL-26, IL-28, and IL-29; while the IL-22R1 subunit itself can also pair an IL-20R2 subunit forming the receptor for IL-20 and IL-24. Taken from Dudakov *et al.*, 2015.

1.5.2 IL-22 function

The primary function of IL-22 is to preserve mucosal barriers and protect the host from microbial pathogens in the skin, respiratory tract and digestive tract (Sonnenberg *et al.*,2011). In addition to its role in infectious disease, the importance of IL-22 in protection and regeneration of epithelial tissues has been widely studied in cancers, inflammatory skin diseases, hepatitis, pancreatitis, colitis, and thymic injury (Dudakov *et al.*,2015).

The role of IL-22 in infectious disease was first described in mouse studies performed with *Citrobacter rodentium* infection, where IL-22 was shown to enhance epithelial cell proliferation

in the gut as IL-22 knockout mice displayed significant damage to colonic epithelial cells (Sugimoto and Ogawa, 2008; Basu *et al.*, 2012). Moreover, infection of IL-22 deficient mice with *C. rodentium* resulted in intestinal epithelial damage, a high bacterial burden and mortality (Zheng *et al.*, 2008). Similarly, in mice infected with *Klebsiella pneumoniae*, IL-22 neutralisation resulted in an exacerbated bacterial dissemination when IL-17 was also absent (Aujla *et al.*, 2008). In influenza infection, IL-22 deficient mice displayed aggravated lung injury (Pociask *et al.*, 2013) and severe impairment in the regeneration of tracheal epithelial cells (Kumar *et al.*, 2013) compared to wild type mice. Although IL-22 has a protective role in several tissues, uncontrolled expression can cause tissue damage and chronic inflammation, which could lead to inflammatory skin diseases such as psoriasis or development of several types of cancer (Schmechel *et al.*, 2008; Zhu *et al.*, 2012).

1.5.3 IL-22 and TB

IL-22 was reported to be non-essential for the control of Mtb in mouse models (Wilson *et al.*, 2010; Khader *et al.*, 2011; Behrends *et al.*, 2013; Segueni *et al.*, 2016), using lab Mtb strains H37Rv and Erdman. IL-22 was shown to be produced mainly by IFN- γ -secreting cells in mice (Behrends *et al.*, 2013). However, a more recent study by Treerat and colleagues described a protective role of IL-22 against a hyper-virulent Mtb strain (HN878) in an aerosol model of infection, where IL-22 knockout mice displayed higher bacterial burdens during the chronic stage of Mtb infection (Treatat *et al.*, 2017). Furthermore, IL-22 produced by NK cells was shown to inhibit the growth of Mtb by enhancing phagolysosomal fusion (Dhiman *et al.*, 2009). Indeed, the administration of IL-22 at the time of BCG vaccination resulted in reduced bacterial burden and enhanced CD4⁺ T cell response after Mtb challenge in mice by inhibiting expansion of induced

Tregs, thus enhancing Ag-specific T cell responses (Dhiman *et al.*, 2012). IL-22 was downregulated in the blood but upregulated in the lungs of rhesus monkeys during severe TB (Qiu *et al.*, 2008). In humans, soluble IL-22 has been detected at sites of pulmonary and extrapulmonary TB (Scriba *et al.*, 2008; Matthews *et al.*, 2011a), and a SNP in the IL-22 promoter has been linked to TB susceptibility (Zhang *et al.*, 2011). Furthermore, isoniazid preventive therapy (IPT) and TB treatment enhanced mycobacteria-specific IL-22 production in the periphery (Zhang *et al.*, 2014; Suliman, Geldenhuys, John L. Johnson, *et al.*, 2016), suggesting that reduction of antigen load in the site of infection by anti-TB drugs leads to restored IL-22 responses in blood. In bovine TB, an IL-22 gene expression signature was the dominant surrogate of BCG vaccine-induced protection following infection with *M. bovis* (Bhujji *et al.*, 2012). Moreover, *in vitro* expression of PPD-specific-IL-22 was higher in cattle naturally infected with *M. bovis* compared to uninfected cattle (Aranday-Cortes *et al.*, 2012).

1.6 The effect of HIV on TB pathogenesis

1.6.1 Alteration of Mtb-specific CD4+ T cells

The risk of developing TB is estimated to be between 16-27 times greater in people living with HIV than among those without HIV infection (WHO, 2019). The obvious reason for increased TB risk is that HIV infection causes progressive loss in CD4+ T cells which is associated with increased risk of developing TB (Sonnenberg *et al.*, 2005). However, the risk of developing TB increases within one year of HIV infection and occurs even at preserved CD4 count (Sonnenberg *et al.*, 2005). Therefore, studies have investigated preferential depletion and dysfunction of Mtb-specific CD4+ T cells in HIV infection.

Geldmacher *et al.* have reported that Mtb-specific peripheral CD4⁺ T cells are preferentially depleted in HIV infection compared to cytomegalovirus (CMV)-specific CD4⁺ T cells (Geldmacher *et al.*, 2010). HIV infection causes a reduction in the frequency and absolute number of circulating CD4⁺ T cells in individuals with preserved CD4 count (Riou, Bunjun, *et al.*, 2016; Bunjun *et al.*, 2017; Day *et al.*, 2017). Several studies have reported a decreased frequency of Mtb-specific responses in bronchoalveolar lavage (BAL) of HIV-infected compared to uninfected individuals (Kalsdorf *et al.*, 2009; Jambo *et al.*, 2011; Bunjun *et al.*, 2017). HIV infection has also been shown to distort the balance of Mtb-specific Th subsets, as lineage-defining transcription factor profiles were skewed in HIV-infected individuals (Riou *et al.*, 2016). Furthermore, SIV infection was reported to alter effector T cell phenotypes and dysregulated T cell homeostasis, which was associated with reactivation of LTBI in macaques (Bucşan *et al.*, 2019). However, a study by Bunjun *et al.* reported that the absolute number of CD4⁺ T cells in bronchoalveolar lavage (BAL) is similar between HIV-infected and uninfected individuals with LTBI, and this could be attributed to infiltration of CD4⁺ T cells into the airways (Bunjun *et al.*, 2017). These data demonstrate that HIV infection causes depletion and dysfunction of Mtb-specific CD4⁺ T cells both in the blood and at the disease sites, thereby compromising Mtb immunity.

1.6.2 Dysfunction of CD8⁺ T cells

CD4⁺ T cells provide help to CD8⁺ T cells (Matloubian, Concepcion and Ahmed, 1994; Serbina, Lazarevic and Flynn, 2001). Therefore, CD4⁺ reduction and dysfunction during HIV infection may result in CD8⁺ T cell dysfunction. HIV infection may result in impaired degranulation and proliferative capacities of Mtb-specific CD8⁺ T cells (Kalokhe *et al.*, 2015). HIV infection also results in chronic activation that may lead to premature exhaustion of CD8⁺ T cells (Breton *et al.*,

2013; Amelio *et al.*, 2019). Further studies to elucidate the impact of HIV on Mtb-specific CD8+ T cells are warranted.

1.6.3 Impairment of innate immunity

Evidence supports the assertion that HIV infection impairs innate immunity, permitting the persistence of Mtb infection. Neutrophils in the blood of ART-naïve HIV-infected individuals were shown to be less effective in restricting Mtb growth compared to HIV-uninfected persons (Lowe *et al.*, 2015). Furthermore, HIV infection impairs the ability of neutrophils to phagocytose Mtb by reducing CD16 and CD35 mediated opsonophagocytosis in human serum (Bangani *et al.*, 2016). In addition, HIV infection impairs neutrophil activation, respiratory burst, phagocytosis, and microbial killing capacity, particularly at low CD4 counts and high viral load (Lowe *et al.*, 2012).

In a study by Jambo *et al.*, HIV was reported to preferentially infect AM as they harboured a substantial amount of HIV in the BAL (Jambo *et al.*, 2014). The phagosomal proteolysis of AM was impaired in HIV-infected cells (Jambo *et al.*, 2014a, 2014b). In TB and HIV co-infected individuals, activation of the innate immune system (monocytes, interleukin-6, tumour necrosis factor- α and colony-stimulating factor) was associated with mortality (Janssen *et al.*, 2017). *In vitro* studies demonstrated that HIV infection can augment mycobacterial virulence factors and impair their ability of macrophages to contain Mtb growth (Imperiali *et al.*, 2001; Pathak *et al.*, 2010). Moreover, HIV accessory protein Nef can interfere with phagosome biogenesis to inhibit macrophage phagocytosis (Mazzolini *et al.*, 2010) and can block the maturation of autophagosomes resulting in the inhibition of autophagy (Kyei *et al.*, 2009; Killian *et al.*, 2012).

Moreover, HIV infection has been reported to inhibit TNF- α dependent macrophage apoptosis by inhibition of TNF- α promoter activation (Patel *et al.*, 2009;).

Of note, HIV and Mtb are both recognized by DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN, CD209) and its homolog, DC-SIGN-related (DC-SIGNR or L-SIGN), and this is likely to influence host immunity and bacterial persistence in co-infected persons (Tailleux *et al.*, 2003; da Silva *et al.*, 2011). Indeed, HIV was shown to interfere with Mtb antigen presentation in an experimental system using human immature DCs coinfecting with HIV and Mtb, as demonstrated by reduced Mtb antigen processing and inhibition of autophagy (Singh *et al.*, 2016).

HIV infection has also been found to deplete human ILCs in blood (Kløverpris *et al.*, 2016). In macaques, SIV infection resulted in loss of ILCs within gut associated lymphocyte tissue (GALT) and a persistent loss of IL-17-producing ILCs in the jejunum (Xu *et al.*, 2010; Li *et al.*, 2014). Moreover, Hueber *et al.* observed a 3-fold depletion of ILC3 in the colon of SIV/SHIV-infected infant rhesus macaques (RM) compared to uninfected (Hueber *et al.*, 2019). In addition, HIV infection was shown to deplete ILC3 in human blood with correlated with HIV disease progression (Zhang *et al.*, 2015). Furthermore, using humanized mice model, Zhang *et al.*, demonstrated that HIV depleted ILC3 through CD95/FasL-mediated apoptosis which required activation of plasmacytoid dendritic cells (pDCs) and induction of IFN (Zhang *et al.*, 2015). It is not known whether ILC depletion occurs in the lungs and may compromise TB immunity.

Interestingly, TB has also been shown to contribute to HIV pathogenesis (Whalen *et al.*, 1995; Badri *et al.*, 2001; Meng *et al.*, 2016). This is supported by reports that show that during active TB, HIV-infected individuals have viral loads increased up to 160-fold compared to non-TB symptomatic HIV-infected patients, suggesting that TB may promote HIV replication in co-infected individuals (Goletti *et al.*, 1996; Toossi *et al.*, 2001). Furthermore, Zhang *et al.* demonstrated an increase in HIV-1 p24 levels in BAL fluid from Mtb-infected lung sites compared with uninfected lung sites in HIV-TB co-infected patients (Zhang *et al.*, 1995). Additionally, it has been shown that active TB causes an increase in expression of HIV coreceptors CXCR4 and CCR5 on CD4+ T cells, thereby increasing the HIV target cells (Juffermans *et al.*, 2001).

1.6.4 IL-22 and HIV

Depletion of IL-22-producing CD4+ T cells has been reported to be one of factors associated with HIV immunopathogenesis. Th22 and Th17 cells were shown to be depleted in blood and tissue mucosa in HIV-infected individuals, and correlated positively with microbial translocation (Kim *et al.*, 2012) and loss of IL-17 and IL-22-producing lymphocytes was found to be associated with damage to the colonic epithelial barrier during SIV infection (Klatt *et al.*, 2012). Microbial translocation is defined as a loss of epithelial integrity and mucosal immune deficiency, allowing bacterial products to enter the circulation, leading to immune activation, the latter playing a key role in the immune-pathogenesis of HIV infection (Brenchley *et al.*, 2006). Further evidence supporting the importance of IL-22-producing CD4+ T cells in maintaining mucosal homeostasis during HIV and SIV infection has been reported. In a longitudinal study performed in rhesus macaques, SIV-infection induced a loss and a severe dysfunction in intestinal IL-17, IL-22, and

IL-17/IL-22 producing CD4⁺ T cells which was not evident in blood and lymph node (Ryan *et al.*, 2016).

It has been shown that CCR6⁺ CD4⁺ T cells, which characterize primarily cells of a Th22 and Th17 phenotype (i.e. cells expressing the cytokines IL-22 and IL-17), express high levels of HIV co-receptor CXCR4 (Alvarez *et al.*, 2013). Kim and colleagues reported that a higher proportion of Th22 cells express the main HIV co-receptor CCR5, compared Th1 cells, as well as integrin $\alpha 4\beta 7$, which has been associated with increased HIV susceptibility (El Hed *et al.*, 2010; Monteiro *et al.*, 2011; Kim *et al.*, 2012; Sivro, Schuetz, Sheward, Joag, Yegorov, Lenine J. Liebenberg, *et al.*, 2018). Furthermore, CCR6⁺ cells lack production of CCR5 ligands (β -chemokines) which have been shown to induce autocrine protection against HIV (El Hed *et al.*, 2010; Alvarez *et al.*, 2013). CD4⁺CCR6 cells have also been associated with enriched HIV DNA and increased HIV replication (Cleret-Buhot *et al.*, 2015; Gosselin *et al.*, 2017; Planas *et al.*, 2017). However, in contrast to these findings, it was recently demonstrated that SIV did not preferentially infect Th17 cells, Th1 cells, Th2 cells, or Tregs, but infected polarized memory CD4⁺ T cells equally *in vivo* (Lai *et al.*, 2019).

1.7 Advances in TB vaccine development

The Bacille Calmette-Guérin (BCG) vaccine, which was developed almost 100 years ago, remains the only licensed vaccine for preventing TB (WHO, 2019). Although effective in preventing childhood and extra-pulmonary TB (60–80% protection), BCG provides inconsistent protection against pulmonary TB and it is not effective when given in adulthood (Fine, 1995; Trunz *et al.*, 2006; Bo *et al.*, 2008). The reasons behind variable protection provided by BCG against pulmonary

TB are unclear. HIV infection further complicates BCG vaccine-induced protection. Indeed, HIV-infected infants had lower CD4 responses after BCG vaccination compared to HIV-uninfected infants (Mansoor *et al.*, 2009). Moreover, BCG is a live attenuated mycobacterial vaccine, thus posing a risk of local or disseminated BCG disease and immune reconstitution inflammatory syndrome (IRIS) (Hesseling *et al.*, 2007). Therefore, the WHO has recommended that HIV-infected infants should no longer be vaccinated with BCG (WHO, 2007). We urgently need an effective TB vaccine that can either replace or supplement BCG (Gupta *et al.*, 2018).

There are currently 12 TB vaccines in development, four in phase 1, eight in phase 2a/b, and three in phase 3 (**Figure 1.5**) (WHO, 2019). Scientists employ different strategies for developing new TB vaccines, to provide for different target groups (infants, adults, individuals with LTBI, or HIV-infected individuals) (Andersen and Urdahl, 2015). These include subunit (*e.g.* H4:IC31), killed mycobacterial whole cells or cell extract (*e.g.* RUTI), live attenuated (*e.g.* MTBVAC), live recombinant (*e.g.* MVA85A) and DNA (*e.g.* Ag85A DNA) vaccines (WHO, 2019). Subunit vaccines usually consist of immunodominant components enhanced using adjuvants. These vaccines have many advantages, including safety, low cost, easy preparation, high yield and high purity. The disadvantages of subunit vaccines are that the short duration of immunogenicity, poor memory immunity, the use of adjuvants and the process of antigen screening to identify immunodominant antigens is complex (Zhu *et al.*, 2018). Killed/inactivated vaccines could potentially be safe and effective against preventing TB disease. However, these vaccines are likely to provide a short period of protective immunity and required multiple inoculations and high doses (Gong *et al.*, 2018). Live attenuated and live recombinant vaccines are advantageous because they can possess a large antigen repertoire and potentially mimic natural infection, therefore they can

potentially elicit broad immune responses. The main drawback of live vaccines is safety, as there is a potential risk of virulence recovery and complications in immunocompromised individuals (Hesseling *et al.*, 2007; Ui *et al.*, 2017). While DNA vaccines are much safer, they still require optimization to obtain the desired efficacy against TB (Zhu *et al.*, 2018).

Recently, a promising vaccine candidate M72/AS01_E demonstrated an efficacy of 54% in the primary analysis and 50% in the final analysis (three years follow-up) of a phase 2b clinical trial conducted in three African countries (Kenya, South Africa, and Zambia) involving HIV-uninfected individuals (Van Der Meeren *et al.*, 2018; Tait *et al.*, 2019). This is a subunit vaccine derived from two immunogenic Mtb antigens (32A and 39A) adjuvanted with AS01_E (Skeiky *et al.*, 1999; Al-Attiyah *et al.*, 2004). M72/AS01_E vaccination induced IgG antibodies and polyfunctional M72-specific CD4⁺ T cell responses (Gillard *et al.*, 2016; Kumarasamy *et al.*, 2018; Van Der Meeren *et al.*, 2018). Although the efficacy of M72/AS01_E remains to be tested in HIV-infected individuals, studies have shown that this vaccine is safe in HIV-infected individuals and induced polyfunctional M72-specific CD4⁺ T cell responses (Thacher *et al.*, 2014; Kumarasamy *et al.*, 2016, 2018). Finally, M72/AS01_E candidate vaccine offers an opportunity to investigate the correlates of immune protection as the lack of understanding of correlates of protection is a major challenge facing development of new TB vaccines (Sakai *et al.*, 2014).

Another interesting advance in TB vaccine development studies is BCG revaccination. A recent study by Nemes *et al.* reported that BCG revaccination of adolescents who had received neonatal BCG vaccine provided an efficacy of 45.4% against Mtb infection as measured by sustained QFT conversion (30 month) and 20.1% efficacy against Mtb infection based on initial QFT conversion (Nemes *et al.*, 2018). However, other studies conducted in Brazil showed no efficacy against Mtb infection or TB disease, indicating that the success of BCG revaccination may be dependent on

study populations, age and the epidemiology of TB (Rodrigues *et al.*, 2005; Barreto *et al.*, 2011). Indeed, effectiveness of primary BCG vaccination has been shown to variable in different populations (Mangtani *et al.*, 2014). The advantage of BCG revaccination is that it is a highly cost-effective intervention as BCG is already licenced.

■ Viral Vector
 ■ Protein/Adjuvant
 ■ Mycobacterial – Killed Whole Cell or Extract
 ■ Mycobacterial – Live Attenuated

Revised 7 October 2019

Phase 1	Phase 2a	Phase 2b	Phase 3
AEC/BC02 Anhui Zhifei Longcom	RUTI Archivel Farma, S.L.		DAR-901 Dartmouth, GHIT
Ad5Ag85A McMaster, CanSino	MTBVAC Biofabri, TBVI, Zaragosa	M72/AS01E GSK	Vaccae™ Anhui Zhifei Longcom
ChAdOx1 85A/MVA85A (ID/IM/Aerosol) Univ of Oxford	ID93 + GLA-SE IDRI, Wellcome Trust, Quratis		VPM1002 SII, Max Planck, VPM, TBVI (Phase 2/3)
	TB/FLU-04L RIBSP	H56:IC31 SSI, IAVI, EDCTP, Valneva	Immuvac ICMR, Cadila Pharmaceuticals
		BCG Revaccination Gates Medical Research Institute (GMRI)	

Figure 1.5: New TB vaccines currently in the global development pipeline. Taken from <https://www.newtbvaccines.org/tb-vaccine-pipeline/>.

Most studies investigate Th1 responses to determine the immunogenicity of TB vaccine candidates (Soares *et al.*, 2008; Tameris *et al.*, 2014; White *et al.*, 2015; Norrby *et al.*, 2017). However, IFN- γ secretion has been shown to be a poor correlate of protection in BCG-vaccinated mice (Majlessi *et al.*, 2006; Mittrücker *et al.*, 2007) and individuals testing positive for IFN- γ release assays (IGRA) have up to 10% of developing TB in their lifetime (O’Garra *et al.*, 2013).

Limited studies have examined vaccine induced IL-22 production. BCG-specific IL-22 responses were detected in BCG-revaccinated adults (Suliman *et al.*, 2016) and BCG-vaccinated infants had

higher BCG-specific IL-22 responses compared to unvaccinated in blood (Nissen *et al.*, 2017). BCG vaccination also induced IL-22 production in bronchoalveolar lavage fluid (Gupta *et al.*, 2018) and recombinant IL-22 was shown to enhance the protective efficacy of BCG vaccine by decreasing the number of immunosuppressive Tregs (Dhiman *et al.*, 2012). These studies indicate that investigating the IL-22 contribution to TB control may lead to new insights that could potentially aid in TB vaccine development.

Overall, there have been exciting advances in TB vaccine development with multiple promising new TB vaccine candidates. There are also vaccines currently in animal models that will enter human trials soon (WHO, 2019). However, many questions remain unanswered, such as different immune correlates of protection, environmental factors that influence the success of TB vaccines, the relationship between the human host and *M. tuberculosis*, the best route of vaccine delivery, and whether it is possible to develop vaccines that will clear Mtb.

1.8 Aims and Objectives

The overall aim of this project was to study T cell immune responses to TB, with a focus on the contribution of Th22 cells to TB immune responses through characterising *Mycobacterium tuberculosis*-specific IL-22-producing CD4+ T cells in detail and examining the impact of TB disease and HIV infection on these cells.

Objective 1: To examine factors that lead to Mtb-specific IL-22 production and further characterise of Mtb-specific Th22 cells

Rationale: In studies performed in our laboratory, we have observed a very low to no IL-22 detection in PBMC, whilst detection is robust in whole blood (Bunjun *et al.*, submitted). We further demonstrated that IL-22 production in PBMC could be recovered when autologous PBMCs were added to whole blood. This suggests that there are factors in blood absent in PBMC which are vital for IL-22 production. Since the characteristics of Th22 cells are not fully defined, we investigated factors that influence Mtb-induced IL-22 production and further characterise Th22 cells. We determined the T cell subsets that produce IL-22 in response to Mtb stimulation, assessed whether IL-22 is produced through “bystander” effect or requires TCR engagement, and investigated soluble factors that could lead to Mtb-specific IL-22 production in PBMCs.

This portion of the study is presented in Chapter 2.

Objective 2: To characterise Mtb-specific Th22 cells and investigate the effect of tuberculosis disease and HIV co-infection

Rationale: A distinct subset of CD4⁺ T cells producing IL-22 have been identified, ‘Th22’ cells. A recent study in mice described a protective role for IL-22 during the chronic stage of Mtb infection against a clinical strain of Mtb. There has been limited research into the effect of HIV and TB co-infection on Th22 cells, which may provide insights into the role they play in human disease. In a previous study performed in our laboratory, we observed high frequencies of mycobacteria-specific Th22 cells, and demonstrated that HIV infection results in the depletion of these Th22 cells (Bunjun et al., submitted). In this study, we sought to extend these investigations to both latent and active TB and HIV co-infection, and compared the quality and quantity of Mtb-specific Th22 cells in persons with HIV infection with active TB disease, HIV infection with latent TB infection, TB disease in the absence of HIV infection, and latent TB infection without HIV infection. We tested the hypothesis that the combination of TB disease and HIV infection would result in extensive reduction on Mtb-specific Th22 cells.

This portion of the study is presented in Chapter 3.

Objective 3: To compare the magnitude and phenotype of CD8⁺ and CD4⁺ responses to Mtb during TB disease and/or HIV infection

Rationale: Whilst many studies have investigated the importance of CD4⁺ T cells in the control of *Mycobacterium tuberculosis*, relatively fewer have characterized CD8⁺ T cells. Evidence suggests that both CD4⁺ and CD8⁺ T cells play a role in successful immune control of

Mycobacterium tuberculosis infection. Therefore, we sought to investigate the contribution of CD8⁺ T cells compared to CD4⁺ T cells in TB immune responses and study the impact of TB disease and HIV infection on CD8⁺ T cells.

This portion of the study is presented in Chapter 4.

CHAPTER 2

Optimization of IL-22 detection and characterization of Mtb-specific CD4+ T cells producing IL-22

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2.1 Introduction

Interleukin-22 (IL-22) is a relatively recently described cytokine that belongs to the IL-10 family of cytokines. IL-22 has been shown to be produced by a range of adaptive and innate cells, including CD4⁺ T cells, $\gamma\delta$ T cells, NKT cells and innate lymphoid cells (ILCs) (Sonnenberg *et al.*, 2011; Ronacher *et al.*, 2018). IL-22 is involved in tissue repair (Pickert *et al.*, 2009; McGee *et al.*, 2013), immunity to bacterial infections (Valeri and Raffatellu, 2016) as well as viral, fungal, and parasitic infections (Dudakov *et al.*, 2015; Brias *et al.*, 2016). IL-22 exerts its function by inducing the production of antimicrobial peptides and proteins such as β -defensins, the S100 family of peptides, Reg3 β , calprotectin and calgranulin A (Lin *et al.*, 2017; Moyat *et al.*, 2017; Tyler *et al.*, 2017). IL-22 signaling also regulates chemokine expression to orchestrate the recruitment of immune cell subsets to sites of infection (Whittington *et al.*, 2004; Aujla *et al.*, 2008). In addition, IL-22 promotes tissue proliferation, regeneration, and healing of epithelial cells (Witte *et al.*, 2010; Sabat *et al.*, 2014; Dudakov *et al.*, 2015).

IL-22 was initially thought to be a Th17 cytokine due to co-expression with IL-17 in mice (Liang *et al.*, 2006). However, human studies showed that that IL-22 is mainly produced by a distinct subset of CD4⁺ T cells named Th22 cells (Duhon *et al.*, 2009; Eyerich *et al.*, 2009; Trifari *et al.*, 2009). The production of IL-22 is thought to be partially regulated by the transcription factor aryl hydrocarbon receptor (AHR) (Trifari *et al.*, 2009; Basu *et al.*, 2012). Exogenous cytokines IL-6, IL-23, TNF and IL-1 β have been shown to promote IL-22 production (Duhon *et al.*, 2009; Trifari *et al.*, 2009; Plank *et al.*, 2017). However, the characterization of IL-22 producing cells, the signals leading to IL-22 production and the range of ways in which IL-22 functions in immunity remains incompletely described, warranting a better understanding of Th22 cells.

In the context of TB infection, there is evidence to suggest a possible role of IL-22 in Mtb immunity (Treerat *et al.*, 2017), but to date Mtb-induced IL-22 production has been understudied in humans. In this Chapter, we describe the optimization of IL-22 detection in human blood, determining factors that contribute to Mtb-specific IL-22 production, and characterizing some aspects of the biology of Mtb-specific IL-22 producing CD4⁺ T cells.

2.2 Material and methods

2.2.1 Study participants

Blood samples were collected from healthy adult donors with LTBI recruited from the University of Cape Town. The exclusion criteria for the study participants were: pregnancy, age <18 years, weight <50kg and any acute or chronic illness. LTBI was diagnosed based on a positive IFN- γ release assay (QuantiFERON®-TB Gold In-Tube) or positive responses to Early secretory antigenic target (ESAT-6) and culture filtrate protein 10kDa (CFP-10) peptides pool and no symptoms of aTB. All participants gave written, informed consent. The study was approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC 051/2018).

2.2.2 Antigens and Mitogens

The following antigens were used in this study:

Bacillus Calmette–Guérin (BCG) is the current licensed vaccine against Mtb and consists of live attenuated *Mycobacterium bovis*. The BCG used in this study was from *M. bovis* Danish strain 1331 (SSI, Denmark). BCG and Mtb have numerous protein antigens in common, however BCG lacks a genomic region (RD-1) that confers virulence to Mtb and virulent *M. bovis* (Mahairas *et al.*, 1996).

ESAT-6/CFP-10 are encoded by the region of difference 1 (RD-1) genes, Rv3875 and Rv3874, respectively. CFP-10 and ESAT-6 are specific to Mtb and play an important role in Mtb virulence (Forrellad *et al.*, 2013). The peptide pools used in this study were made from 15mer peptides

overlapping by 10 amino acids and covering the entire length of Rv3874 and Rv3875. Seventeen peptides made up ESAT-6 and 16 made up CFP-10 (Peptide Synthetics, UK).

Mycobacterium tuberculosis lysate (MtbL) from Mtb strain H37Rv (BEI Resources) contains the cell contents from log-phase Mtb. MtbL is a mixture of all the proteins, lipids and carbohydrates present in the cell.

Gamma-irradiated Mtb whole cells (γ -irr Mtb) from Mtb strain H37Rv (BEI Resources) contains Mtb bacilli grown to late-log phase in glycerol-alanine-salts medium and inactivated by exposure to 2.4 megaRads of ionizing gamma irradiation using a ^{137}Cs source.

Phorbol myristate acetate/Ionomycin (PMA/Iono): PMA is a specific activator of Protein Kinase C (PKC) that is commonly used with ionomycin, a membrane permeable calcium ionophore that promotes transport of calcium ions Ca^{2+} into treated cells, to activate the transcription factors NF- κB and nuclear factor of activated T cells (NFAT), thus inducing cytokine production (Schindler and Baichwal, 1994; Pinton *et al.*, 2008).

Staphylococcus enterotoxin B (SEB) is an enterotoxin produced by the gram-positive bacteria *Staphylococcus aureus*. SEB is also a superantigen that induces polyclonal T cell activation and cytokine production (McLeod *et al.*, 1998).

Phytohemagglutinin (PHA) is a lectin from the bean *Phaseolus vulgaris*, that triggers calcium dependent signaling pathways leading to NFAT activation and cytokine production, by binding to sugars on glycosylated surface proteins and crosslinking them (Fortin *et al.*, 2001).

These antigens and mitogens were used at concentrations previously reported (Hanekom *et al.*, 2004) or were titrated in our laboratory for specific assays, and final concentrations used are described below.

2.2.3 Blood collection and stimulation

Blood was collected in sodium heparin tubes and processed within four hours of collection. Unless stated otherwise, whole blood assays were performed according the protocol described previously (Hanekom *et al.*, 2004). Briefly, 0.5 ml of heparinized whole blood was stimulated with Mtb cell lysate (strain H37Rv, 20 µg/ml, BEI Resources), an Mtb-specific peptide pool [(4 µg/ml), consisting of 17 and 16 peptides covering the entire ESAT-6 and CFP-10, respectively], live bacillus Calmette-Guerin (BCG) (MOI of 4, *Mycobacterium bovis* Danish strain 1331, SSI), gamma-irradiated Mtb whole cells (Strain H37Rv, 600 µg/ml, BEI Resources), Phorbol Myristate Acetate (PMA; 0.01 µg/ml; Sigma-Aldrich) and Ionomycin (25 µg/ml; Sigma-Aldrich), Staphylococcal Enterotoxin B (SEB; 5 µg/ml; Sigma-Aldrich) or Phytohemagglutinin (PHA; 10 µg/ml; Thermo Scientific) at 37 °C for a total of 12 h in the presence of anti-CD28 and anti-CD49d (1 µg/ml each; BD Biosciences) co-stimulatory antibodies. After 7 h, brefeldin A (10 µg/ml; Sigma-Aldrich) was added to block cytokine release by inhibiting protein transport from the Golgi apparatus to the endoplasmic reticulum. Red blood cells were lysed with Alternative Lysing Solution (ALS; 150 mM NH₄Cl, 10 mM KHCO₃, 1 mM Na₄EDTA) and washed twice with 15 ml

PBS. The cell pellet was subsequently stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Molecular Probes) or LIVE/DEAD® Fixable Near-Infrared Stain (Invitrogen) and cells washed twice with 15 ml PBS. Cells were then fixed using FACS Lysing solution (BD Biosciences) for 10 min and stained immediately.

2.2.4 Peripheral blood mononuclear cells (PBMC) isolation and stimulation

Heparinized blood collected in BD Vacutainer® sodium heparin tubes was separated using Ficoll-Hypaque (Sigma Aldrich) density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC). Briefly, room temperature Ficoll-Hypaque (Sigma-Aldrich) was added to Leucosep tubes (Greiner) and centrifuged at 900 x g for 1 min to pass the Ficoll through the separation disc. Whole blood was then added to Leucosep tubes containing Ficoll and centrifuged at 900 x g for 10 min to allow for separation. The layer containing PBMC was transferred to 50 ml tubes and washed twice with RPMI 1640 (Sigma) supplemented with 1% fetal calf serum (FCS, HyClone). PBMC were then rested for 4 h at 37 °C, 5% CO₂ in RPMI 1640 supplemented with 10% FCS (R10). The cells were then washed with R10 before transferring to 5 ml FACS tubes for stimulation. PBMC (2 x10⁶) were stimulated with Mtb cell lysate (strain H37Rv, 25 µg/ml, BEI Resources) in 500 µl of R10 or conditioned media (see **Section 2.2.5**) for a total of 16 h with BFA addition after 7 h. All assays were performed on freshly isolated PBMC.

2.2.5 Treating PBMC with conditioned media

Whole blood was stimulated as described in **Section 2.2.3**. Blood was centrifuged and the plasma from the stimulated or non-stimulated blood was added to RPMI to prepare 20% Mtb-stimulated plasma (Mtb/plasma) or 20% non-stimulated plasma (NS/plasma), respectively. Freshly isolated

PBMC were then resuspended in R10, RPMI with autologous 20% Mtb/plasma or RPMI with 20% NS/plasma prior to stimulation with MtbL as described in **Section 2.2.4** above.

2.2.6 Flow Cytometry

Antibodies

The antibodies listed in **Table 2.1** were used for surface or intracellular staining. All antibodies used were titrated to identify the optimal concentration for detection of each marker. This titer was selected based on saturation, the signal to noise ratio and staining index. A fluorescence minus one (FMO) control (*i.e.* staining with all antibodies except the one of interest) was included to guide our gating strategy. Antibody titrations were performed for every new lot of antibody.

Surface and Intracellular staining

For surface staining, fixed cells were transferred to 96-well v-bottom plates and washed three times with 200 μ l of FACS wash buffer (2% FCS in phosphate buffered solution (PBS)). The plate was centrifuged at 800 x g for 5 min at 4 °C and the supernatant discarded. Cells were then resuspended in 50 μ l of a surface antibody mix in FACS wash buffer and incubated in the dark for 20 min at room temperature, followed by washing the cells three times with 200 μ l of FACS wash buffer again. Cells were then resuspended in 200 μ l of Perm/Wash buffer (BD Biosciences) and incubated in the dark for 30 min at room temperature. The plate was centrifuged at 800 x g for 5 min at 4 °C, the supernatant discarded, and the cells resuspended in 50 μ l of an intracellular antibody staining mix in Perm/wash buffer and incubated in the dark for 30 min at room temperature. Cells were washed three times with Perm/Wash buffer and resuspended in 150 μ l of Cell Fix (BD Biosciences).

Table 2.1 Antibodies used for flow cytometry

Markers	Fluorophore	Clone	Manufacturer	Function	Staining
Viability marker	APC-Cy7		Invitrogen	Exclusion	Surface
Viability marker	Pacific Blue		Invitrogen	Exclusion	Surface
CD14	PE-Cy5.5	Tuk4	Invitrogen	Lineage	Surface
CD3	BV650	OKT3	BD Biosciences	Lineage	ICS
CD4	ECD	T4	Beckman Coulter	Lineage	Surface
CD8	Qdot 705	3B5	Life Technologies	Lineage	Surface
CD56	PE-Cy7	NCAM 16.2	BD Biosciences	Lineage	Surface
CD161	APC	HP-3G10	eBioscience	Lineage	Surface
IFN- γ	Alexa fluor 700	B27	BD Biosciences	Cytokine	ICS
IL-22	eFluor 450	22URTI	eBioscience	Cytokine	ICS
IL-22	PE	22URTI	eBioscience	Cytokine	ICS
IL-22	APC	22JOP	eBioscience	Cytokine	ICS
IL-22	APC	142928	R & D systems	Cytokine	ICS
IL-17	FITC	BL168	Biolegend	Cytokine	ICS
IL-17	Alexa fluor 647	N49-653	BD Biosciences	Cytokine	ICS
Pan $\gamma\delta$	PE	IMMU510	Beckman Coulter	Lineage	Surface
TCR V α 7.2	BV510	3C10	Biolegend	Lineage	Surface
TCR V β 11	FITC	C21	Beckman Coulter	Lineage	Surface
TCR V α 24	PE	C15	Beckman Coulter	Lineage	Surface

Acquisition and data analysis

Stained cells were acquired on a four-laser BD Fortessa (**Figure 2.1**) and analyzed using FlowJo (v10, TreeStar). A positive cytokine response was defined as at least twice the background (*i.e.*

unstimulated cells) and a minimum of 10 events. To define the phenotype of cytokine-producing cells, a cutoff of 20 events was used.

Compensation

In order to accurately determine primary fluorescent signals for each detector, we determined the fluorescent contribution of each fluorophore in each detector and calculated the compensation matrix (*i.e.* the fluorescent signal percentages subtracted from each primary detector).

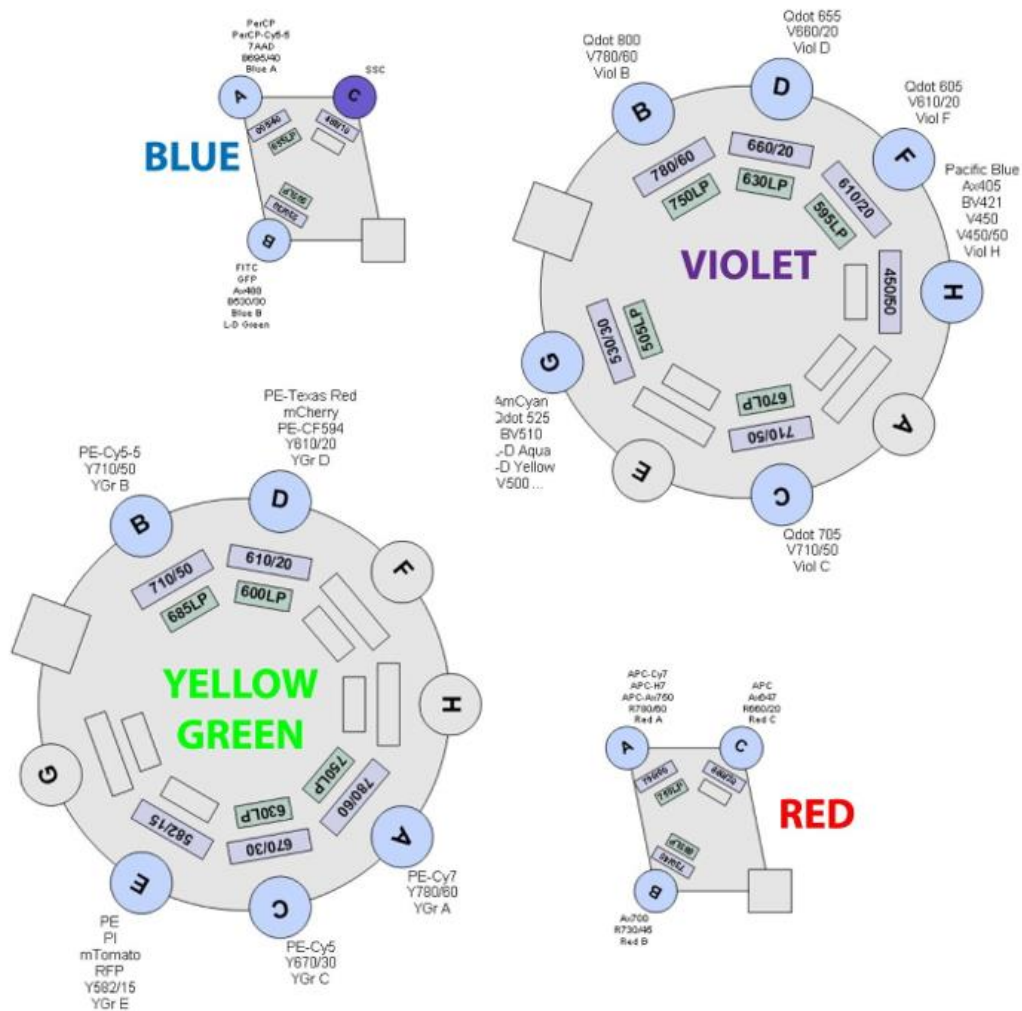


Figure 2.1: The filter configuration of the BD Fortessa used in the study. The filter arrangement is shown for each of the four lasers, with two octagons and two trigons.

Briefly, individually stained compensation beads (anti-mouse Ig- κ or anti-rat Ig- κ beads; BD Biosciences) corresponding to each antibody-conjugate used in the flow cytometry panel were prepared for each flow cytometry run. One drop of compensation beads (~ 40 μ l) was incubated with each antibody (at the optimally determined titer) in a 5 ml polystyrene tube for 20 min at room temperature. The excess antibody was washed off by adding 3 ml PBS and centrifuging at 800 x g for 5 min. The supernatant was decanted, and the beads resuspended in 200 μ l of Cell Fix buffer (BD Biosciences).

2.2.7 Determining the TCR V β repertoire of cytokine-positive cells

The IOTest® Beta Mark TCR V β Repertoire Kit (Beckman Coulter) was used to determine the TCR V β repertoire of Mtb-specific IFN- γ or IL-22-producing CD4+ T cells. Briefly, cells from Mtb lysate stimulated whole blood were stained with CD3 BV650 (OKT3; BD Biosciences), CD4 PerCP-Cy5.5 (L200; BD Biosciences), IFN- γ Alexa700 (B27; BD Biosciences) and IL-22 APC (22JOP; eBioscience™), and each of the eight vials containing mixtures of conjugated TCR v β antibodies corresponding to 24 different specificities that represent up to 70% of normal human TCR v β Repertoire (**Table 2.2**).

Table 2.2: List of TCR V β - antibodies in tubes A-H (Beckman Coulter)

Tube	V β family	Fluorochrome	Clone
A	V β 5.3	PE	3D11
	V β 3	FITC	CH92
	V β 7.1	PE + FITC	ZOE
B	V β 9	PE	FIN9
	V β 16	FITC	TAMAYA1.2
	V β 17	PE + FITC	E17.5F3
C	V β 18	PE	BA62.6
	V β 20	FITC	ELL1.4
	V β 5.1	PE + FITC	IMMU157
D	V β 13.1	PE	IMMU222
	V β 8	FITC	56C5.2
	V β 13.6	PE + FITC	JU74.3
E	V β 5.2	PE	36213
	V β 12	FITC	VER2.32
	V β 2	PE + FITC	MPB2D5
F	V β 23	PE	AF23
	V β 21.3	FITC	IG125
	V β 1	PE + FITC	BL37.2
G	V β 11	PE	C21
	V β 14	FITC	CAS1.1.3
	V β 22	PE + FITC	IMMU546
H	V β 13.2	PE	H132
	V β 7.2	FITC	ZIZOU4
	V β 4	PE + FITC	WJF24

2.2.8 Lymphocyte-specific protein tyrosine kinase (Lck) inhibition

Whole blood from healthy individuals was incubated with increasing concentrations (1 μ M to 60 μ M) of Lck inhibitor (7-cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d] pyrimidin-4-ylamine, Sigma) for 30 min prior to cell stimulation (Rapecki *et al.*, 2002; Schade *et al.*, 2008.). Whole blood was then stimulated as described in **Section 2.2.4.**, followed by analysis of cytokine production. To determine the percentage inhibition, we used the following formula: frequency of cytokine-producing CD4+ T cells at a given Lck inhibitor concentration divided by frequency of cytokine-producing CD4+ T cells without Lck inhibitor, multiplied by 100.

2.2.9 Statistical analysis

Statistical tests were performed using Prism (v6; GraphPad). Nonparametric tests were used for all comparisons. The one-way ANOVA Friedman test and Kruskal-Wallis test with Dunn's Multiple Comparison test was used for multiple comparisons and the Mann-Whitney U test and Wilcoxon matched pairs test were used for unmatched and paired samples, respectively. Chi-square tests were used to analyze categorical data to compare proportions of individuals with detectable cytokine responses. A p value of < 0.05 was considered statistically significant. SPICE version 5.1 was used to analyze cytokine co-expressing populations and to perform permutation tests. Pestle was used for background subtraction and generating SPICE data.

2.3 Results

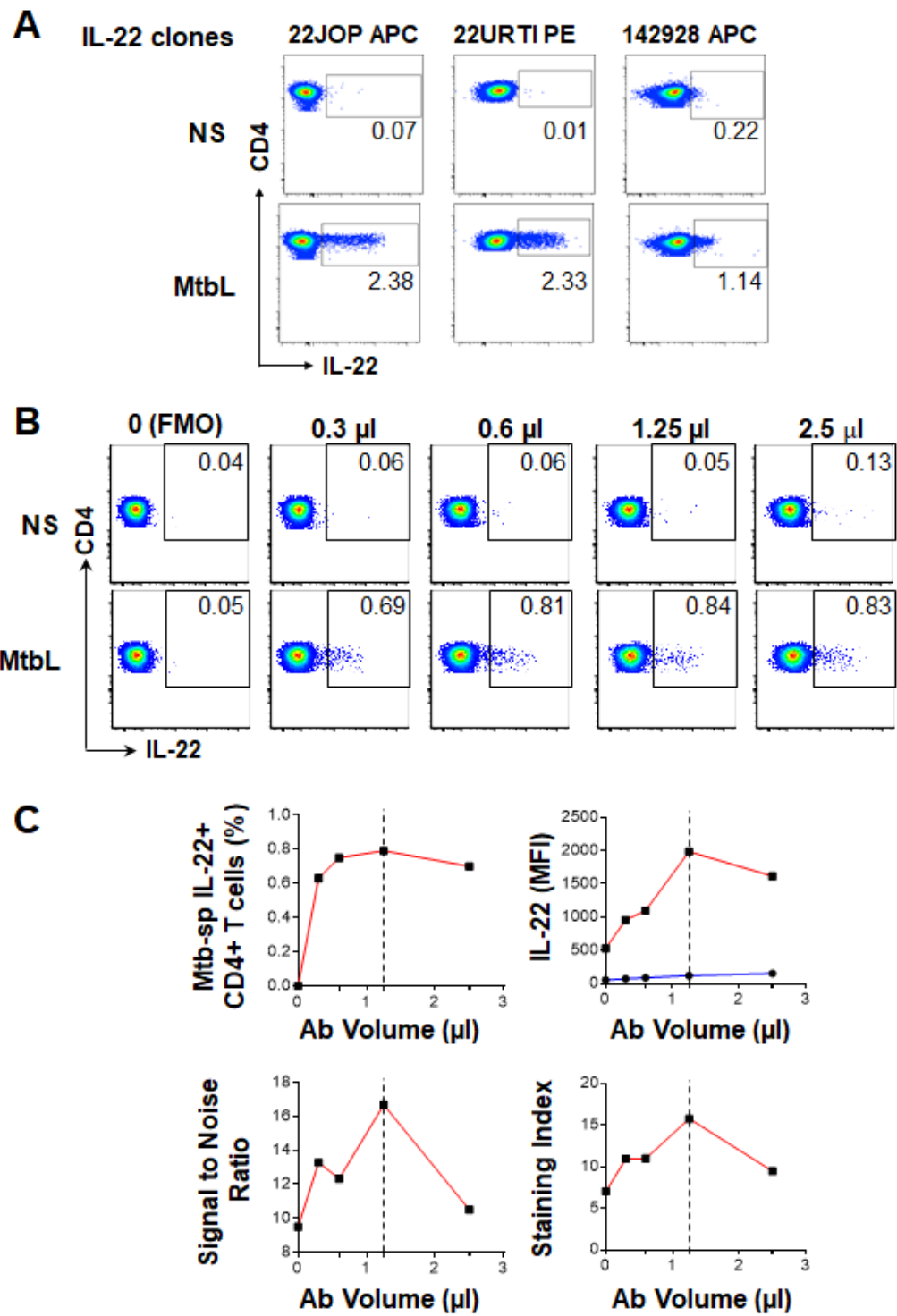
2.3.1 Optimization of IL-22 cytokine production in whole blood

To optimize detection of IL-22 production, we first compared which IL-22 antibody clones provide the optimal detection (**Figure 2.2A**). Blood stimulated with MtbL was stained with three IL-22

antibody clones: IL-22 PE (22URTI; eBioscience™), IL-22 APC (22JOP; eBioscience™), and IL-22 APC (142928; R & D) (**Figure 2.1A**). We detected higher frequencies of IL-22-producing CD4⁺ T cells using IL-22 antibody clones 22URTI and 22JOP, compared to clone 142928 (frequency: 2.33%, 2.38% vs. 1.14%, respectively). We selected IL-22 antibody clone 22URTI to use for all subsequent experiments.

To determine the optimal concentration of antibody, we titrated all antibodies included in flow cytometry panels. **Figure 2.2B** and **C** shows titration of IL-22 antibody, cells were stained with increasing volume of the antibody (0 μ l - 2.5 μ l) and no stimulation and FMO controls were included. We then compared the frequency and median fluorescent intensity (MFI) of IL-22-producing CD4⁺ T cells in response to Mtb lysate stimulation at different IL-22 titers and calculated the staining index $((\text{MFI pos} - \text{MFI neg}) / 2 * \text{SD MFI neg})$ and signal to noise ratio $(\text{MFI pos} / \text{MFI neg})$ to select the optimal concentration of antibody (**Figure 2.2B&C**).

Figure 2.2 (next page): Comparison of IL-22 antibody clones and titration of IL-22 antibodies. (A) Representative flow cytometry plots showing IL-22 production by CD4⁺ T cells using different antibody clones. Antibody clones tested and the conjugated fluorochromes used are indicated on top of each set of plots. (B) Representative flow cytometry plots showing frequencies of IL-22-producing CD4⁺ T cells at different antibody titers after whole blood stimulation with MtbL. (C) Determining optimal antibody titer. The dotted line indicates the chosen titer. Ab, antibody, MFI: median fluorescence intensity, SD: standard deviation, SI: staining index, S/N: signal to noise, MtbL: *Mycobacterium tuberculosis* lysate, NS: no stimulation, FMO: Fluorescent Minus One. Calculations: Signal to noise ratio = $\text{MFI pos} / \text{MFI neg}$; Staining index = $(\text{MFI pos} - \text{MFI neg}) / 2 * \text{SD MFI neg}$



Next, we investigated the ability of different mitogens including Phorbol myristate acetate/Ionomycin (PMA/Iono), Staphylococcus enterotoxin B (SEB) and phytohemagglutinin (PHA) to induce IFN- γ , IL-22 and IL-17 production to identify an optimal positive control for each cytokine, particularly IL-22 (**Figure 2.3**). **Figure 2.3A** shows representative flow cytometry plots showing MtbL-, PMA/Iono-, SEB- and PHA-responding IFN- γ , IL-22 and IL-17 responses. The highest IFN- γ production was detected when blood was stimulated with PMA/Ionomycin (median: 8.1%) compared to SEB (median 1.3%, $p = 0.005$) or PHA (median 1.03%, $p=0.002$). We detected similar and generally low frequencies of IL-22-producing CD4+ T cells when blood was stimulated with PMA/Ionomycin (median: 0.17%) and SEB (median: 0.19%) and both responses were significantly higher than PHA responses (median 0.03%; $p = 0.004$ and $p = 0.004$, respectively). In contrast to the poor IL-22 responses induced, PHA and PMA/Iono induced robust production of IL-17 (medians: 0.7% and 0.6%; respectively), with significantly lower CD4+ cells producing IL-17 in response to SEB (median 0.1%; $p=0.002$ and $p=0.01$, respectively). As PMA/Ionomycin was the only mitogen able to induce production of all tested cytokines (IFN- γ , IL-22 and IL-17), albeit at low levels for IL-22, this mitogen was chosen as a positive control. It is interesting that similar IL-22 responses were detected when blood was stimulated with PMA and SEB. This is surprising as SEB is known to trigger only 20% of TCRs through src kinase Fyn, while PMA/IONO bypasses TCR and activates PKC, NF- κ B and NFAT (Schindler and Baichwal, 1994; Pinton *et al.*, 2008; Janik *et al.*, 2015), thus PMA would be expected to induce higher IL-22 production compared to SEB.

In our experiment we included stimulation with MtbL and measurement of IFN- γ , IL-22 and IL-17 responses to MtbL (**Figure 2.3**). Interestingly, we observed a similar magnitude of Mtb-specific IFN- γ and IL-22 producing CD4+ T cells in response to this stimulation (median: 0.59% and

0.62%; respectively). MtbL also induced the highest frequencies of IL-22-producing cells, greater than any of the mitogens tested. In contrast, IL-17 producing CD4+ T cells were poorly detectable in response to MtbL stimulation compared to IFN- γ and IL-22 (median: 0.01%; $p = 0.0003$ and $p = 0.0009$; respectively) (**Figure 2.3B**).

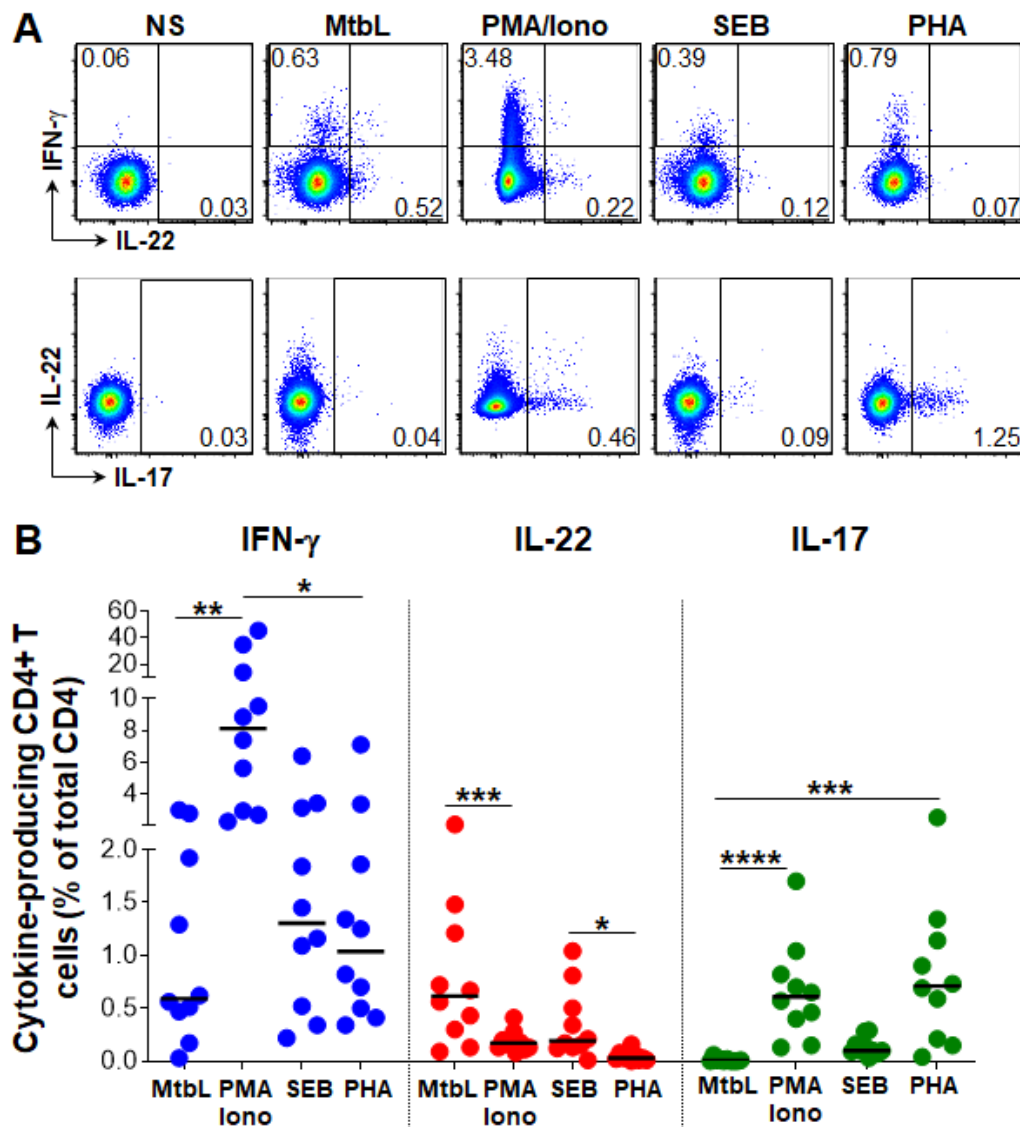


Figure 2.3: Identifying an optimal positive control for detecting IL-22 production from CD4+ T cells. (A) Representative flow cytometry plots showing IFN- γ , IL-22 and IL-17 responses after stimulation with MtbL, PMA/Iono, SEB and PHA. (B) Comparison of the frequency of IFN- γ , IL-22 and IL-17 when stimulated with different mitogens and MtbL ($n=10$). Medians are depicted. NS: no stimulation. Statistical comparisons were performed using a one-way ANOVA Friedman test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

It is interesting that higher IL-22 responses were detected after stimulation with Mtb lysate compared to PMA/IONO. One plausible explanation would be that IL-22 detected after Mtb lysate stimulation is resulting from cytokine production induced in other cell types, however, experiments in chapter 3 section 2.4, page 61 indicate that Mtb induced IL-22 responses are indeed specific and do not represent bystander activation.

We next sought to determine whether similar cytokine profiles were generated in response to different types of Mtb antigens, and thus compared blood stimulated with gamma-irradiated whole cell Mtb (γ -irr Mtb), live mycobacteria (*M. bovis* BCG) or an Mtb peptide pool based on ESAT-6 and CFP-10 proteins. No differences in the magnitude of IL-22, IFN- γ or IL-17 responses were observed between the different “complex” mycobacterial antigens tested (*i.e.* Mtb cell lysate, gamma-irradiated Mtb and BCG) (**Figure 2.4A&B**). In contrast, IL-22 production was barely detectable in response to Mtb peptide stimulation (**Figure 2.4C**), with only 2/11 donors with latent Mtb infection exhibiting an IL-22 response, at a frequency of <0.05%. All donors were positive for IFN- γ responses (median 0.18%) and 3/11 donors had IL-17 responses. These data indicate that IL-22 is readily detectable to complex mycobacterial antigen preparations but poorly detectable when using Mtb peptides, suggesting that antigen processing and presentation requirements for IL-22 may differ from IFN- γ induction and that IL-22 production may be mediated through non-TCR stimulation of other cell types. This is expanded on further in chapter 3.

2.3.2 Factors that contribute to Mtb-specific IL-22 production in PBMC compared to whole blood

MtbL stimulation leads to a similar magnitude of IL-22 and IFN- γ production when employing a whole blood assay. However, previous data from our laboratory shows that IL-22 is poorly detectable in PBMC compared to whole blood (Bunjun *et al.*, submitted). Interestingly, addition of fluorescently-labeled PBMC into autologous whole blood resulted in full recovery of IL-22 responses from CD4⁺ T cells derived from PBMC (Bunjun *et al.*, submitted). This suggests that PBMC do indeed contain the cells that are able to produce IL-22 in response to Mtb stimulation, but lack the physiological environment for optimal recall of IL-22 responses when stimulated with antigen.

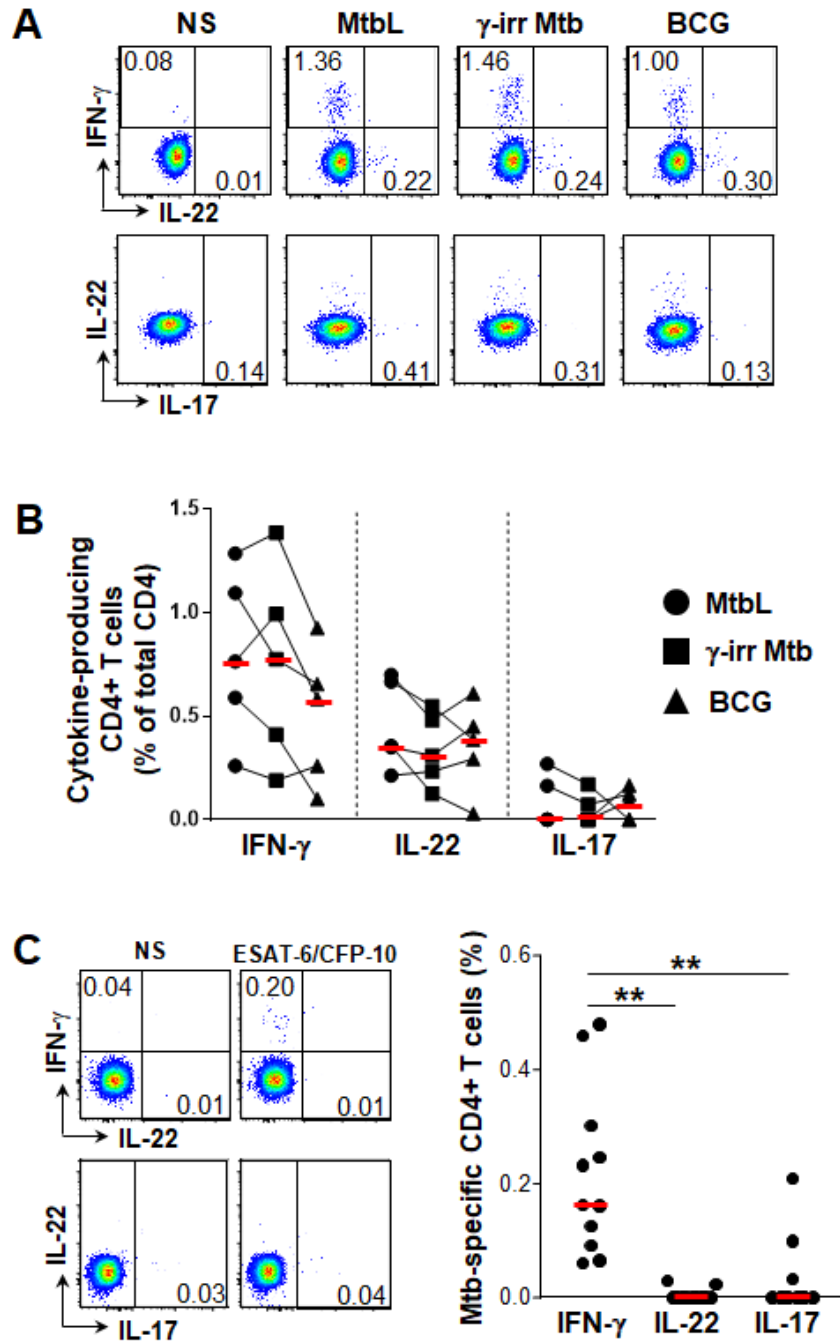
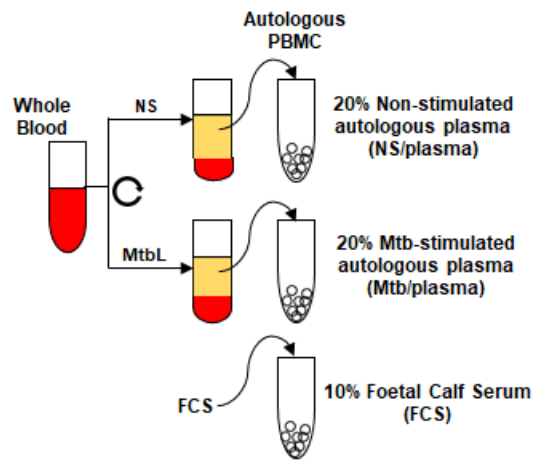
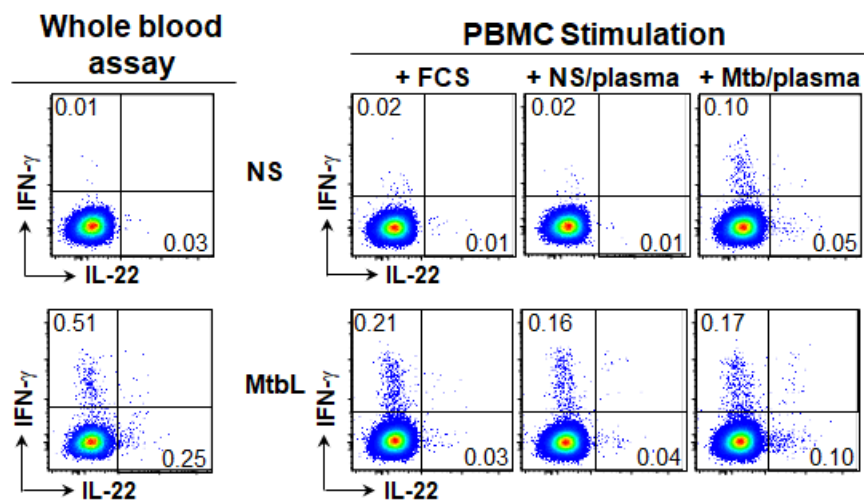
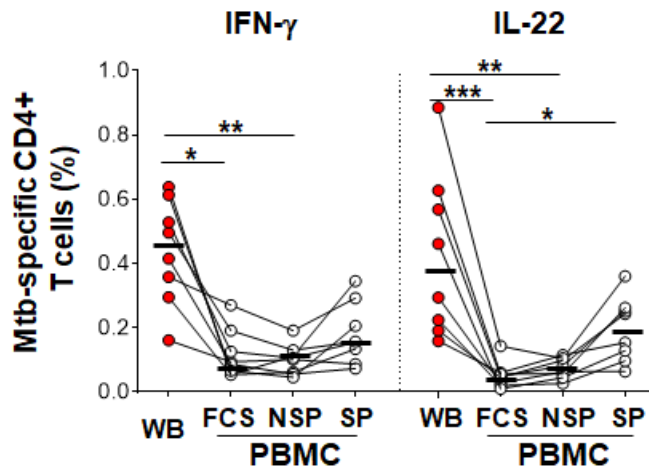


Figure 2.4: Comparison of the frequency of IFN- γ , IL-22 and IL-17-producing CD4+ T cells in response to different mycobacterial antigen formulations. (A) Representative flow cytometry plots showing IFN- γ , IL-22 and IL-17 responses after stimulation with MtbL, γ -irr Mtb, and BCG. (B) Comparison of the frequency of IFN- γ , IL-22 and IL-17 responses to different antigens (n=5). (C) Comparison of the frequency of IFN- γ , IL-22 and IL-17 after stimulation with ESAT-6/CFP-10 peptides (n=11). NS: no stimulation. The bar represents the median. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. ** $p < 0.01$.

To investigate whether soluble mediators present in whole blood that may be absent in PBMC are responsible for inducing IL-22 production from CD4⁺ T cells, we used conditioned media to determine whether IL-22 production requires plasma proteins present in whole blood but not in PBMC. We measured the magnitude of IFN- γ and IL-22-producing CD4⁺ T cells detected using a whole blood assay compared to PBMC stimulation when PBMC are supplemented with conditioned media consisting of either 20% Mtb-stimulated plasma (Mtb/plasma), 20% non-stimulated plasma (NS/plasma) or 10% FCS in RPMI (**Figure 2.5A**). The whole blood assay enabled robust detection of cytokines (IFN- γ and IL-22) in response to MtbL stimulation in all eight donors (**Figure 2.5B&C**). However, the frequency of IFN- γ producing CD4⁺ T cells was significantly lower (median ~5 fold) when PBMC were stimulated with MtbL (median: 0.46% vs 0.09%, respectively; $p = 0.0117$) (**Figure 2.5C**). The difference was even more dramatic for IL-22 responses in PBMC, where Mtb-specific CD4⁺ T cells producing IL-22 were approximately ~8 fold lower (median: 0.38% in blood vs 0.05% in PBMC; $p = 0.0002$) (**Figure 2.5C**). Supplementing PBMC with conditioned media did not significantly improve IFN- γ production (median: 0.11% for NSP and 0.16% for SP) (**Figure 2.5C**). Interestingly, we observed a slight restoration of IL-22 production when PBMC were supplemented with Mtb/plasma conditioned media compared to R10 (median: 0.20% vs 0.05%; ~ 4-fold increase $p = 0.0161$). This increase was not observed when PBMC were supplemented with NS/plasma (median: 0.07% for NSP vs 0.05% for FCS; $p = ns$) (**Figure 2.5C**).

Figure 2.5 (next page): Frequency of Mtb-specific CD4⁺ T cells in PBMC compared to whole blood. (A) Schematic representation of method (B) Flow cytometry plots of IFN- γ and IL-22 production after stimulation with MtbL. NS: no stimulation. (C) Summary graph of the frequency of cytokine responses. Medians are depicted. Statistical comparisons were performed using a one-way ANOVA Friedman test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. FCS: fetal calf serum, NSP: non-stimulated plasma, SP: Mtb-stimulated plasma.

A**B****C**

To ensure that the partial restoration of IL-22 production observed in the presence of Mtb/conditioned plasma was not solely due to additional MtbL present in the stimulated plasma, we defined the dose-response curve of IL-22 production in response to increasing concentrations of MtbL (from 10 to 40 $\mu\text{g/ml}$) in three donors. Maximal IL-22 production was observed at 10 $\mu\text{g/ml}$, thus the partial restoration of IL-22 responses observed when conditioned media was used is not due to increased MtbL antigen availability (**Figure 2.6**). These results suggest that Mtb antigen-induced soluble factors secreted by cells present in whole blood but absent in PBMC may influence IL-22 production, although restoration remains partial and it is likely that additional factors influence IL-22 induction in PBMC.

Altogether, these experiments show that IL-22-producing CD4⁺ T cells constitutes a substantial portion of Mtb immune responses in whole blood. However, these cells are hardly detectable in PBMC. In addition, unlike IFN- γ responses, IL-22 responses were not induced following stimulation with Mtb peptides. These data raise several questions: (i) Does IL-22 production originate from unconventional T cells? and (ii) Is IL-22 production mediated via a T cell receptor (TCR)-independent pathway, rather than being induced by CD4⁺ T cells upon engagement of cognate antigen presented to the TCR? (Boyman *et al.*, 2010).

In order to address these questions, we next determined whether IL-22 production was mediated through TCR triggering (*i.e.* Mtb antigen being directly recognized by the TCR on responding CD4⁺ T cells), we inhibited the TCR pathway by blocking Lck, a tyrosine kinase critical for early propagation of TCR signalling, using increasing concentrations of the Lck inhibitor 7-cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-d] pyrimidin-4-ylamine. Using whole blood from five healthy donors with latent Mtb infection, we found that both IFN- γ and IL-22 expression were

inhibited upon Lck inhibition, in a dose-dependent manner. Interestingly, the ED₅₀ (efficient dose causing 50% of maximum effect) for IL-22 inhibition was 2.6 times lower compared to the ED₅₀ for IFN- γ inhibition (7.5 μ M and 19.5 μ M, respectively; **Figure 2.7**). Of note, cytokine co-expression profile indicates that on a small proportion of IL-22 is co-expressed with IFN-g, and this is further explored in chapter 3. The observed inhibition is most likely specific as it has been shown that lck blocking with 7-Cyclopentyl-5-(4-phenoxyphenyl) 7Hpyrrolo[2,3-d] pyrimidin-4-ylamine leads to reduction of aCD3/CD28 induced cytokine production but not PMA/IONO induced cytokine production (Rapecki *et al.*, 2002; Schade *et al.*, 2008).” These data demonstrate that IL-22 production from CD4+ T cells is TCR-dependent.

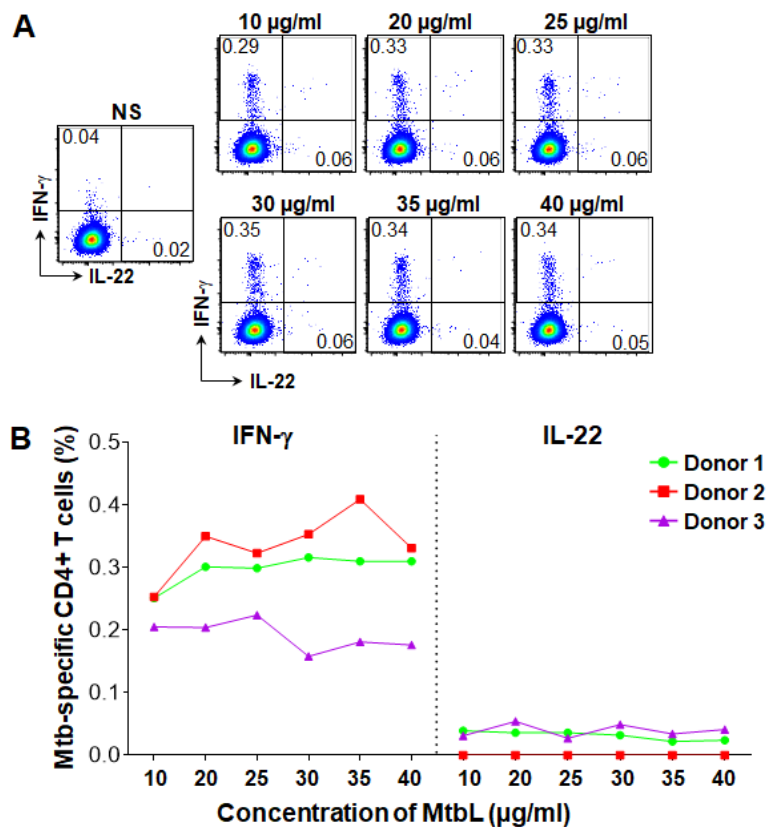


Figure 2.6: Titration of MtbL in PBMC. (A) Representative flow cytometry plots showing CD4+ IFN- γ and IL-22 responses when PBMC are stimulated with different concentrations of MtbL. NS: no stimulation. (B) Summary graph (n=3).

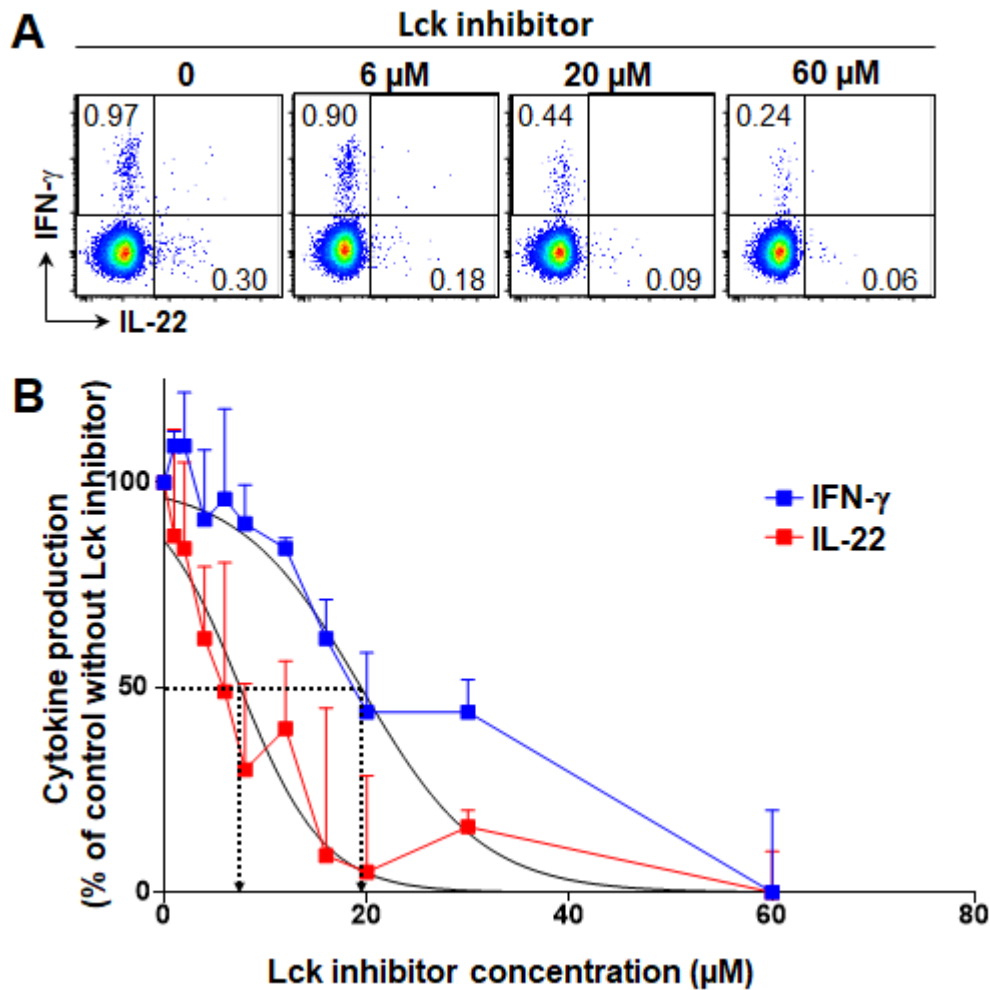


Figure 2.7: Impact of TCR blocking on IFN- γ and IL-22 production. (A) Representative flow cytometry plots showing Mtb-specific IFN- γ and IL-22 responses in the presence of different concentrations of Lck inhibitor 7-Cyclopentyl-5-(4-phenoxyphenyl) 7Hpyrrolo[2,3-d] pyrimidin-4-ylamine. (B) Summary graph (n=5) showing the proportion of IFN- γ or IL-22 producing CD4⁺ T cells relative to whole blood stimulation in the absence of Lck inhibitor. Medians and interquartile ranges are depicted. Vertical arrows show ED₅₀. Nonlinear regression curve fit used.

2.3.3 Characteristics of Mtb-specific IL-22 producing CD4 T cells

It has been shown that a variety of immune cells including conventional T cells (recognizing peptide antigens presented by MHC molecules) and unconventional T cells (recognizing nonpeptide antigens, functioning as a bridge between innate and adaptive responses) have the ability to produce IL-22 (Sonnenberg *et al.*, 2011; Ronacher *et al.*, 2018). Thus, we investigated

IL-22 and IFN- γ production from a range of T cell subsets in whole blood, including mucosal-associated invariant T (MAIT) cells, $\gamma\delta$ T cells, Natural Killer T (NKT) cells, CD4⁺ T cells and CD8⁺ T cells. Cells were gated on live lymphocytes that were CD3⁺, and then defined as NKT (CD56⁺), $\gamma\delta$ (CD56- $\gamma\delta$ TCR⁺), MAIT (CD56- $\gamma\delta$ TCR-V α 7.2+CD161⁺), CD4 (NKT- $\gamma\delta$ TCR-MAIT-CD8-CD4⁺) and CD8 (NKT- $\gamma\delta$ TCR-MAIT-CD4-CD8⁺) T cells (**Figure 2.8A**). In 10 healthy donors tested for cytokine responses, all cell subsets assessed produced IFN- γ when stimulated with Mtb lysate. In contrast, in most donors (7 out of 10), IL-22 was exclusively produced by CD4⁺ T cells, with only 3 donors demonstrating a minor contribution of either MAIT or $\gamma\delta$ cells to the total IL-22 response from CD3⁺ cells (**Figure 2.8B**). Of note, the majority of MAIT and $\gamma\delta$ T cells are CD4⁻ (**Figure 2.8C**), indicating that our gating strategy used in previous experiments did not include IL-22 from these T cell sources. Thus, conventional CD3⁺CD4⁺ T cells appear to be the major source of IL-22 in response to Mtb antigens.

Lastly, we compared the V β repertoire usage of IL-22 and IFN- γ producing CD4⁺ T cells in blood from five healthy donors with latent Mtb infection using IOTest® Beta Mark TCR V β Repertoire Kit (Beckman Coulter). **Figure 2.9A** shows representative flow plots of nine out of 24 v β measured. The panel of 24 monoclonal antibodies to TCR V β families stained approximately 61% of CD4⁺, 40% of CD4⁺IFN- γ ⁺ and 62% of CD4⁺IL-22⁺. We observed a similar distribution of TCR V β subsets between Mtb-specific IL-22 and IFN- γ producing CD4⁺ T cells as well as in the total CD4⁺ compartment. TCR V β usage ranged from median values of 0.2% for V β 23 to 13.6% for V β 2, indicating non-random V β usage. Interesting, in all donors, V β 2 and V β 5.1 accounted for more than 5% of the total V β usage in Mtb-specific IL-22 and IFN- γ producing CD4⁺ T cells (median: 13.6% and 10.4 for V β 2, 8.5% and 6.7% for V β 5.1, respectively; **Figure 2.9B**).

Similarly, V β 2 and V β 5.1 were highly expressed in total CD4 compartment (median: 10.0% for V β 2 and 7.2% for V β 5.1, **Figure 2.9C**). Cumulative data (**Figure 2.9C**) shows similar TCR usage between IFN- γ and IL-22-producing CD4⁺ T cells, although there are slight differences on an individual level (**Figure 2.9C**). The similar distribution of TCR between IFN-g and IL-22 producing CD4⁺ T cells is interesting as these cells represent distinct subsets of CD4⁺ T cells (discussed in chapter 3 below) with minimal co-expression of IFN- γ and IL-22. There was also preferential usage of V β 1, V β 8, V β 17 and V β 22 by Mtb-specific IL-22 and IFN- γ producing CD4⁺ T cells, as well as total CD4⁺ T cells, with at least two out of five donors expressing more than 2.5% of these TCR V β families.

These data show that IL-22 and IFN- γ -producing CD4⁺ T cells belong to the same families of $\alpha\beta$ T cells as indicated by a similar TCR usage. Moreover, there were no differences in TCR V β usage between Mtb-specific CD4⁺ T cells and total CD4⁺ T cells, indicating that Mtb does not induce biased usage of TCR V β .

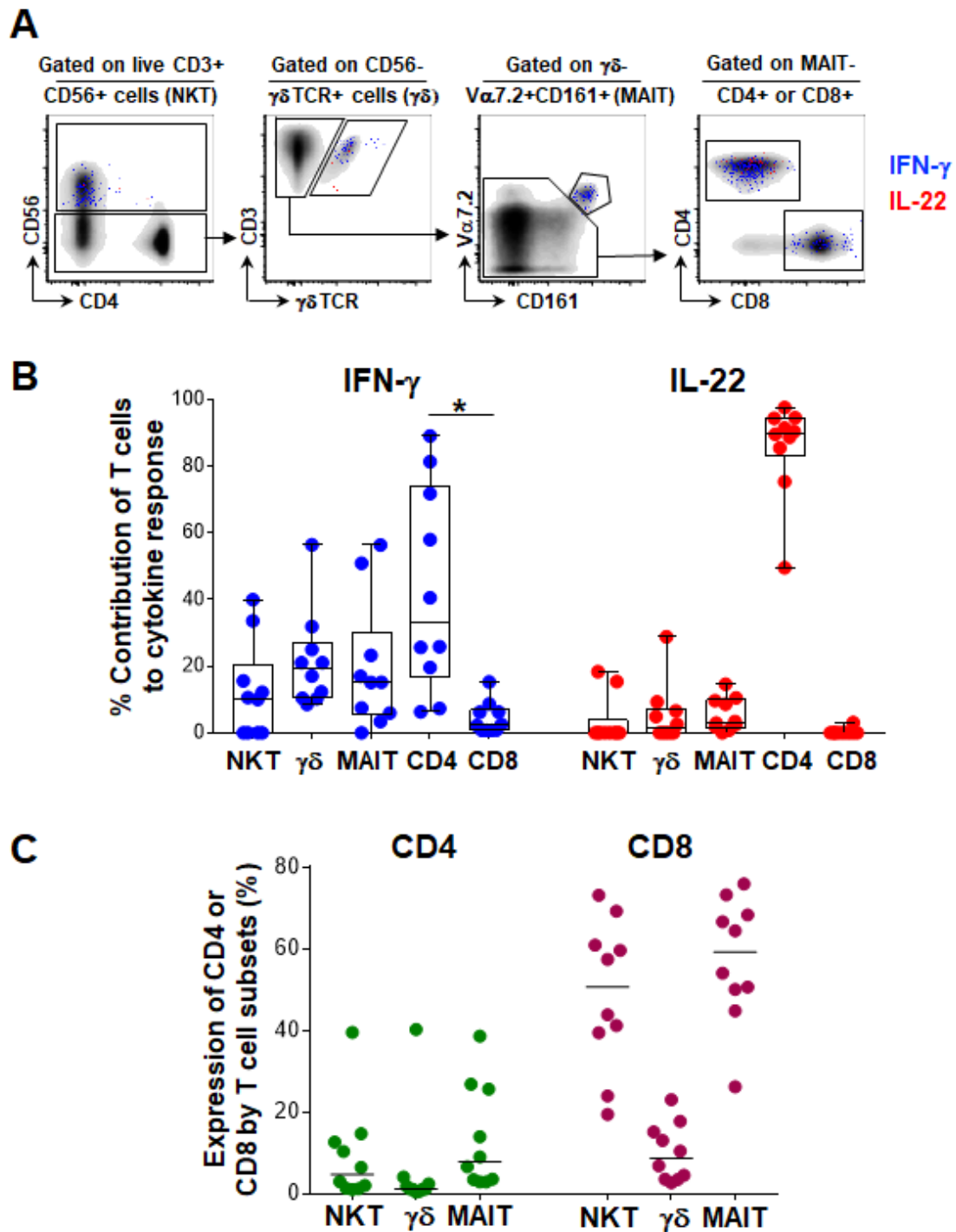


Figure 2.8: Contribution of different T cell subsets to Mtb-specific response. (A) Representative flow cytometry overlay plots showing IFN- γ and IL-22 production in different T cell (CD3+) subsets: NKT (CD56+), $\gamma\delta$ (CD56- $\gamma\delta$ TCR+), MAIT (CD56- $\gamma\delta$ TCR-V α 7.2+CD161+), CD4 (NKT- $\gamma\delta$ TCR-MAIT-CD8-CD4+) and CD8 (NKT- $\gamma\delta$ TCR-MAIT-CD4-CD8+) cells. (B) Summary graph of the contribution of distinct T cell subsets to cytokine responses. Medians and interquartile ranges are depicted. (C) Expression of CD4 and CD8 in different T cell subsets. Medians are depicted.

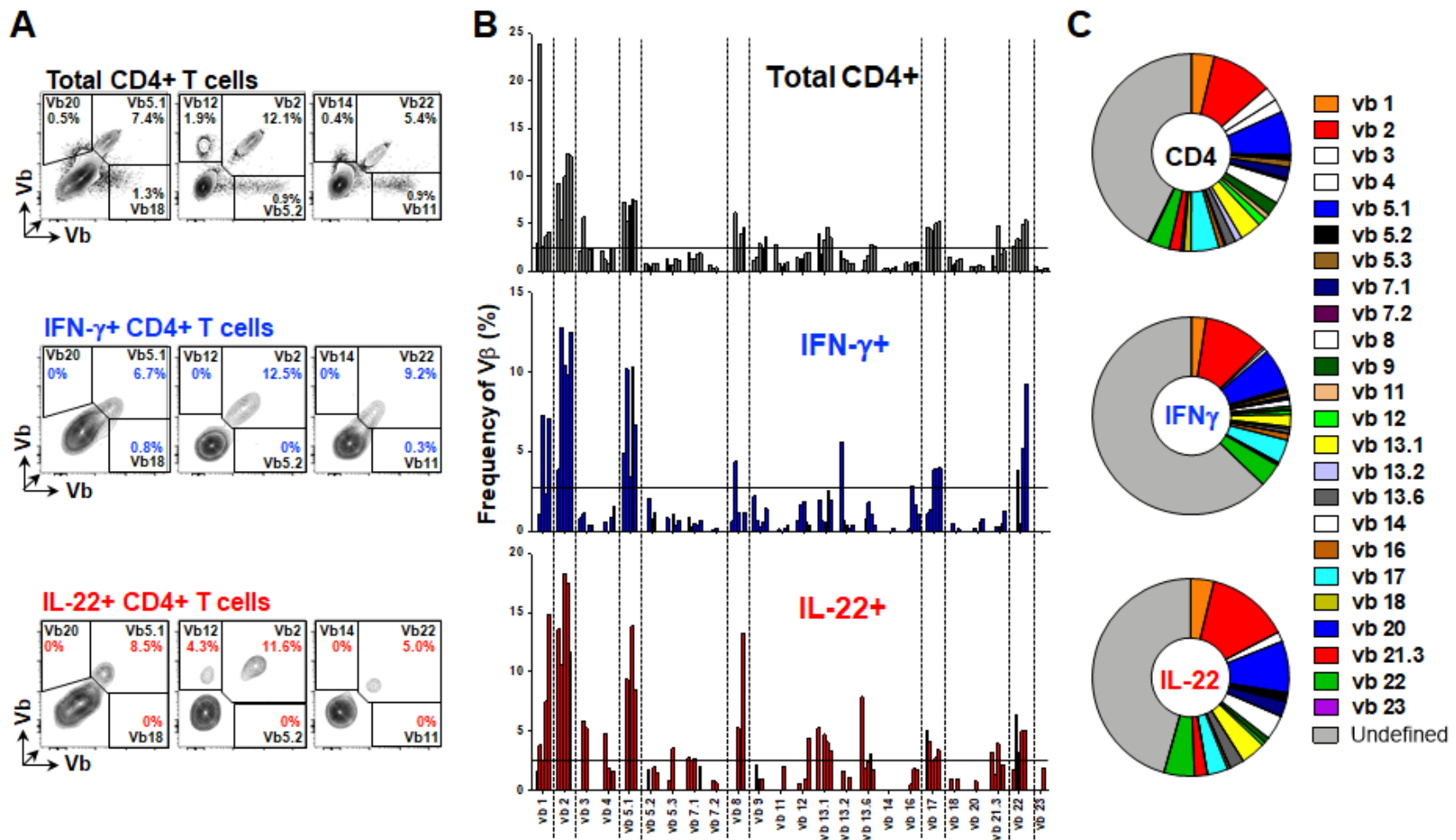


Figure 2.9: TCR Vβ repertoire of IFN-γ and IL-22 producing CD4+ T cells. (A) Representative flow cytometry plots showing 9/24 Vβ antibodies on total CD4+ T cells, IFN-γ and IL-22 producing CD4+ T cells (B) Bar graph showing TCR distribution on each individual and (C) Summary pie chart showing expression of 24 Vβ antibodies by total CD4+ T cells, IFN-γ and IL-22 producing CD4+ T cells (n = 5). Means are depicted.

2.4 Discussion

Peripheral blood mononuclear cell (PBMC) are typically used to investigate cellular immune responses. While PBMC-based assays allow cryopreservation of sample for later use, these types of assays require larger volumes of blood samples, as well as extensive processing, that could affect certain cell populations (Kuleshova *et al.*, 2001). Thus, whole blood assays are an attractive alternative, requiring smaller blood volumes, being easy and rapid to process, and maintaining a more physiological environment (Silberer *et al.*, 2008; Deenadayalan *et al.*, 2013; Silva *et al.*, 2013). However, one disadvantage of whole blood assay is that the samples require immediate stimulation, although they can be fixed and stored after stimulation, for later batch staining (as was performed in Chapter 3 and 4). Previous studies have reported differences in cytokine production from PBMC compared to whole blood assays (Silberer *et al.*, 2008; Deenadayalan *et al.*, 2013; Silva *et al.*, 2013). Here, the whole blood assay allowed robust detection of IFN- γ (median: 0.46%) and IL-22 (median: 0.38%)-producing CD4⁺ T cells in response to Mtb stimulation. IFN- γ responses were 5-fold lower in PBMC compared to whole blood but were still detectable. However, we observed extremely low to absent IL-22 production in PBMC (median: 0.05%). The lack of IL-22 production in PBMC could be explained by multiple factors: i) IL-22 producing cells might be lost/impaired during PBMC extraction (Grievink *et al.*, 2016), ii) plasma proteins or Mtb antigen-induced soluble factors from cell types in whole blood not present in PBMC could be necessary to induce IL-22 production and iii) particular antigen-presenting cells and/or costimulatory signals from innate cells may be required to induce Th22 responses. Our laboratory recently demonstrated that adding fluorescently-labeled PBMC to whole blood before Mtb stimulation resulted in the recovery of IL-22 responses in PBMC, indicating that lack of IL-22 responses was not attributed to loss of CD4⁺ T cells producing IL-22 in PBMC (Bunjun *et al.*, submitted). Moreover, we showed here that the supplementation of PBMC culture media with autologous

plasma from Mtb-stimulated blood only modestly improved IL-22 detection from PBMC, suggesting that bystander soluble factors are not sufficient to trigger IL-22 responses (**Figure 2.5**). However, it is also possible that insufficient plasma was used, resulting in a reduced effect.” (**Figure 2.5**). Further studies are required elucidate which physiological environment is needed for optimal IL-22 production.

A range of cell types of the innate and adaptive immune response have been reported to produce IL-22 (Dhiman *et al.*, 2009; Toussiroot *et al.*, 2018; Cella *et al.*, 2019). In our work, we show that conventional ($\alpha\beta$) CD4⁺ T cells are the predominant source of Mtb-specific IL-22. Interestingly, whilst IL-22 responses were readily detectable upon stimulation with complex mycobacterial antigens (*i.e.* Mtb cell lysate, gamma-irradiated Mtb and BCG), Mtb peptides (ESAT-6/CFP-10) did not elicit IL-22 responses, further suggesting that adequate antigen processing and presentation (Magee *et al.*, 2012) and/or cytokine co-stimulation may be necessary to induce IL-22 secretion (Boyman, 2010). A previous study in our laboratory also demonstrated that PPD stimulation results in higher responses than ESAT6 and CFP10 but lower responses than live BCG stimulation (Bunjun *et al.*, under review). It is also possible that non-peptide moieties, only present in complex antigen (such as lipids or lipoproteins), may mediate IL-22 production. Indeed, it has been reported that the production of IL-22 by CD4⁺ T cells may be induced upon CD1a triggering in humans (De Jong *et al.*, 2010; Otsuka *et al.*, 2018). Of note, group 1 (CD1a, b and c) CD1-restricted T cells specific for microbial lipid antigens display similar $\alpha\beta$ TCR to peptide specific T cells (Grant *et al.*, 1999). Our data showing that Mtb-specific IL-22 production is TCR-dependent and that the TCR V β usage in IL-22-producing cell is comparable to IFN- γ -producing cells suggest that conventional (although not necessarily classically-restricted) CD4⁺ T are the source of IL-22. To comprehensively define the activation pathway necessary to induce IL-22 production requires

further experiments. For example, to determine which cell subsets are required to induce the production of IL-22 in response to Mtb, cells such as neutrophils or dendritic cells abundant in whole blood, these cells could be depleted before stimulation or PBMC could be supplemented with these cells, to determine the effect on IL-22 production. To further investigate the restriction of Th22 cells, experiments blocking MHC Class II or CD1 molecules could be conducted.

Overall, the data presented in this chapter highlights novel features regarding Th22 cells compared to Th1 cells. It is possible that Mtb-specific Th22 cells recognize non-peptide or modified peptide antigen, or require a specific activation pathway and other requirements for the detection of these cells *in vitro*, a reason why they have been relatively understudied compared to Th1 and Th17 cells. Understanding these unusual features will be key to studying these cells further, determining the role they may play in TB immunity, and how we may harness them using vaccines or immunotherapy.

CHAPTER 3

Characterization of *Mycobacterium tuberculosis*-specific Th22 cells and the effect of tuberculosis disease and HIV co-infection

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3.1 Introduction

TB is the leading cause of death from an infectious disease worldwide, with 10 million TB cases per year and 1.6 million deaths in 2018 (WHO, 2019). South Africa is one of the eight countries that together account for two thirds of all TB cases globally (WHO, 2019). Although TB is curable, the development of drug-resistant TB is a public health crisis and WHO estimates that 3.5% of new TB cases and 18% of previously treated cases in 2018 had multidrug resistant (MDR)-TB (WHO, 2019). Moreover, approximately one quarter of the world's population is latently infected with *Mycobacterium tuberculosis* (WHO, 2019). In immunocompetent individuals, the risk of progression from infection to active TB disease is 2–10% in a lifetime, illustrating that the human immune system can control Mtb in most cases (O'Garra *et al.*, 2013). Bacillus Calmette-Guérin (BCG) is the only licensed TB vaccine and protects against disseminated TB in children but provides variable protection against highly prevalent pulmonary tuberculosis (PTB) in adults (Fine, 1995; Trunz *et al.*, 2006; Bo *et al.*, 2008). Therefore, there is an urgent need for new and effective TB vaccines, and recent progress in clinical trials is promising (Nemes *et al.*, 2018; Tait *et al.*, 2019).

Understanding the immune responses required to control Mtb will aid in development of improved vaccines against TB (Sakai *et al.*, 2014). It is well established that CD4⁺ T cells, particularly Th1 cells producing the cytokines IFN- γ and TNF- α , are critical for immunity against Mtb (Jasenosky *et al.*, 2015). It is also clear that Th1 immunity alone is not sufficient, as IFN- γ has been reported to be a poor correlate of BCG vaccination-induced protection against tuberculosis in mice (Majlessi *et al.*, 2006; Mittrücker *et al.*, 2007). Furthermore, IFN- γ -independent mechanisms of CD4⁺ T cell mediated control of Mtb infection have been documented (Wozniak *et al.*, 2010b; Gallegos *et al.*, 2011; Lu *et al.*, 2019). Thus, other CD4⁺ T helper subsets beyond Th1 cells may be essential for protection against TB.

There is growing interest in the cytokine IL-22 and its role in TB immunity. IL-22 belongs to the IL-10 family of cytokines and its receptor is composed of two heterodimeric subunits, IL-22R1 and IL-10R2 (Logsdon *et al.*, 2004). IL-22 mainly targets non-hematopoietic cells, namely epithelial cells and fibroblasts in tissues (Dudakov *et al.*, 2015), but expression of the IL-22 receptor has also been reported to be upregulated on macrophages (Dhiman *et al.*, 2009; Treerat *et al.*, 2017). IL-22 was initially reported to be dispensable for Mtb control in mouse models (Wilson *et al.*, 2010; Behrends *et al.*, 2013). Recently, however, a protective role for IL-22 in TB immunity was described in a murine model, where IL-22 deficient mice displayed greater bacterial burdens after aerosol infection with a virulent clinical strain of Mtb, HN878 (Treerat *et al.*, 2017). In humans, soluble IL-22 has been detected at sites of extra-pulmonary tuberculosis (Matthews *et al.*, 2011a), and a higher concentration of IL-22 was observed in bronchoalveolar lavage fluid (BALF) of individuals with active TB compared to healthy donors (Scriba *et al.*, 2008). Moreover, IL-22 has been shown to inhibit intracellular *M. tuberculosis* growth in macrophages (Dhiman *et al.*, 2009), and a polymorphism in the IL-22 promoter has been linked to TB susceptibility (Zhang *et al.*, 2011).

IL-22 is produced by a variety of cells, including T cells (Th17, Th1 and $\gamma\delta$ T cells) and a range of innate cells (ILCs and NK cells) (Dhiman *et al.*, 2009; Witte *et al.*, 2010; Toussiro *et al.*, 2018; Cella *et al.*, 2019). In humans, IL-22 is mainly produced by a distinct subset of CD4⁺ T cells, named Th22 cells (Duhon *et al.*, 2009; Eyerich *et al.*, 2009; Trifari *et al.*, 2009). Our laboratory recently showed that IL-22-producing CD4⁺ T cells contribute a substantial portion to the mycobacterial response during latent TB infection (LTBI; Bunjun *et al.*, submitted). These mycobacteria-specific Th22 cells were depleted during HIV infection to a similar extent as Th1 cells, emphasizing their potential importance in protective immunity to TB. In this study, we wished to further characterize Mtb-specific Th22 cells during TB disease and HIV

co-infection. Our aims were to investigate the contribution of Th22 cells to TB immune responses, characterize Th22 cells further to gain insights into their function, and examine the impact of TB disease and HIV infection on Th22 cells, in comparison to Th1 and Th17 cells. Our findings confirm that Mtb-specific IL-22 is produced by Th22 cells and makes up a substantial contribution to TB immune responses. We demonstrate for the first time that IL-22 production is dependent on TCR engagement but may require alternative co-stimulatory molecules or antigen presenting cells. Strikingly, Mtb-specific Th22 cells were severely diminished in patients with both HIV infection and TB disease.

3.2 Material and methods

3.2.1 Study participants

Blood samples were collected from 72 individuals recruited from the Ubuntu Clinic, Khayelitsha, Cape Town, South Africa. Participants were classified into four groups according to their HIV-1 and TB status, being LTBI or having newly-diagnosed active TB disease (aTB): HIV-/LTBI (n = 19), HIV+/LTBI (n = 18), HIV-/aTB (n = 19), and HIV+/aTB (n = 16). The clinical characteristics for each group are presented in **Table 3.1** and **Figure 3.1**. LTBI was diagnosed based on a positive IFN- γ release assay (QuantiFERON®-TB Gold In-Tube), no symptoms of aTB and no detection of Mtb in sputum by GeneXpert. Diagnosis of aTB was based on clinical symptoms and positive sputum test. All HIV-infected individuals were antiretroviral treatment (ART) naïve and all TB cases were drug sensitive and TB treatment-naïve at the time of enrolment. This study was approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC 051/2018).

Table 3.1: Clinical characteristics of the study groups.

	LTBI		Active TB	
	HIV-	HIV+	HIV-	HIV+
<i>n</i>	19	18	19	16
CD4 count (cells/mm ³) ^a	923 [687-1051]	547 [463-626]	648 [593-778]	153 [75-207]
Log ₁₀ HIV viral load (RNA copies/ml) ^a	n.a	4.61 [3.72-4.97]	n.a	5.06 [4.68-5.54]
Age ^a	25 [19-31]	33 [29-38]	31 [26-40]	34 [29-46]
Sex (F/M)	9/10	16/2	2/17	9/7

^aInterquartile ranges are indicated in brackets. F: female; M: male.

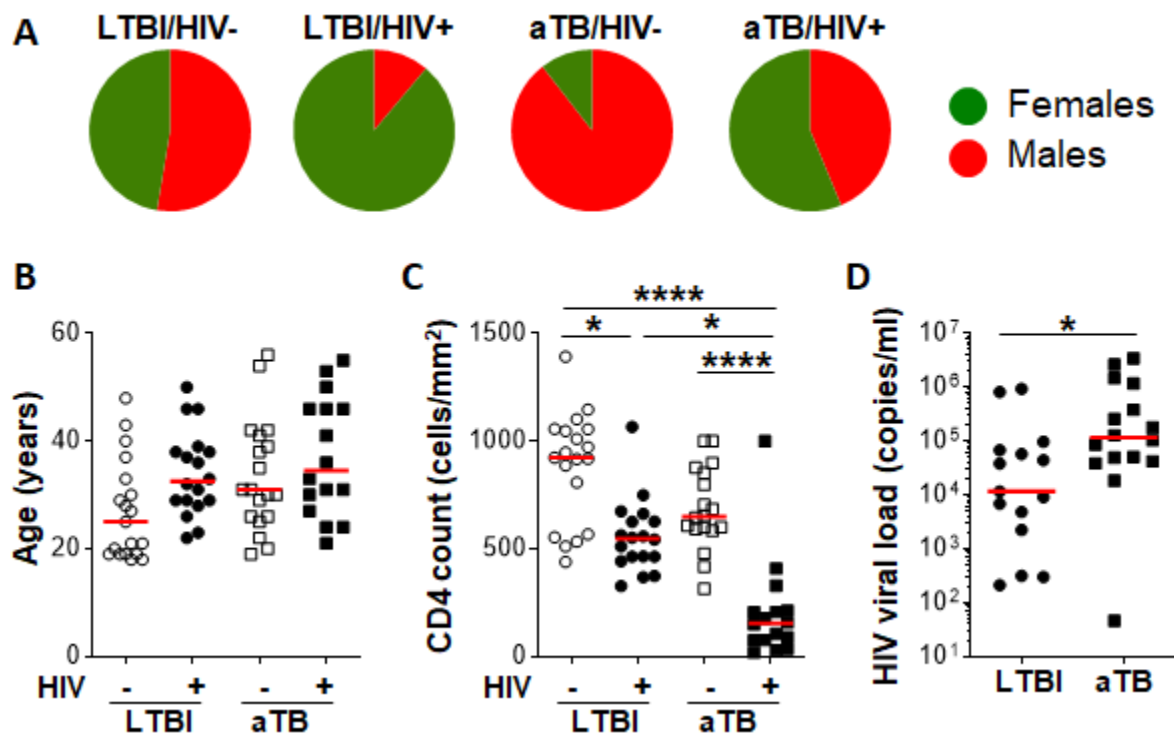


Figure 3.1: Clinical characteristics of the study groups. (A) Pie charts showing sex distribution in each clinical group. Summary graph of (B) the age (C) CD4 count in LTBI/HIV- (n=19), aTB/HIV- (n=18), LTBI/HIV+ (n=19), and aTB/HIV+ (n=16) and (D) HIV viral load in LTBI and aTB individuals. Medians are depicted. Statistical comparisons were performed using a one-way ANOVA Kruskal-Wallis Dunn's multiple comparisons test. * $p < 0.05$, **** $p < 0.0001$.

3.2.2 Blood collection and whole blood stimulation

Blood was collected and stimulated as described in **Section 2.2.4** in the previous chapter. Mtb whole cell lysate (strain H37Rv, 20 µg/ml, BEI Resources) was used to stimulate 0.5 ml of heparinized whole blood.

3.2.3 Flow Cytometry

Cryopreserved cells were thawed, washed and then permeabilized with Perm/Wash buffer (BD Biosciences). Cells were incubated at 4°C for 1 h with the following antibodies: CD19 Pacific Blue (6D5; Biolegend), CD14 Pacific Blue (M5E2; Biolegend), CD3 BV650 (OKT3; BD Biosciences), CD4 PerCP-Cy5.5 (L200; BD Biosciences), CD45RA BV570 (HI100; Biolegend), CD27 PE-Cy5 (1A4CD27; Beckman Coulter), CCR7 PE-CF594 (3D12; BD Biosciences), HLA-DR APC-Cy7 (L243; BD Biosciences), KLRG1 PE-vio770 (REA261; Miltenyi Biotec), CD26 FITC (M-A261; BD Biosciences), IFN- γ Alexa700 (B27; BD Biosciences), IL-17 Alexa 647 (N49-653; BD Biosciences), IL-22 PE (22URTI; e-Bioscience). Stained cells were acquired on a BD Fortessa and analyzed using FlowJo (v10, TreeStar). A positive cytokine response was defined as at least twice the background (i.e. unstimulated cells) and more than 10 events. To define the phenotype of cytokines-producing cells, a cutoff of 20 events was used. These cut-off values were determined empirically and have been shown to be reliable in other studies (Strickland *et al.*, 2017; Riou *et al.*, 2017). The gating strategy is illustrated in **Figure 3.2**.

3.2.4 Statistical analysis

Statistical tests were performed as described in **Section 2.2.6**.

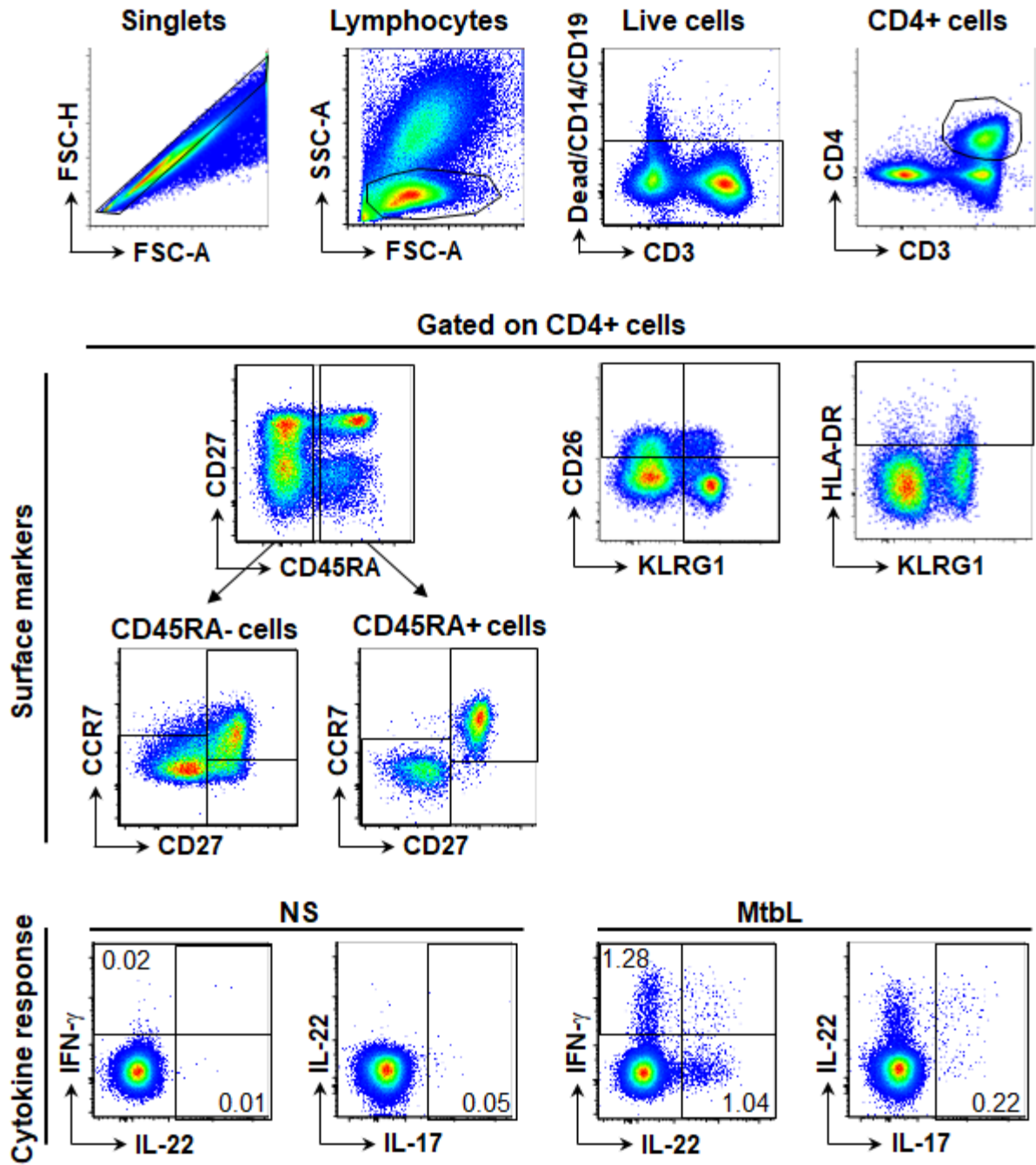


Figure 3.2: Gating strategy. Gating strategy used to determine the phenotype, memory profile and functional properties of CD4+ T cells. NS: no stimulation, MtbL: *Mycobacterium tuberculosis* lysate.

3.3 Results

3.3.1 Characterization of CD4 Th22 cells in the immune response to Mtb in

HIV-/LTBI individuals

A subset of CD4⁺ T cells that produces the cytokine IL-22 in response to mycobacterial antigen stimulation has been described (Scriba *et al.*, 2008). We sought to further characterize Mtb-specific IL-22 production and define the relative contribution of IL-22 compared to more classically measured responses, namely IFN- γ and IL-17. We first compared the magnitude of Mtb-specific IL-22 producing CD4⁺ T cells to IFN- γ and IL-17 responses, in whole blood from healthy individuals with latent Mtb infection (n = 19; **Figure 3.3A**). Mtb-specific CD4⁺ T cell responses (producing any of the measured cytokines) were detected in all individuals (median: 1.34%, interquartile range (IQR): 0.97-2.52) (**Figure 3.3B**). The highest frequency was observed for IFN- γ responses (median: 0.93%, IQR: 0.40-1.60). Although lower, the magnitude of IL-22⁺ Mtb-specific CD4⁺ T cells was not significantly different to the IFN- γ response (median: 0.46%, IQR: 0.22-0.96). In contrast, IL-17 producing CD4⁺ T cells were detectable at much lower frequencies (median: 0.06%, IQR: 0.04-0.11), ~16 to ~8 fold lower than IFN- γ (p < 0.0001) and IL-22 (p = 0.0002) responses, respectively (**Figure 3.3B**). Using a Boolean gating strategy, we next assessed all possible cytokine combinations to determine the co-expression profile of IFN- γ , IL-22 and IL-17 in Mtb-specific CD4⁺ T cells. **Figure 3.3C** shows that the single IFN- γ response accounted for a median of 60% of the total Mtb response, and single IL-22-producing cells contributed a median of 37% to Mtb response. Cytokine co-expression was marginal, with only a small proportion of IFN- γ /IL-22 co-expressing cells observed (median: 3.6%, IQR: 2-6). Only three out of 19 individuals displayed Mtb-specific CD4⁺ T cell responses that produced IL-17 alone that contributed more than 5% to the total Mtb response (**Figure 3.3C**). These results show that IL-22 contributes a substantial portion to the Mtb response, and these cells are a subset distinct from Th1 or Th17 cells.

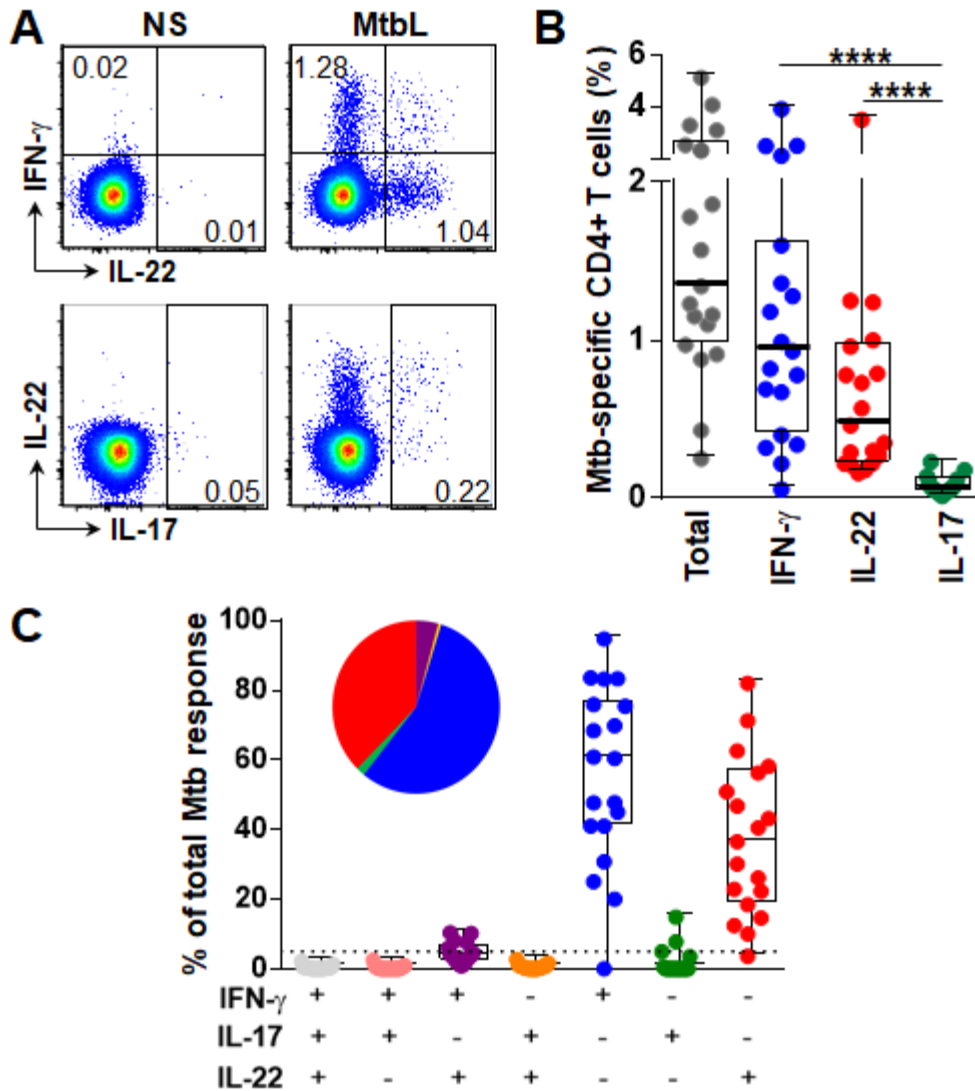


Figure 3.3: Contribution of IFN- γ , IL-22 and IL-17 to Mtb-specific CD4+ T cell responses in LTBI individuals. (A) Representative flow cytometry plots of IFN- γ , IL-22 and IL-17 production after stimulation with MtbL. NS: no stimulation. (B) Summary graph of the frequency of cytokine responses. Statistical comparisons were performed using a one-way ANOVA Kruskal-Wallis Dunn's multiple comparisons test. **** $p < 0.0001$. (C) Proportion of different combinations of IFN- γ , IL-22 and IL-17 in response to MtbL stimulation. Medians and interquartile ranges are depicted. The pie chart above the graph corresponds to the matching colour of the cytokine combination.

3.3.2 Mtb-specific Th22 cells exhibit distinct memory and activation profiles compared to Th1 and Th17 cells

To further describe the phenotypic characteristics of Mtb-specific Th22 cells and compare them

to those of Th1 and Th17 subsets, we compared their memory (CD45RA, CD27 and CCR7), activation profile (HLA-DR) and homing potential using Killer cell lectin-like receptor G1 (KLRG1) and Dipeptidyl peptidase IV (CD26) in healthy individuals with latent Mtb infection. During Mtb infection, KLRG1-expressing cells appear to be retained within lung blood vasculature, while KLRG1- cells migrate to the lung parenchyma (Sakai *et al.*, 2014). CD26 is involved in enzymatic chemokine modification that enhances T cell migration (Iwata *et al.*, 1999; Ikushima *et al.*, 2002). We focused our analysis on IFN- γ single producing cells (Th1), IL-22 single producing cells (Th22) and IL-17 single producing cells (Th17), since the proportion of cytokine co-expressing cells was negligible (**Figure 3.3C**).

The measurement of CD45RA, CD27 and CCR7 enabled the detection of five distinct memory subsets, namely naïve (CD45RA+CD27+CCR7+), central memory (CM: CD45RA-CD27+CCR7+), transitional memory (TM: CD45RA-CD27+CCR7-), effector memory (EM: CD45RA+CD27-CCR7-), and effector cells (Eff: CD45RA-CD27-CCR7-) (**Figure 3.4A**). The memory profile of Mtb-specific CD4+ T cells varied depending on their Th polarization. Th1 cells were significantly enriched in differentiated EM cells compared to Th22 and Th17 cells (median: 50%, 32% and 19%, respectively). The majority of Th22 cells were evenly distributed between the CM and EM subset (33 and 32%, respectively). The Th22 subset was also characterized by a low proportion of cells exhibiting a TM phenotype compared to the Th1 or Th17 subsets (median: 6%, 17%, 18%, respectively; **Figure 3.4B**).

When assessing the activation profile of Mtb-specific CD4+ Th subsets (**Figure 3.5A**), we observed that Th22 cells were characterized by a significantly lower expression of HLA-DR compared to both Th1 and Th17 cells (median 1.1%, compared to 7.3% and 9.3%, respectively; **Figure 3.5B**). Similarly, KLRG1 expression was nearly absent on Th22 cells compared to Th1

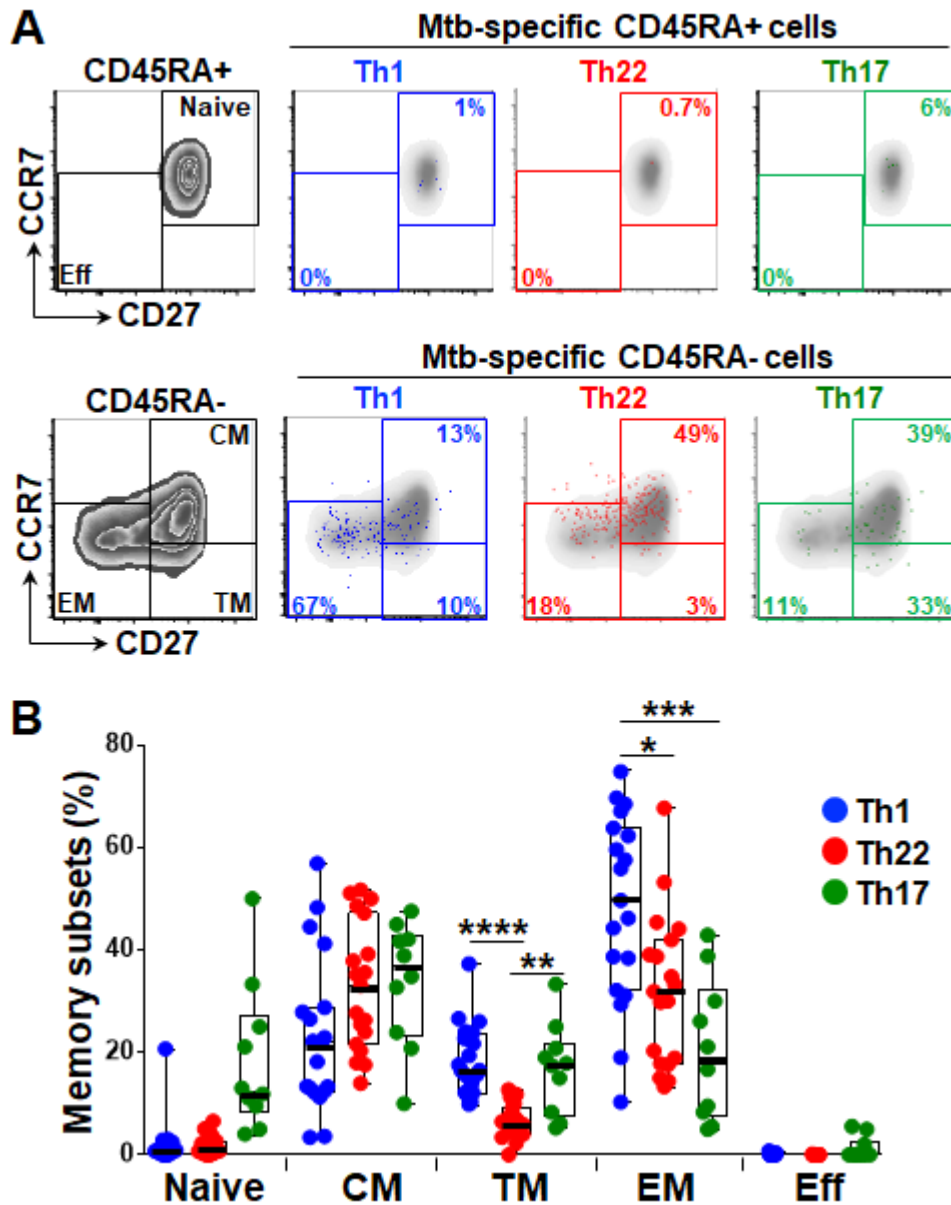


Figure 3.4: Comparison of the memory differentiation profiles of Mtb-specific Th1, Th22 and Th17 cells in LTBI individuals. (A) Representative overlay plots showing memory subsets in total CD4+ T cells (grey), IFN- γ + (blue), IL-22+ (red) and IL-17+ (green) cells in response to MtbL. (Naive: CD45RA+CD27+CCR7+; central memory (CM): CD45RA-CD27+CCR7+; transitional memory (TM): CD45RA-CD27+CCR7-, effector memory (EM): CD45RA-CD27-CCR7- and effector cells (Eff): CD45RA+CD27-CCR7-). (B) Summary graph depicting medians and interquartile ranges of the memory phenotype of Mtb-specific Th1, Th22 and Th17 cells. Only cytokine responses of more than 20 events were analyzed. Each dot represents an LTBI individual. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

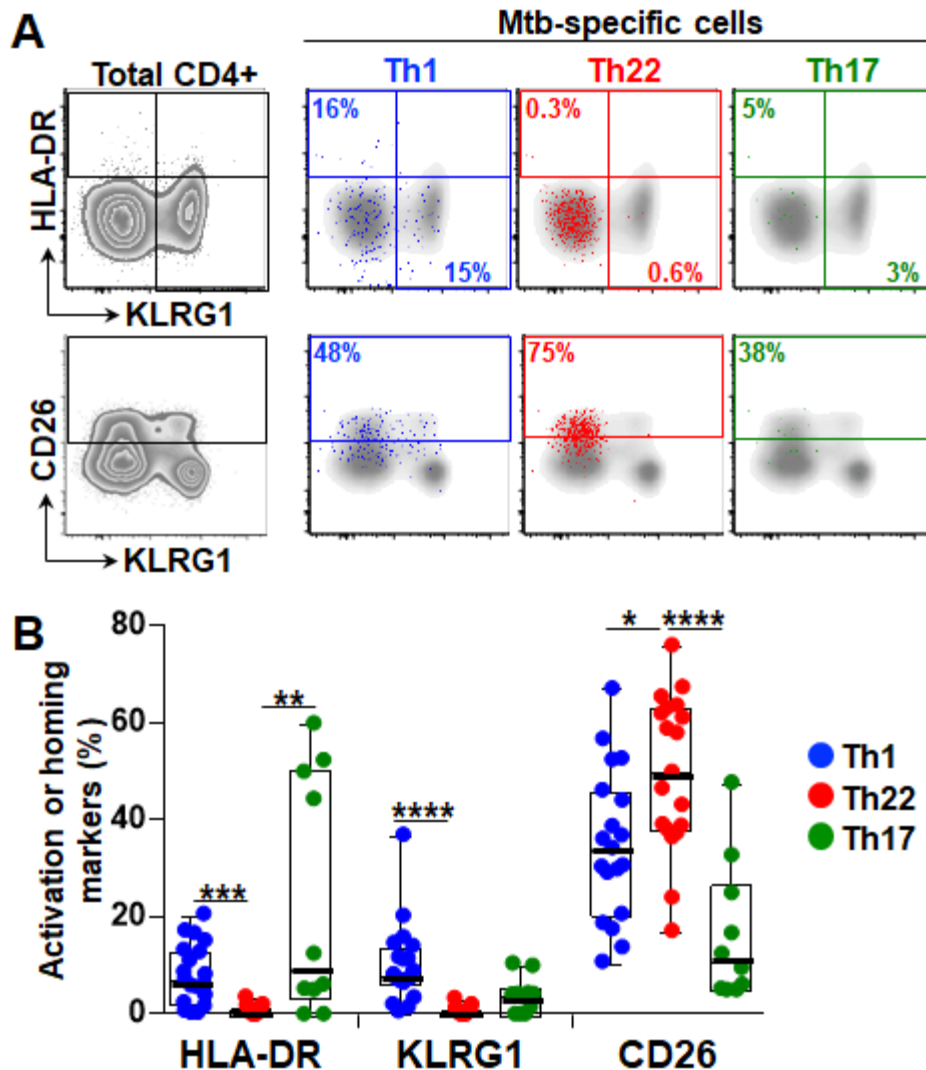


Figure 3.5: Comparison of the activation profiles of Mtb-specific Th1, Th22 and Th17 cells in LTBI individuals. (A) Representative overlay plots showing HLA-DR, KLRG1 and CD26 expression in total CD4+ T cells (grey), IFN- γ + (blue), IL-22+ (red) and IL-17+ (green) cells in response to MtbL. (B) Summary graph depicting medians and interquartile ranges of the expression of HLA-DR, KLRG1 and CD26 on Mtb-specific Th1, Th22 and Th17 cells. Only cytokine responses of more than 20 events were analyzed. Each dot represents an LTBI individual. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

and Th17 cells (0.6%, 8.2% and 4.1%; respectively) (Figure 3.5B). In contrast, 50% of Th22 cells expressed CD26, compared to 34% of Th1 cells and 11% of Th17 cells ($p = 0.048$ and $p < 0.0001$, respectively) (Figure 3.5B). Overall, these results indicate that Mtb-specific Th22 cells exhibit distinct memory, activation profiles and homing potential compared to Th1 and Th17 cells.

3.3.3 HIV infection and TB disease alters the distribution of Mtb-specific CD4+ Th subsets

To define the impact of HIV infection and TB disease on the distribution of Mtb-specific Th subsets, we next compared the magnitude of Th1, Th22 and Th17 cells in 72 participants classified into four groups according to their HIV-1 and TB status: HIV-/LTBI ($n = 19$), HIV+/LTBI ($n = 18$), HIV-/aTB ($n = 19$), and HIV+/aTB ($n = 16$). The clinical characteristics of each group are summarized in **Table 3.1** and **Figure 3.1**. In participants with LTBI, HIV infection led to a significant reduction (median ~3.6 fold) in the frequency of Mtb-specific Th1 cells (median: 0.23% for and 0.84% for HIV-infected and HIV-uninfected, respectively; $p = 0.026$; **Figure 3.6A**). While not statistically significant, the magnitude of the Mtb-specific Th22 response also decreased in HIV-infected participants compared to HIV-uninfected subjects (median: 0.18% and 0.45%, respectively). When assessing the effect of TB disease, we found that whilst TB disease did not significantly alter the magnitude of Th1 or Th22 responses in HIV-uninfected individuals, in HIV-infected persons the Mtb-specific Th profile was markedly distorted during TB compared to LTBI (**Figure 3.6A**). The magnitude of Th1 responses was significantly higher in aTB/HIV+ individuals compared to LTBI/HIV+ participants (median: 1.21% vs 0.23%, respectively, $p = 0.0005$). In contrast, Mtb-specific Th22 cells were significantly lower in aTB/HIV+ compared to LTBI/HIV+ (median: 0.08% vs 0.23%, $p < 0.0460$). No differences were observed for Mtb-specific Th17 cells in all four groups, which were of consistently low magnitude.

To take into account the significant variation in absolute CD4+ T cell counts among participants (**Figure 3.1**), the absolute number of Mtb-specific CD4+ T cells was calculated

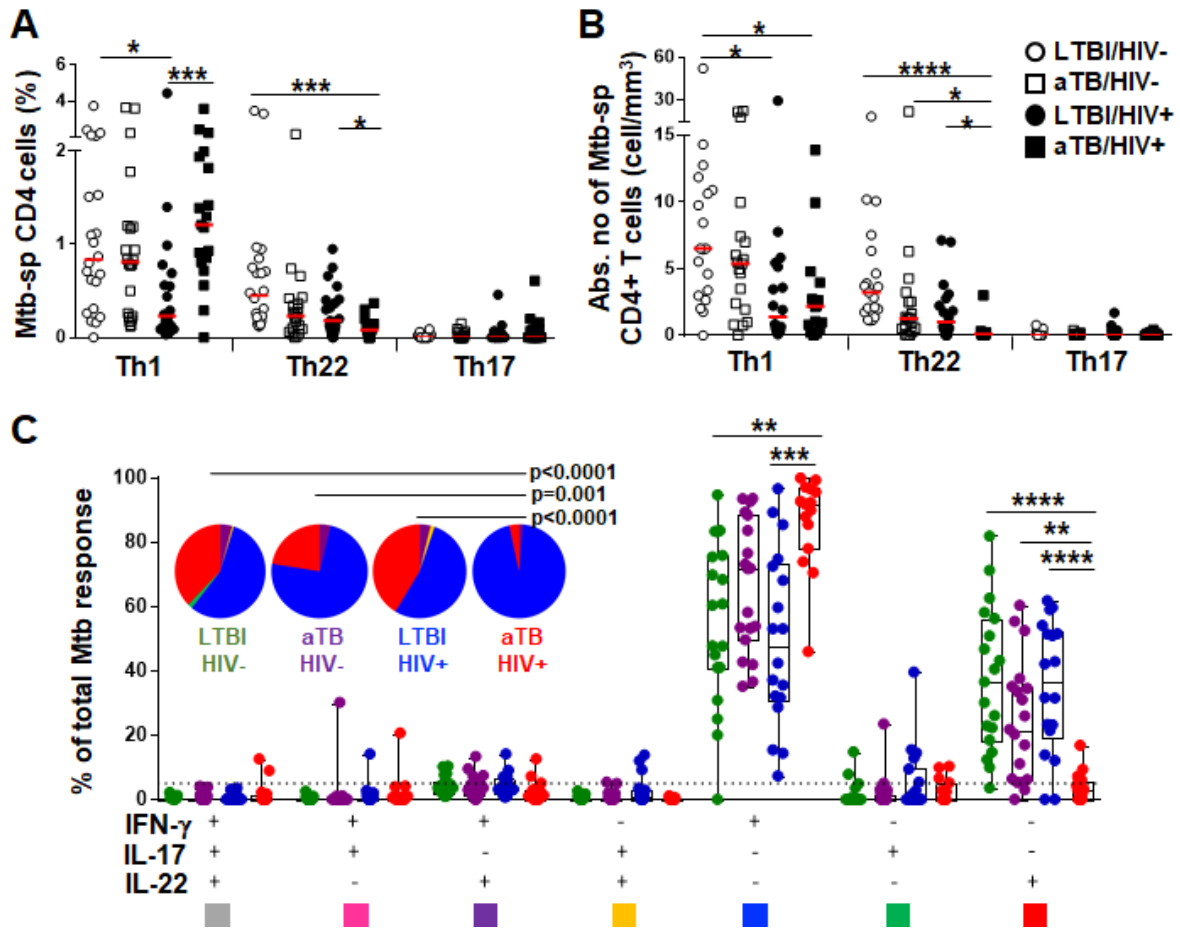


Figure 3.6: Comparison of the frequency and functional profile of Mtb-responding CD4+ T cells in LTBI/HIV- (n=19), aTB/HIV- (n=18), LTBI/HIV+ (n=19), and aTB/HIV+ (n=16). (A) Frequency and (B) Absolute number of Th1, Th22 and Th17 responses to MtbL. Red bars represent the medians. (C) Distribution of combinations of Mtb-specific cytokine responses as a proportion of the total cytokine response. Each section of the pie chart represents median proportions of specific combination of cytokines, as indicated by the color at the bottom of the graph. The median and interquartile range are indicated by the box and whisker plots. The dotted line indicates 5% of the response. Only cytokine responses of more than 20 events were analyzed. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

and compared between each clinical group. As expected, HIV co-infection resulted in reduced Mtb-specific Th1 absolute cell numbers regardless of TB status (median: 6.5 vs 1.4 cells/mm³ in LTBI and 5.4 vs 2.2 cells/mm³ in aTB; **Figure 3.6B**). A comparable profile was observed for Th22 cells, and due to the decreased frequency of Th22 cells in aTB/HIV+ compared to LTBI/HIV+, combined with low CD4 counts in the former group, the absolute number of

circulating Mtb-specific Th22 cells was markedly reduced in aTB/HIV+ individuals (median: 0.09 cells/mm³ in aTB, compared to 1.02 cells/mm³ in LTBI; **Figure 3.6B**). Of note, for HIV-infected individuals, we found no relationship between the frequency of any of the Th cytokine responses with absolute CD4 count or HIV viral load, regardless of TB status (**Figure S1**).

Next, we examined whether HIV infection and/or active TB alters the relative contribution of Th subsets to the total Mtb response. Whilst there were no significant differences in the contribution of Th1 and Th22 cells to the Mtb response in LTBI/HIV-, LTBI/HIV+ and aTB/HIV- groups (medians for Th1: 60%, 48% and 72%; and medians for Th22: 37%, 37% and 22%, respectively), in the aTB/HIV+ group, the Mtb-specific response consisted almost exclusively of Th1 cells (>90%), with Th22 cells representing less than 5% of the total Mtb response (**Figure 3.6C**).

3.3.4 TB disease and HIV co-infection differentially influence memory and activation profiles of Mtb-specific CD4+ Th subsets

Finally, we performed phenotypic characterization of Mtb-specific CD4+ Th subsets during HIV infection and TB disease. Comparing first the memory differentiation phenotype of Th subsets, we showed that active bacterial replication, irrespective of HIV co-infection, promotes the differentiation of Mtb-specific Th1 cells. The proportion of Th1 cells exhibiting an EM phenotype was significantly higher in aTB compare to LTBI (69% vs 50% for HIV-, $p = 0.0295$ and 77% vs 45% for HIV+, $p = 0.0004$; **Figure 3.7A**). In contrast, no alteration of the memory profile of Th22 or Th17 cells was observed in aTB (**Figure 3.7B&C**). Interestingly, Mtb-specific CD4+ Th subsets displayed a consistently different memory phenotype in all clinical groups, with Th1 cells displaying more differentiated (EM) compared to Th22 and Th17 cells (**Figure 3.8**). In contrast, Th22 and Th17 cells appeared more evenly distributed in the CM and EM subsets, regardless of clinical group (**Figure 3.7B&C**).

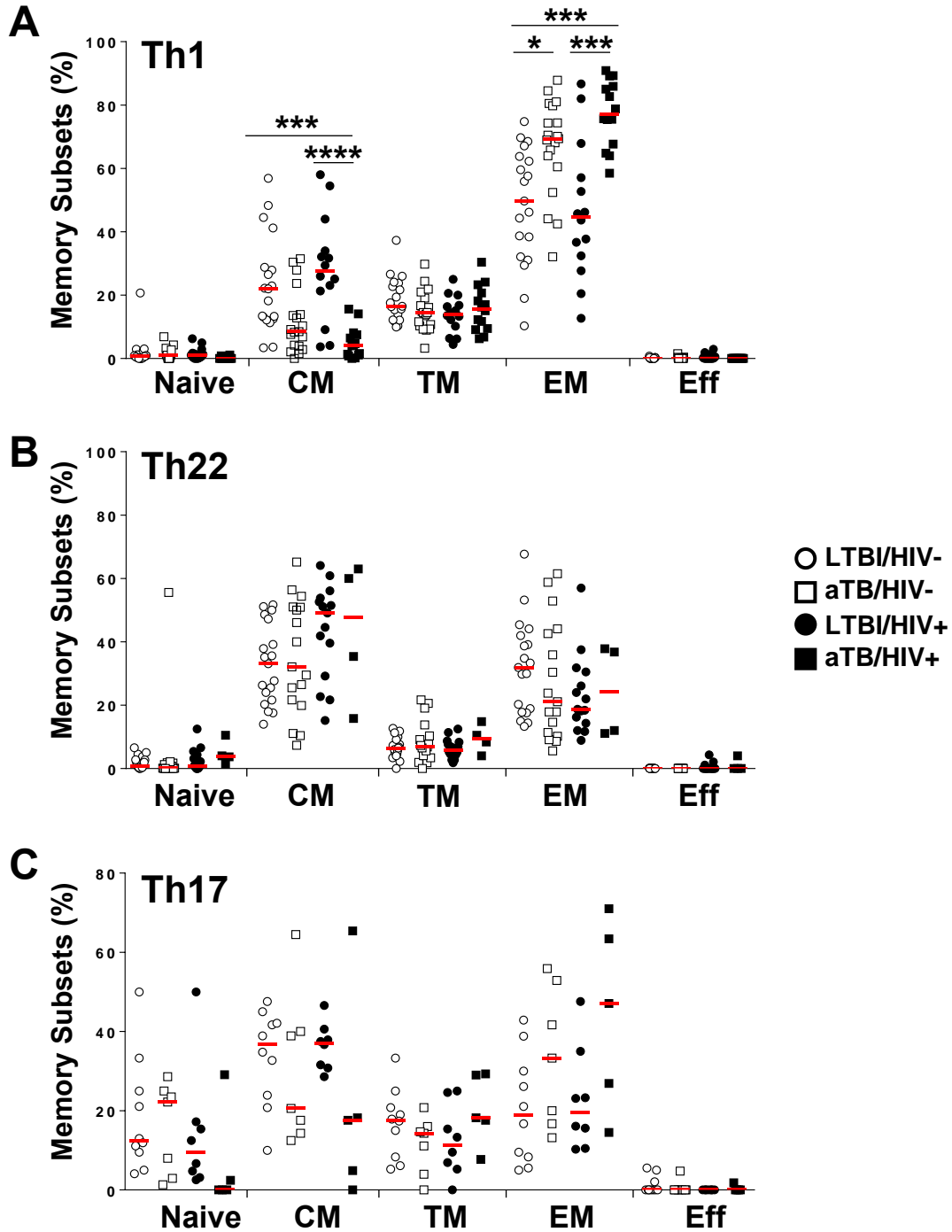


Figure 3.7: Memory phenotypes of Mtb-specific CD4⁺ T cells in individuals with distinct HIV and tuberculosis (TB) disease states. Memory phenotype of (A) Th1, (B) Th22 and (C) Th17 cells in LTBI/HIV⁻, aTB/HIV⁻, LTBI/HIV⁺ and aTB/HIV⁺. Red bars represent the median. Only cytokine responses of more than 20 events were analyzed. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

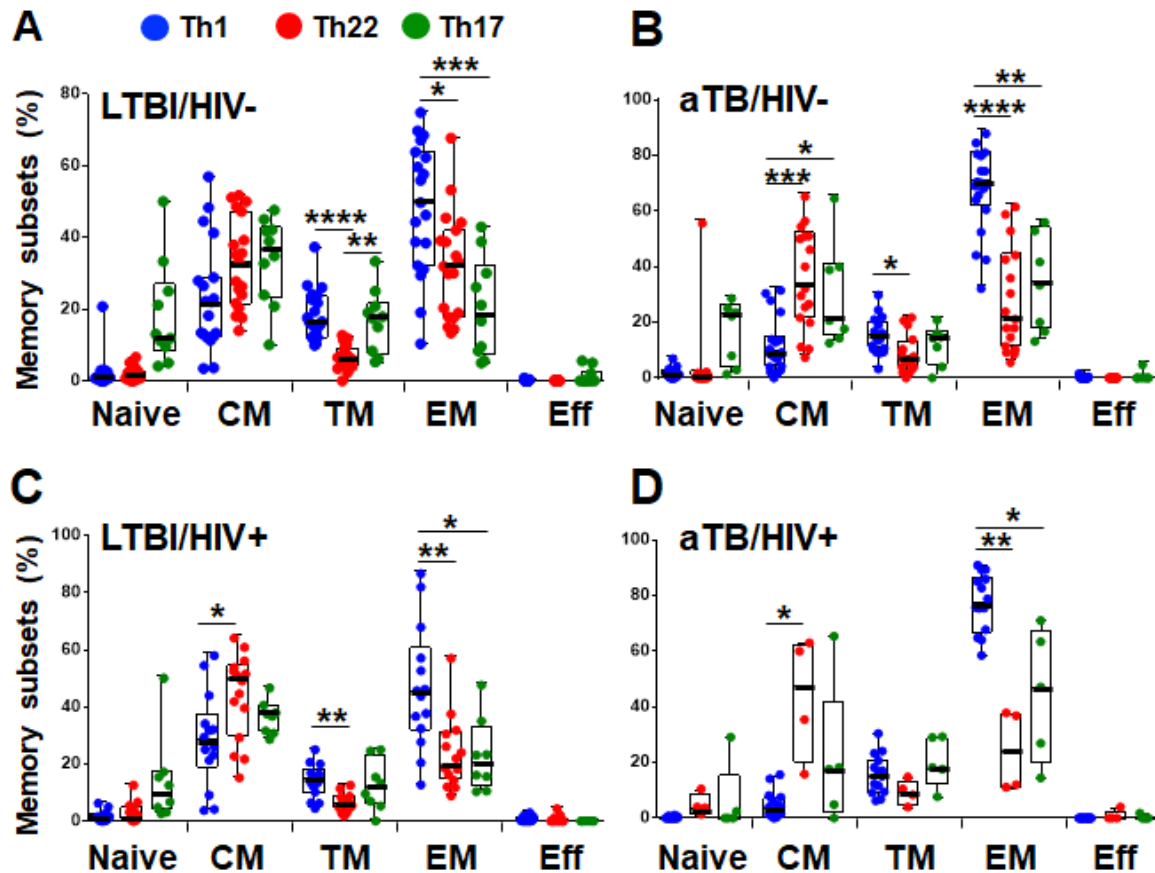


Figure 3.8: Comparison of the memory differentiation profiles of Mtb-specific Th1, Th22 and Th17 cells and the effect of HIV infection and active TB disease. Summary graph of (A) LTBI/HIV-, (B) aTB/HIV-, (C) LTBI/HIV+, and (D) aTB/HIV+ individuals. Box and whisker plots indicate the median and interquartile range of the phenotype of the different Th subsets. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * < 0.05 ** < 0.01, *** < 0.001, **** < 0.0001.

We next assessed the effect of HIV infection and aTB on the activation and homing profiles of Mtb-specific CD4+ Th subsets. As previously described (Riou *et al.*, 2017), irrespective of HIV infection, Mtb-specific Th1 cells in aTB were characterized by significantly higher expression of HLA-DR when compared to persons with LTBI (median: 45% vs 7%, respectively for HIV-, $p = 0.0004$ and 60% vs 8% for HIV+, $p = 0.0009$) (Figure 3.9A). For Th22 cells, the frequency of HLA-DR-expressing cells was significantly higher in HIV-associated LTBI and aTB (Figure 3.9B). However, this increase in HLA-DR expression in

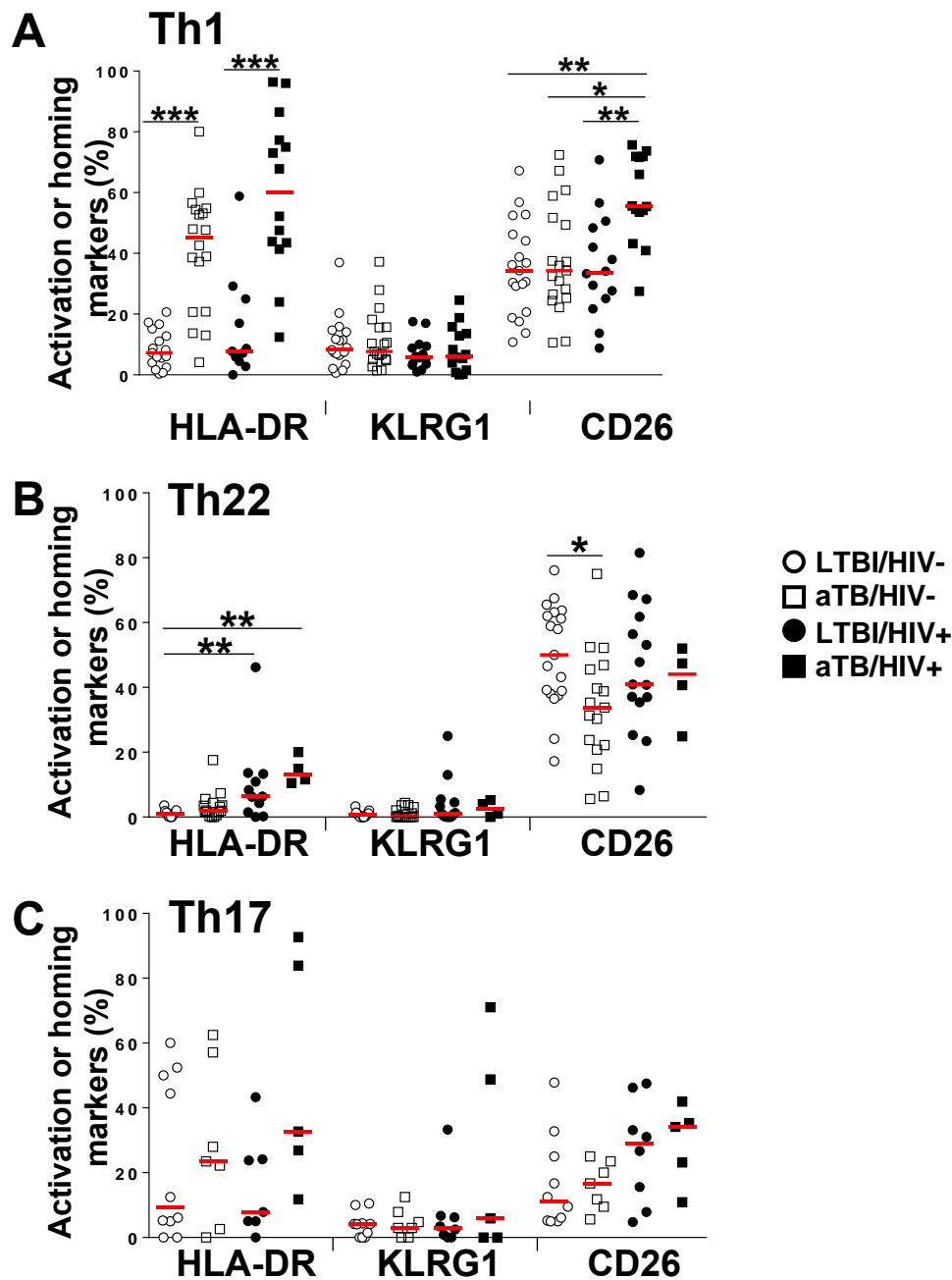


Figure 3.9: Activation phenotypes of Mtb-specific Th1, Th22 and Th17 cells in individuals with distinct HIV and tuberculosis (TB) disease states. Activation phenotype of (A) Th1, (B) Th22 and (C) Th17 cells in LTBI/HIV⁻, aTB/HIV⁻, LTBI/HIV⁺ and aTB/HIV⁺. Red bars represent the median. Only cytokine responses of more than 20 events were analyzed. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Th22 cells observed during HIV infection was most likely due to increased activation in total CD4⁺ T cells caused by HIV infection, as there was a strong correlation between HLA-DR expression in Th22 cells and total CD4⁺ cells (**Figure S1**). One interpretation of Th22 cells not expressing HLA-DR would be that Th22 responses are not specific for Mtb, however, TCR blocking data from Chapter 2 demonstrate that these responses are indeed specific. Of note, there were lower frequencies of Th22 cells expressing HLA-DR compared to both Th1 and Th17 cells in all clinical groups (**Figure 3.10**). Similar to Th22 cells, no differences were observed in HLA-DR expression in Th17 cells during aTB (**Figure 3.9C**).

We did not observe any differences in the homing potential of Mtb-specific CD4⁺ Th subsets based on KLRG1 expression during HIV infection and/or TB disease (**Figure 3.9A, B&C**). In contrast, CD26 expression on Th1 cells was higher in aTB/HIV⁺ (median: 55%) compared to aTB/HIV⁻ (median: 34%, $p = 0.0112$), LTBI/HIV⁺ (median: 34%, $p = 0.0083$), and LTBI/HIV⁻ (median: 34%, $p = 0.0037$) (**Figure 3.9A**). On Th22 cells, expression of CD26 was lower in aTB/HIV⁻ individuals compared to LTBI/HIV⁻ (median: 41% and 51%, respectively; $p = 0.0373$) (**Figure 3.9B**). We did not observe any differences in homing potential for Th17 responses (**Figure 3.9C**), which is likely due to the infrequent Th17 responses detected. CD4⁺ T helper cells also displayed differences in CD26 and KLRG1 expression during aTB and HIV infection (**Figure 3.10**).

These data confirm that active Mtb replication causes memory differentiation of Mtb-specific Th1 cells. Consequently, Th1 cells are highly activated during active TB, irrespective of HIV infection. In contrast, TB disease does not alter memory or activation phenotype profile of Mtb-specific Th22 cells but may affect their homing potential. Indicating that TB differentially modulate Mtb-specific Th1 and Th22 subsets.

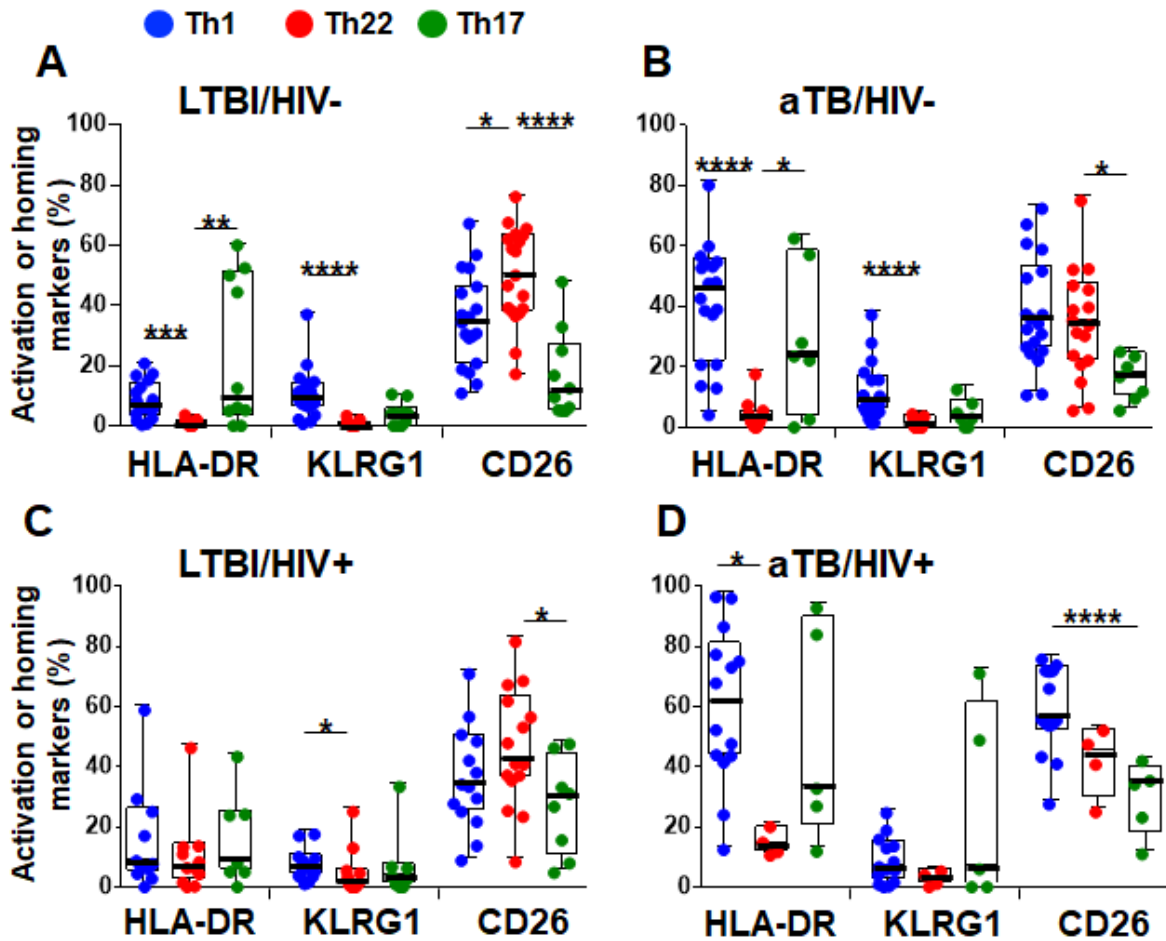


Figure 3.10: Comparison of the activation and homing profiles of Mtb-specific Th1, Th22 and Th17 cells and the effect of HIV infection and active TB disease. Summary graph in (A) LTBI/HIV-, (B) aTB/HIV-, (C) LTBI/HIV+, and (D) aTB/HIV+ individuals. Box and whisker plots indicate the median and interquartile range of the phenotype of the different Th subsets. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * < 0.05 ** < 0.01, *** < 0.001, **** < 0.0001.

3.4 Discussion

The importance of CD4⁺ Th1 (IFN- γ) and Th17 (IL-17) responses in protective immunity to Mtb is well established (Jasenosky *et al.*, 2015; Shen and Chen, 2018). The emerging role of IL-22 and Th22 cells in TB immunity is less well studied, and we sought to address this knowledge gap. To better understand the contribution of Th22 cells to Mtb immune responses, we examined the dynamics of this subset in TB disease, and the effect of HIV co-infection, by comparing the magnitude, differentiation and activation profiles of CD4⁺ T cells producing IFN- γ , IL-22 and IL-17. We extend our previous observations from latent Mtb infection, where we demonstrated that IL-22 contributes substantially to the total CD4⁺ T cell response to Mtb, to show for the first time that Th22 cells displayed distinct memory and activation profiles compared to Th1 and Th17 cells, and that Mtb-specific Th22 were severely depleted in the context of HIV infection and TB disease.

Recently, we reported that Th22 cells constituted up to 50% of mycobacterial responses in Mtb-exposed individuals (Bunjun *et al.*, submitted), in line with previous studies (Scriba *et al.*, 2008). In the current study, we confirmed that IL-22 contributes a substantial portion to Mtb immune response, with the magnitude of Th22 responses to Mtb at a median of 37% of the total Mtb response measured, with Th1 cells contributing 60% and Th17 cells less than 5% to the response. These data demonstrate that Th22 cells make up a large portion of the immune responses to TB and it remains to be shown whether Th22 cells play a role in TB immunity. Indeed, IL-22 was found to play a protective role against hypervirulent Mtb clinical strain HN878 (Treerat *et al.*, 2017). This and other studies also demonstrated that IL-22 receptors are expressed on macrophages within the granuloma, and IL-22 was shown to inhibit Mtb growth directly through induction of TNF- α to activate macrophages or by enhancing phagolysosomal fusion in Mtb infected macrophages through calgranulin A expression (Dhiman *et al.*, 2009;

2012; Treerat *et al.*, 2017). IL-22 also acts on lung epithelial cells to induce antimicrobial proteins or recruits other immune cells (Liang *et al.*, 2006; Treerat *et al.*, 2017). Further studies are required to elucidate how IL-22 contributes to Mtb control.

IL-22 was thought to be a Th17 cytokine due to co-expression with IL-17 in mouse studies and shared function between the two cytokines (Liang *et al.*, 2006). Consistent with previous studies, our co-expression profile of IFN- γ , IL-22 and IL-17 confirmed that IL-22 is mostly produced independently of IFN- γ and IL-17 (Duhon *et al.*, 2009; Trifari *et al.*, 2009). This implies that IFN- γ , IL-22 and IL-17 might play different roles during an immune response (Ouyang *et al.*, 2008). Indeed, IL-22 was required for Mtb control at the chronic stage of Mtb infection, while IL-17 was important in the acute stage of Mtb infection in mice (Gopal *et al.*, 2014; Treerat *et al.*, 2017). Although IL-22 and IL-17 have some overlapping functions, such as induction of antimicrobial peptides, regulating chemokine expression for immune cell recruitment to sites of infection and promoting tissue proliferation, regeneration, and healing (Liang *et al.*, 2006; Gopal *et al.*, 2014; Sabat *et al.*, 2014; Treerat *et al.*, 2017), IL-22 may also mediate mycobacterial control by inducing TNF- α production to activate macrophages (Dhiman *et al.*, 2012; Treerat *et al.*, 2017), a function that has previously been associated with IFN- γ (Flynn *et al.*, 1993).

Consistent with our recent findings (Bunjun *et al.*, submitted), HIV infection resulted in a lower frequency of Th1 and Th22 cells in Mtb-exposed individuals, albeit non-significantly in the present study for Th22 cells. The majority of Th22 cells express CCR6 (Duhon *et al.*, 2009) and CD4⁺ CCR6⁺ cells have been linked to increased permissiveness to HIV infection (Gosselin *et al.*, 2010; Monteiro *et al.*, 2011; Alvarez *et al.*, 2013), are enriched in HIV DNA (Cleret-Buhot *et al.*, 2015; Gosselin *et al.*, 2017), and preferentially support HIV replication

(Planas *et al.*, 2017). Indeed, CD4+CCR6+ cells have been shown to express high levels of HIV co-receptors CCR5 and CXCR4 important for viral entry, and integrin $\alpha 4\beta 7$ which has been associated with increased HIV susceptibility (El Hed *et al.*, 2010; Monteiro *et al.*, 2011; Alvarez *et al.*, 2013; Sivro *et al.*, 2018). Furthermore, CCR6+ cells lack CCR5 ligands which have been shown to induce autocrine protection against HIV (El Hed *et al.*, 2010; Alvarez *et al.*, 2013).

The frequency of mycobacteria-specific Th1 and Th22 cells has also been shown to be lower in individuals with TB compared to healthy individuals (Scriba *et al.*, 2008). Here, we observed no differences in Mtb-specific Th1 response between individuals with latent TB infection and TB patients. However, Mtb-specific Th22 responses were 50% lower in individuals with TB compared to Mtb exposed individuals. Since IL-22-producing cells express CCR6, this may signal that during active TB disease these cells migrate to the lungs from the circulation (Annunziato *et al.*, 2007; Acosta-Rodriguez *et al.*, 2007). Soluble IL-22 is elevated at the site of infection during pulmonary and extra-pulmonary TB (Scriba *et al.*, 2008; Matthews *et al.*, 2011a) and IL-22 producing cells have been detected in the lungs and granulomas of rhesus monkeys with TB (Qiu *et al.*, 2008; Yao *et al.*, 2010). Of note, isoniazid preventive therapy (IPT) resulted in an increase in frequencies of mycobacteria-specific IL-22 producing CD4+ T cells in blood (Suliman, Geldenhuys, John L Johnson, *et al.*, 2016), suggesting a restoration in blood after sterilization of bacteria at the site of infection. Zhang *et al.* reported an increase in Mtb-specific soluble IL-22 in blood of TB patients after TB treatment, which was associated with a decrease in the frequencies of regulatory B cells (CD19+CD5+CD1d+) (Zhang *et al.*, 2014), suggesting that reduction of antigen load in the site by anti-TB drugs leads to restored IL-22 responses in blood. A major, novel finding from our study was that Mtb-specific Th22 cells were barely detectable (0.08%) in individuals with both HIV infection and TB disease.

Based on the reasons stated above, we speculate that in addition to depletion of Mtb-specific CD4⁺ Th22 cells as a result of HIV infection, that during active TB, Mtb-specific Th22 cells migrate out of blood into the lungs, resulting in Th22 cells being diminished in the blood during HIV-TB co-infection.

We confirmed that Th1 cells mostly consist of effector memory subset (50%) while Th22 cells displayed similar distribution between central (33%) and effector (32%) memory subsets. By characterizing memory subsets using CD45RA, CCR7 and CD27 expression, we were able to provide a more detailed definition than previously reported (Bunjun *et al.*, submitted). Central memory cells home to secondary lymphoid organs and have delayed effector function, while effector memory subset possess an immediate effector function (Sallusto *et al.*, 1999). This suggests that Th22 cells are potentially longer lived with the ability to migrate to the site of infection but may possess less rapid effector function compared to Th1 cells. We also observed differences in the activation profiles between Th22, Th1 and Th17 cells. Only 1% of Th22 cells expressed HLA-DR, which was significantly lower than Th1 cells (7%; $p = 0.0006$) and Th17 cells (9%; $p = 0.0026$). This could suggest that other activation markers (e.g. CD25, CD69 or CD26) may be required to detect the activation status of Th22 cells (Rea, McNerlan and Alexander, 1999). Indeed, we observed CD69 and CD25 upregulation on IL-22-producing cells upon Mtb stimulation (Wendoh, Keeton, Burgers, unpublished). Th22 cells did not demonstrate upregulation of HLA-DR even in TB disease, while Th1 cells expressed high levels of HLA-DR in the context of TB disease, irrespective of HIV status (Adekambi *et al.*, 2015; Riou *et al.*, 2017). Indeed, the increased level of activation on Th22 cells observed during HIV infection, unlike Th1, merely mirrored the background activation in due by HIV, demonstrated by a strong correlation with activation on total CD4⁺ T cells (data not shown).

In conclusion, our study builds on previous work that Th22 cells contribute substantially to the immune response to Mtb during both infection and disease, and that these cells are reduced in blood during HIV co-infection. We hypothesise that Th22 cells can migrate to the lungs for an effective response to provide protection against TB. Further investigation of the role of Th22 cells in TB immunity is warranted, to generate insights that could contribute to the development of an effective vaccine against TB.

CHAPTER 4

Comparison of CD8+ and CD4+ responses to Mtb peptides during TB disease and/or HIV infection

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4.1 Introduction

The importance of CD4⁺ T cells in protective tuberculosis (TB) immunity has been extensively studied and it is widely accepted that these cells play a critical role in *Mycobacterium tuberculosis* (Mtb) control (Leveton *et al.*, 1989; Selwyn *et al.*, 1989; Caruso *et al.*, 1999; Lawn *et al.*, 2002; Lin *et al.*, 2012). However, the role of CD8⁺ T cells in controlling Mtb is still controversial.

Since Mtb is a facultative intracellular pathogen, infection typically leads to processing via vacuolar pathways and MHC II presentation of Mtb antigens to CD4⁺ T cells. However, secreted Mtb antigens can be processed by cytosolic pathways and presented by MHC class I molecules to elicit Mtb-specific CD8⁺ responses (Lewinsohn *et al.*, 2006). CD8⁺ T cells specific for ESAT-6/CFP-10 have been detected in TB patients and Mtb-exposed individuals (Lalvani *et al.*, 1998). Upon activation, CD8⁺ T cells can produce IL-2, IFN- γ , and TNF- α , which may contribute to effective immunity against Mtb (Silva *et al.*, 2014).

In mouse models, some studies have shown that mice deficient in CD8⁺ T cells had a higher Mtb burden and a slight decrease in survival (Sousa *et al.*, 2000; van Pinxteren *et al.*, 2000; Badri *et al.*, 2001; Moguees *et al.*, 2001). In contrast, other murine studies have reported that depletion of CD8⁺ T cells does not affect Mtb bacterial load or survival (Leveton *et al.*, 1989; Flory *et al.*, 1992). In non-human primate (NHP) models, depleting CD8⁺ T cells in bacille Calmette-Guérin (BCG)-vaccinated rhesus macaques resulted in higher levels of bacteria in bronchoalveolar lavage (BAL) fluid and lung lobes after pulmonary Mtb infection (Chen *et al.*, 2009). Moreover, a loss of immune control of TB following re-infection was evident in CD8⁻ depleted rhesus macaques that had previously been cured from Mtb (Chen *et al.*, 2009), suggesting a role for CD8⁺ responses in the control of Mtb infection. Indeed, evidence shows

that unlike CD4⁺ responses, Mtb-specific CD8⁺ T cells may be more important during the latent phase of Mtb infection than acute phase of infection (van Pinxteren *et al.*, 2000; Moguees *et al.*, 2001). This was demonstrated in mouse models, where *in vivo* depletion of CD8⁺ T cells resulted in higher bacterial burden in the lung during the chronic stage of Mtb infection, while CD4⁺ T cell depletion resulted in higher bacterial burden in the lung during the acute phase of Mtb infection (van Pinxteren *et al.*, 2000; Moguees *et al.*, 2001).

In humans, the anti-TNF therapeutic infliximab used for treatment of rheumatoid arthritis (RA) has been shown to promote TB progression, in part by depleting a subset of effector memory CD8⁺ T cells that contributes to the killing of Mtb (Bruns *et al.*, 2009). In a study by Yang *et al.*, CD8⁺ T cell lines that recognized the Mtb immunodominant epitope TB10.4 did not recognize Mtb-infected macrophages nor inhibit the growth of Mtb (Yang *et al.*, 2018). However, TB10.4-specific CD8⁺ T cells were reported to mediate protection against Mtb through IFN- γ release and transporters associated with antigen processing (TAP)-dependent antigen presentation (Nunes-Alves *et al.*, 2015).

In vitro, Mtb-specific CD8⁺ T cells can aid in killing Mtb-infected cells by secreting IFN- γ and TNF- α cytokines (Stenger *et al.*, 1997; Lalvani *et al.*, 1998; Cho *et al.*, 2000), or producing cytotoxic molecules such as perforin and granulysin (Lalvani *et al.*, 1998; Cho *et al.*, 2000). Using Mtb-specific T cell clones, Lewinsohn and colleagues found that CD8⁺ T cells preferentially recognize heavily Mtb-infected cells, suggesting that CD8⁺ T cells may also play a role of surveillance of cells with higher numbers of intracellular bacilli (Lewinsohn *et al.*, 2003).

HIV infection has been shown to impair CD4⁺ immunity to TB by progressive depletion of CD4⁺ T cell numbers (Sonnenberg *et al.*, 2005), preferentially depleting Mtb-specific CD4⁺ T cells (Geldmacher *et al.*, 2010), and altering the equilibrium of Mtb-specific CD4⁺ subsets

(Riou *et al.*, 2016). HIV infection also impairs the CD8⁺ compartment, skewing the memory differentiation profile of CD8⁺ T cells, with the accumulation of pre-terminally differentiated CD45RA⁻CCR7⁻ cells instead of terminally differentiated CD45RA⁺CCR7⁻ cells (Champagne *et al.*, 2001). This pre-terminally differentiated memory phenotype is associated with CD8⁺ T cells expressing low levels of perforin and possessing impaired cytolytic activity in HIV disease (Appay *et al.*, 2000; Andersson *et al.*, 2007). Thus, HIV is thought to induce loss of cytolytic effector molecules in pathogen-specific cytotoxic CD8⁺ T cells (CTLs).

The impact of HIV-1 co-infection on Mtb-specific CD8⁺ T cells is relatively understudied. One study with participants with latent TB showed that ESAT-6/CFP-10-specific CD8⁺ T cells from HIV-coinfected people exhibited decreased expression of the degranulation marker CD107a and impaired proliferative capacity (Kalokhe *et al.*, 2015). Thus, as for CD4⁺, it is plausible that HIV promotes premature exhaustion of Mtb-specific CD8⁺ T cells, affecting their functional capacity and/or survival potential.

In this study, we sought to define the magnitude and phenotypic profile of Mtb-specific CD8⁺ T cell responses, compare them to CD4⁺ responses and investigate the impact of HIV infection and TB disease on Mtb peptide-specific CD8⁺ responses.

4.2 Material and methods

4.2.1 Study participants

Blood samples were collected from 70 participants divided in four groups according to their HIV-1 and TB status: HIV-/LTBI (n = 19), HIV+/LTBI (n = 17), HIV-/aTB (n = 19), and HIV+/aTB (n = 15). Detailed clinical characteristics of study participants are described in **Section 3.2.1, Table 3.1 and Figure 3.1.**

4.2.2 Blood collection and whole blood stimulation

Blood was collected and stimulated as described in **Section 2.2.4.** Mtb-specific peptide pool [(4 µg/ml), consisting of 17 and 16 peptides covering the entire ESAT-6 (early secretory antigenic target) and CFP-10 (culture filtrate protein) was used to stimulate 0.5 ml of heparinized whole blood.

4.2.3 Flow Cytometry

Cryopreserved cells were thawed, washed and then permeabilized with Perm Wash buffer (BD Biosciences). Cells were incubated at 4 °C for 1 h with the following antibodies: CD19 Pacific Blue (6D5; Biolegend), CD14 Pacific Blue (M5E2; Biolegend), CD3 BV650 (OKT3; BD Biosciences), CD4 PerCP-Cy5.5 (L200; BD Biosciences), CD8 BV510 (RPA-T8; Biolegend), CD45RA BV570 (HI100; Biolegend), CD27 PE-Cy5 (1A4CD27; Beckman Coulter), CCR7 PECF594 (3D12; BD Biosciences), KLRG1 PE-vio770 (REA261; Miltenyi Biotec), HLA-DR APC-Cy7 (L243; BD Biosciences), IFN-γ Alexa700 (B27; BD Bioscience), TNF-α BV785 (MAb11; Biolegend), IL-17 Alexa 647 (N49-653; BD Biosciences), IL-22 PE (22URTI; e-Bioscience). Stained cells were acquired on a BD Fortessa and analyzed using FlowJo (v10, TreeStar). A positive cytokine response was defined as at least twice the background (*i.e.*

unstimulated cells) and more than 10 events. To define the phenotype of cytokines-producing cells, a cutoff of 20 events was used. The gating strategy is illustrated in **Figure 4.1**.

4.2.4 Statistical analysis

Statistical tests were performed as described in **Section 2.2.6**.

4.3 Results

4.3.1 The profile of Mtb-specific CD8+ and CD4+ T cell responses during TB disease and/or HIV infection

To define the contribution of CD8+ T cells to Mtb immune responses, we first measured and compared the magnitude of cytokine (IFN- γ , TNF- α , IL-22 and IL-17)-producing cells in the CD4+ and CD8+ compartment. Blood was stimulated with ESAT-6/CFP-10 peptide pool in 70 participants divided into four groups according to their HIV-1 and TB status: HIV-/LTBI (n = 19), HIV+/LTBI (n = 17), HIV-/aTB (n = 19), and HIV+/aTB (n = 15) (described in **Table 3.1, Figure 3.1** in the previous Chapter). As negligible amount of IL-22 and IL-17 were detected in all groups from both CD4+ and CD8+ T cells in response to peptide pool stimulation (**Figure S2**), we focused our analysis on Th1 cytokines (*i.e.* IFN- γ and TNF- α). **Figure 4.1** shows representative flow cytometry plots of IFN- γ and TNF- α production from one donor in each clinical group. Responders were defined as individuals producing any combination of IFN- γ and/or TNF- α .

We first investigated the impact of HIV infection and TB disease on the proportion of individuals with detectable Mtb peptide-specific CD4+ and CD8+ T cells (**Figure 4.2B**). In LTBI individuals, T cell response to Mtb peptides were more prevalent in the CD4+ compartment compared to the CD8+ compartment irrespective of HIV co-infection (58% vs

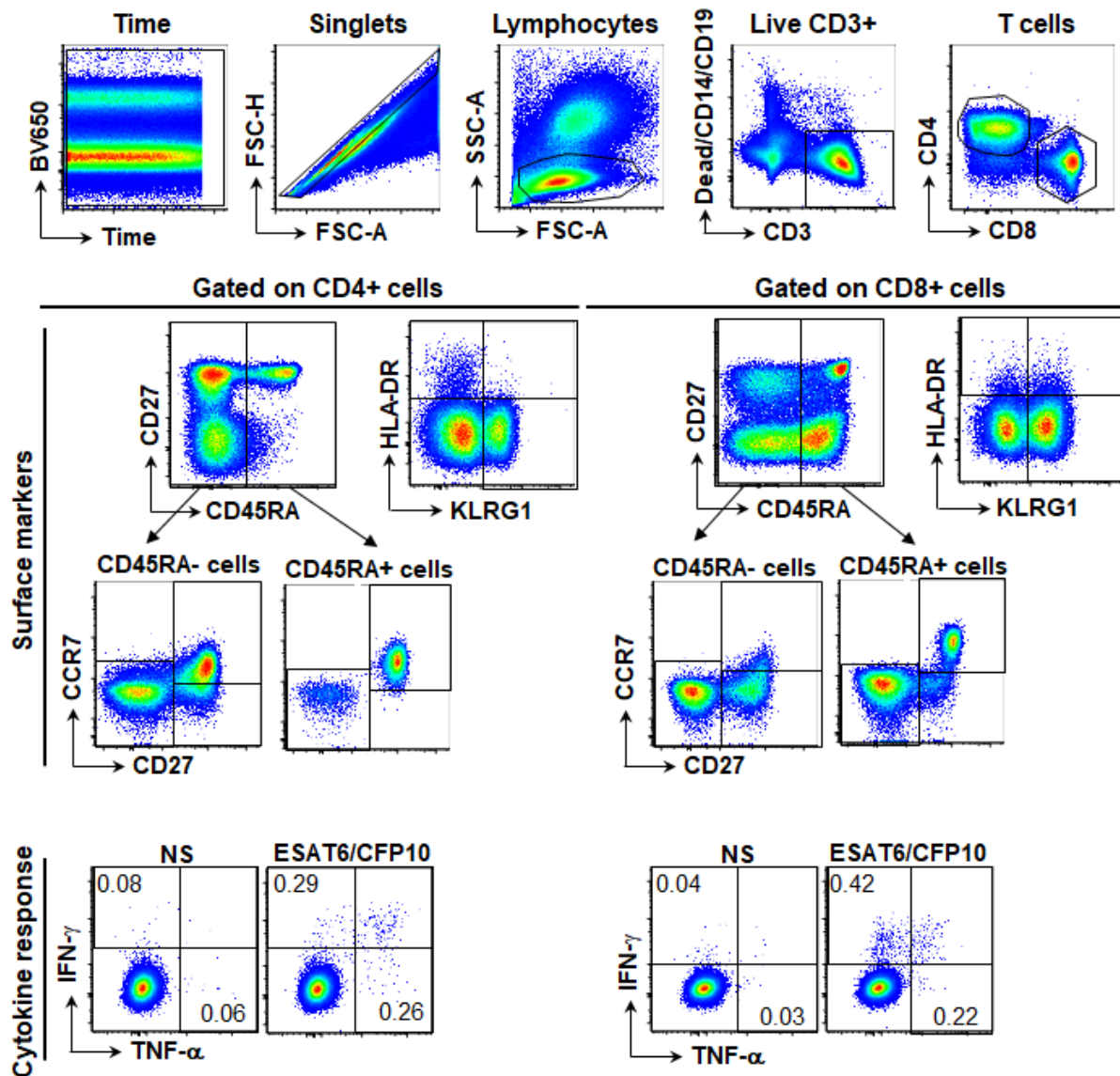


Figure 4.1: Gating strategy. Gating strategy used to determine the phenotype, memory profile and functional properties of CD4+ and CD8+ T cells. NS: no stimulation.

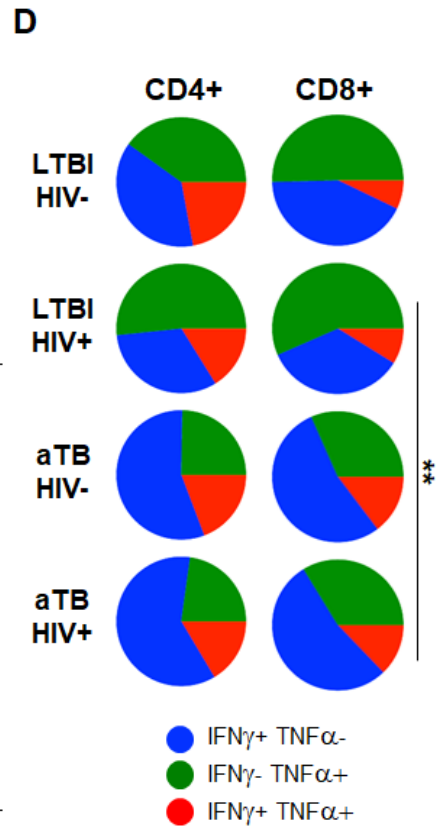
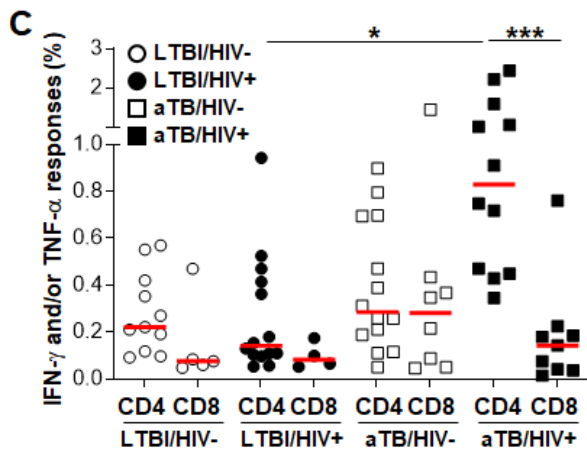
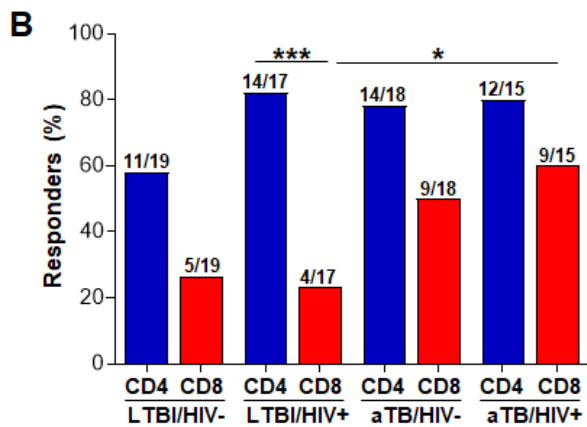
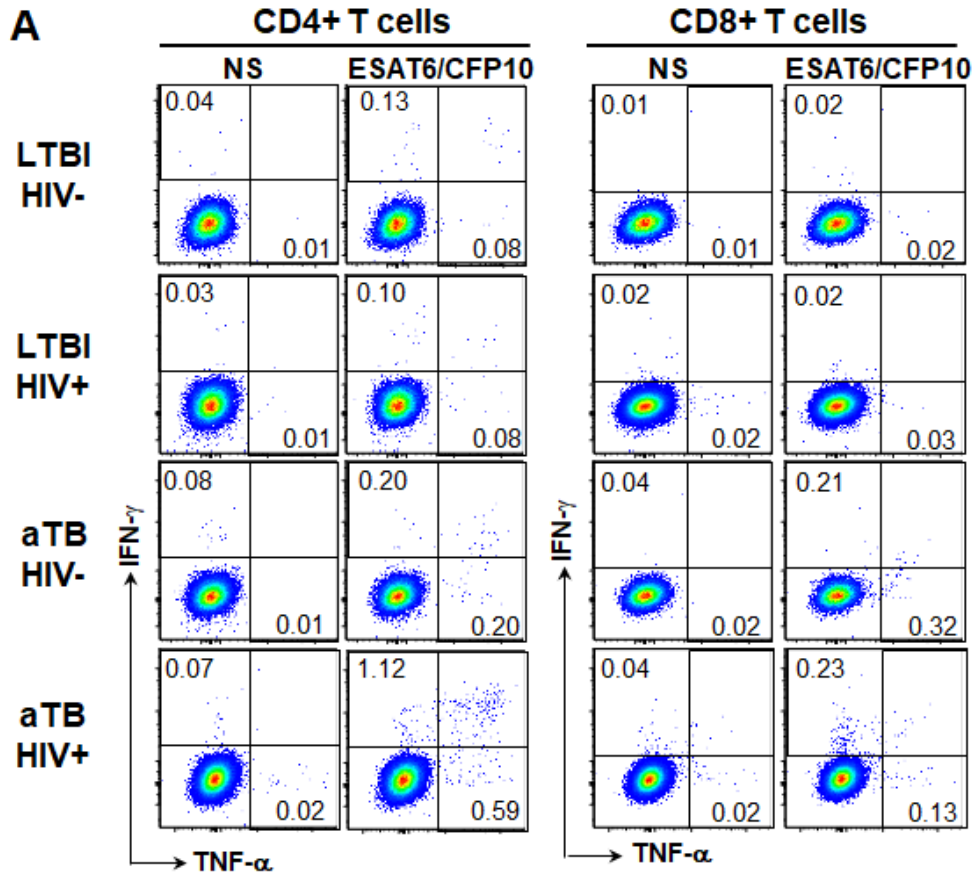
26%; $p = 0.0487$ in HIV-uninfected individuals and 82% vs 24%, $p = 0.0006$ in HIV-infected individuals; respectively). There were no significant differences in the proportion of individuals with detectable Mtb peptide-specific CD4+ T cells between HIV-/LTBI, HIV+/LTBI, HIV-/aTB, and HIV+/aTB groups (58%, 82%, 78%, and 80%; respectively).

Conversely, the proportion of individuals exhibiting an Mtb-specific CD8+ response was elevated in active TB compared to LTBI in both HIV-uninfected and HIV-infected persons (60% vs 24% for HIV+, $p = 0.036$; and 50% vs 26% for HIV-, $p = 0.14$). It is not surprising

that the effect observed in the magnitude of CD4 ESAT6/CFP10 response in LTBI/HIV- vs LTBI/HIV+ is less than that observed in Figure 3.6 where Mtb lysate was used, as this is consistent with a previous study (Riou *et al.*, 2017). These data are in accordance with previously published data (Rozot *et al.*, 2013; Amelio *et al.*, 2018).

Next, we compared the magnitude of IFN- γ and/or TNF- α responses to Mtb peptides in the CD4+ and CD8+ compartment in individuals with detectable responses and defined the effect of HIV infection and active TB on these responses (**Figure 4.2C**). While the frequency of Mtb-specific CD4+ and CD8+ responses were comparable in the HIV-/LTBI, HIV+/LTBI and HIV-/aTB groups, the magnitude of CD4+ responses was significantly higher compared to CD8+ responses in the HIV+/aTB group (medians: 0.83% and 0.14%; $p = 0.0009$, respectively). Although active TB resulted in a higher proportion of CD8 responders in HIV-infected individuals, no differences were observed in the frequency of CD8+ responses between any of the disease groups (medians: HIV-/LTBI = 0.076%, HIV+/LTBI = 0.082%, HIV-/aTB = 0.282%, and HIV+/aTB = 0.143%). However, in HIV-infected individuals, TB disease significantly altered cytokine co-expression of Mtb-specific CD8+ T cells ($p = 0.007$, **Figure 4.2D**). Regarding CD4+ responses, our results show that whilst aTB did not significantly alter the magnitude of CD4+ responses in HIV-uninfected individuals (medians: 0.29% for aTB and 0.22% for LTBI), in HIV-infected persons with aTB the Mtb peptide-specific CD4+ responses were significantly higher compared to LTBI (medians: 0.73% and 0.14%; $p = 0.006$, respectively). These results are consistent with the CD4+ Th1 response profile observed upon Mtb lysate stimulation reported in **Section 3.3.3 (Figure 3.6A)** in the previous chapter.

Figure 4.2 on the next page: Comparison of the frequency of Mtb peptide-specific CD4+ and CD8+ T cells in LTBI/HIV- (n=19), aTB/HIV- (n=18), LTBI/HIV+ (n=19), and aTB/HIV+ (n=15). (A) Representative flow cytometry plots showing IFN- γ and TNF- α production by CD4+ and CD8+ T cells in response to stimulation with ESAT-6/CFP-10 peptide pool. (B) Proportions of individuals with detectable Mtb-specific CD4+ and CD8+ T cells. (C) Frequency of Mtb-specific CD4+ and CD8+ T cells producing any combination of IFN- γ and TNF- α in four clinical groups. Only individuals with detectable responses are shown. The bars represent the median. (D) Pie charts showing different combinations of IFN- γ and TNF- α . Medians are depicted. Statistical comparisons were performed using chi-square test (B), one-way ANOVA Kruskal–Wallis test (C) and permutation test using the SPICE software (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



4.3.2 The effect of HIV infection and TB disease on Mtb-specific CD4+ and CD8+ T cell memory profile

We next characterized and compared the memory profile of Mtb peptide-specific CD4+ and CD8+ responses during HIV infection and aTB disease (**Figure 4.3**). As for Mtb lysate-specific Th1 responses (presented in **Section 3.3.3, Figure 3.7**), we found that during aTB, Mtb peptide-specific CD4+ T cells displayed a more differentiated phenotype (EM) compared to HIV-infected individuals with LTBI, but not HIV-uninfected individuals (medians: 32% vs 68% for HIV+, $p = 0.025$ and 48% vs 67% for HIV-, $p = ns$) (**Figure 4.3B**). Concomitantly, a lower proportion of CD4+ T cell responses with a central memory phenotype was observed in aTB compared to LTBI in HIV-infected individuals, but not HIV-uninfected individuals (medians: and 36% vs 9% for HIV+, $p = 0.025$ and 15% vs 15% for HIV-, $p = ns$) (**Figure 4.3B**). Although we did not observe any significant changes in the memory phenotype of Mtb peptide-specific CD8+ T cells during HIV infection and/or TB disease, there was a trend towards an increased proportion of transitional memory (TM) (CD45RA-CD27+CCR7+) cells in HIV-infected TB patients compared to LTBI individuals (medians: 50% vs 17%, respectively; $p = 0.058$) (**Figure 4.3B**).

In LTBI individuals, Mtb-specific CD8+ T cells displayed a more differentiated memory phenotype compared to Mtb-specific CD4+ T cells, where Mtb-specific CD8+ T cells were enriched in cells with an effector phenotype compared to Mtb peptide-specific CD4+ T cells (**Figure 4.3B**). This was also observed in the entire CD8+ compartment compared to the CD4+ compartment in all clinical groups (**Figure 4.4**) indicating that CD8+ T cells are inherently different to CD4+ T cells in their memory differentiation profiles. These results suggest that active Mtb replication leads to the memory differentiation of Mtb-specific CD4+ T cells and may also impair Mtb-specific CD8 memory maturation.

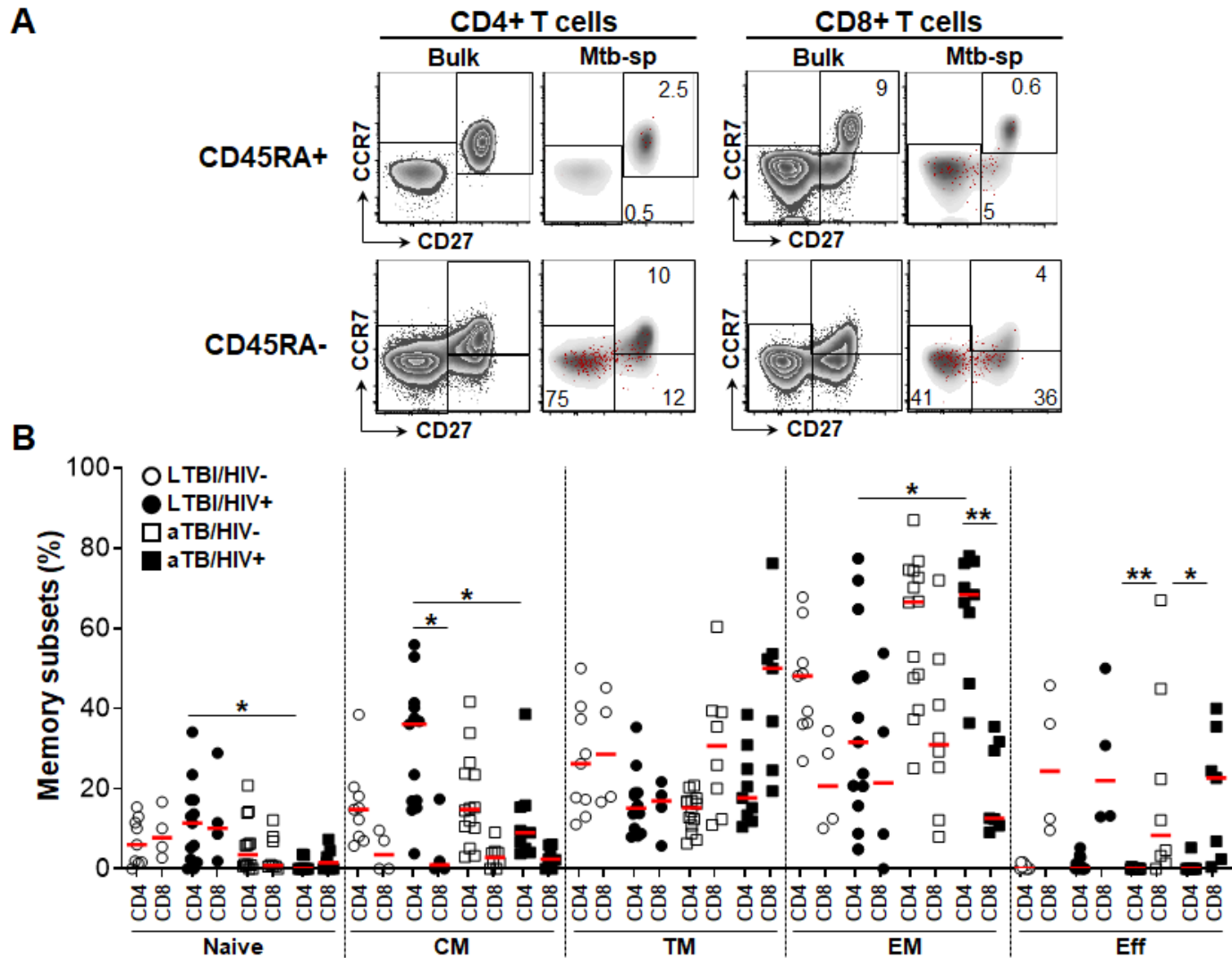


Figure 4.3: Comparison of the memory differentiation profile of Mtb peptide-specific CD4⁺ and CD8⁺ cells in individuals with distinct HIV and TB disease states. (A) Representative overlay plots showing memory subsets in total CD4⁺ and CD8⁺ T cells (grey), IFN- γ ⁺ and/or TNF- α ⁺ cells (brown) in response to ESAT-6/CFP-10 peptides pool. (Naive: CD45RA⁺CD27⁺CCR7⁺; central memory (CM): CD45RA⁻CD27⁺CCR7⁺; transitional memory (TM): CD45RA⁻CD27⁺CCR7⁻, effector memory (EM): CD45RA⁻CD27⁻CCR7⁻ and effector cells (Eff): CD45RA⁺CD27⁻CCR7⁻). (B) Summary graph. Medians are depicted. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$.

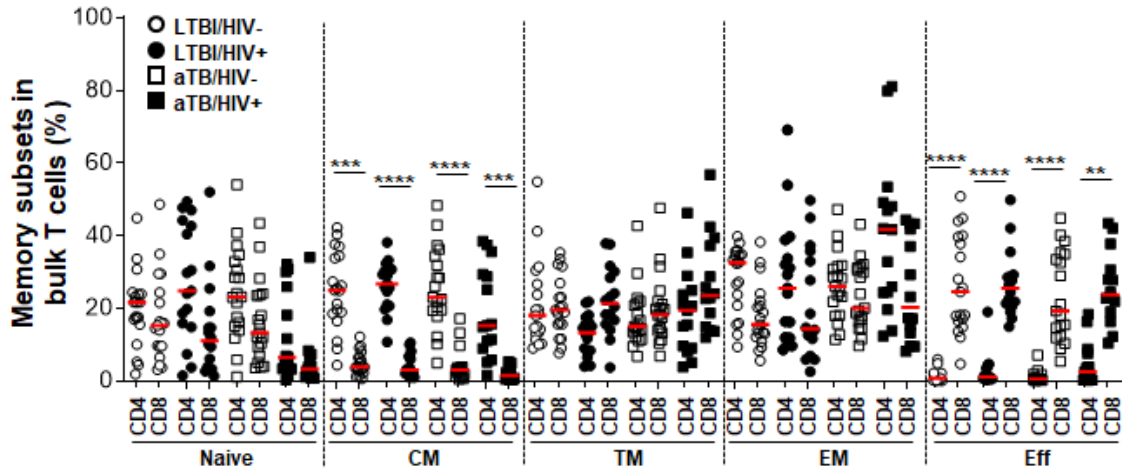


Figure 4.4: Comparison of the memory differentiation profile of total CD4+ and CD8+ T cells in individuals with distinct HIV and TB disease states. Medians are depicted. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test., ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

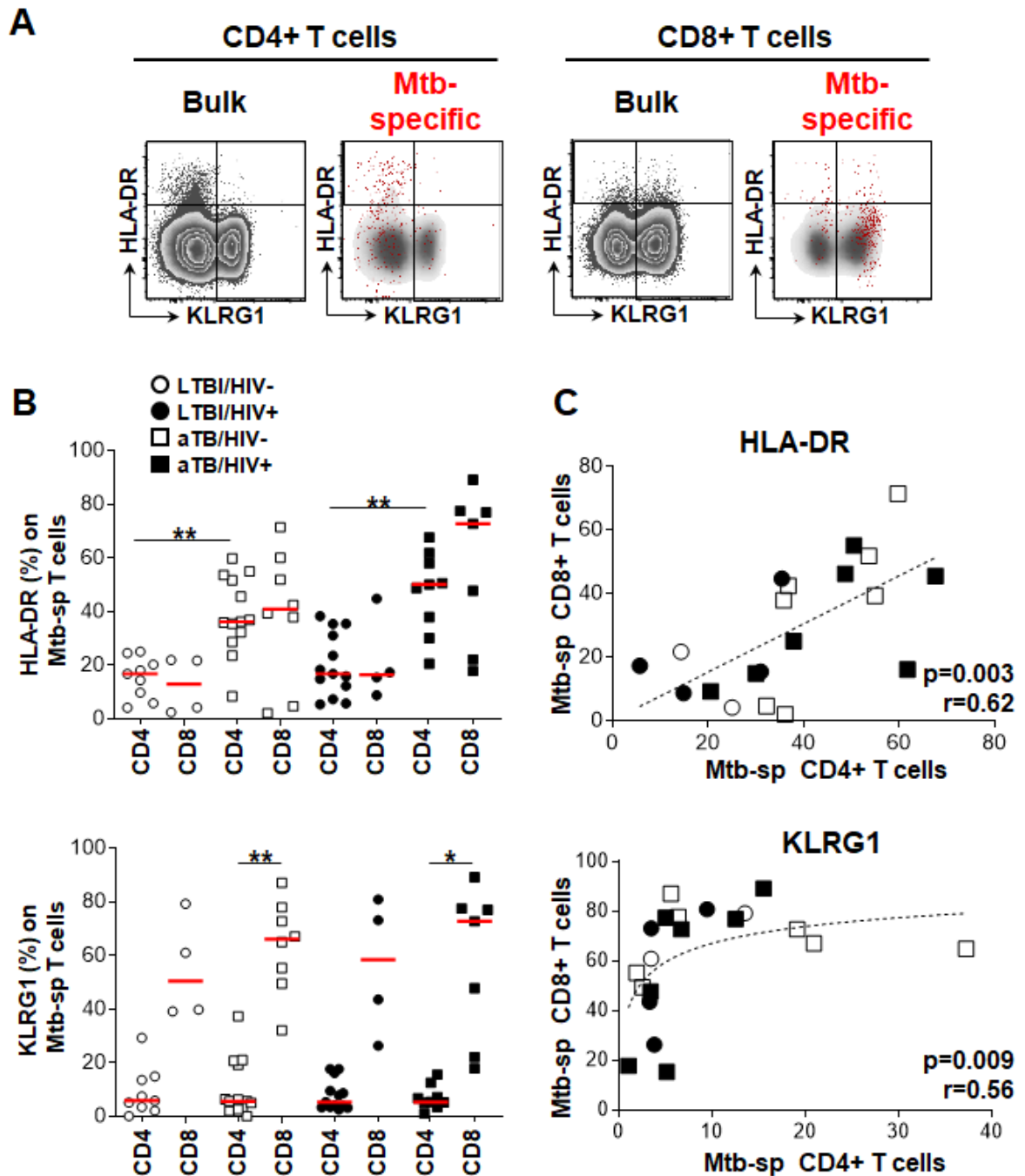
4.3.3 The effect of HIV infection and TB disease on Mtb-specific CD4+ and CD8+ T cells activation profile

Lastly, we assessed the activation profile of Mtb peptide-specific CD4+ and CD8+ responses during HIV infection and aTB disease using HLA-DR and KLRG1 (**Figure 4.5**). As previously described in **Figure 3.8** in the previous Chapter, Mtb peptide-specific CD4+ T cells were characterized by significantly higher expression of HLA-DR in aTB compared to LTBI (median: 36% vs 17%, for HIV-, $p = 0.009$ and 50% vs 17% for HIV+, $p = 0.003$, respectively). While not reaching significance because of low number of CD8 responders in LTBI groups, there was a higher frequency of HLA-DR-expressing Mtb peptide-specific CD8+ T cells in active TB compared to LTBI (median: 41% vs 13%, for HIV-, $p = 0.794$ and 73% vs 16% for HIV+, $p = 0.201$, respectively), with HLA-DR frequencies being comparable between Mtb peptide-specific CD8+ T and Mtb peptide-specific CD4+ T cells in all clinical groups (**Figure 4.5B**). In fact, the activation profile of Mtb peptide-specific CD8+ T cells mirrored that

observed in Mtb peptide-specific CD4⁺ T cells, as illustrated by the positive linear relationship ($r = 0.62$; $p = 0.003$) (**Figure 4.5C**). Interestingly, HLA-DR expression was also comparable between total CD4⁺ or CD8⁺ in all groups, and TB disease did not distort the activation phenotype of total CD4⁺ and CD8⁺ T cells (**Figure 4.6A**), suggesting that elevated activation observed during aTB is restricted to CD4⁺ and CD8⁺ T cells targeting Mtb peptides and most probably driven by pathogen replication.

HIV infection and/or TB disease did not induce any changes in KLRG1 expression on both Mtb peptide-specific CD4⁺ T cells and Mtb peptide-specific CD8⁺ T cells (**Figure 4.5D**). However, Mtb peptide-specific CD8⁺ T cells were characterized by elevated KLRG1 expression compared to Mtb peptide-specific CD4⁺ T cells in all groups (medians: 50% vs 6% for LTBI/HIV⁻, $p = 0.19$ and 58% vs 5% for LTBI/HIV⁺, $p = 0.09$; 66% vs 6% for aTB/HIV⁻, $p = 0.003$ and 73% vs 5% for aTB/HIV⁺, $p = 0.021$; respectively) (**Figure 4.5D**). Nevertheless, there was a positive association of KLRG1 expression between Mtb-specific CD8⁺ T cells and Mtb-specific CD4⁺ T cells ($r = 0.56$; $p = 0.009$) (**Figure 4.5E**). Similarly, total CD8⁺ T cells expressed more KLRG1 compared to CD4⁺ T cells in all disease groups (medians: 60% vs 11% for LTBI/HIV⁻, $p < 0.0001$, 72% vs 13% for LTBI/HIV⁺, $p < 0.0001$, 57% vs 10% for aTB/HIV⁻, $p < 0.0001$ and 58% vs 12% for aTB/HIV⁺, $p = 0.006$; respectively) (**Figure 4.6B**).

Interestingly, there was a positive association of HLA-DR and KLRG1 expression between Mtb-specific CD4⁺ T cells and total CD4⁺ T cells ($r = 0.42$; $p = 0.006$ for HLA-DR and $r = 0.39$; $p = 0.008$ for KLRG; **Figure 4.7**). Similarly, although not reaching significance, there was a positive correlation between Mtb-specific CD4⁺ T cells and total CD8⁺ T cells expressing HLA-DR and KLRG1 ($r = 0.41$; $p = 0.055$ and $r = 0.41$; $p = 0.056$, respectively; **Figure 4.7**).



HLA-DR

Mtb-sp CD8+ T cells

Mtb-sp CD4+ T cells

p=0.003
r=0.62

KLRG1

Mtb-sp CD8+ T cells

Mtb-sp CD4+ T cells

p=0.009
r=0.56

Figure 4.5: Comparison of the activation phenotype of Mtb-specific CD4+ and CD8+ cells in individuals with distinct HIV and TB disease states. (A) Representative overlay plots showing HLA-DR and KLRG1 expression in total CD4+ and CD8+ T cells (grey), IFN- γ + and/or TNF- α + cells (brown) in response to ESAT-6/CFP-10 peptides pool. **(B)** Summary graph. Medians are depicted. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$. **(C)** The relationship between Mtb peptide-specific CD4+ and CD8+ T cells expressing HLA-DR and KLRG1. Each dot represents an individual. Statistical analyses were performed using a non-parametric Spearman rank correlation.

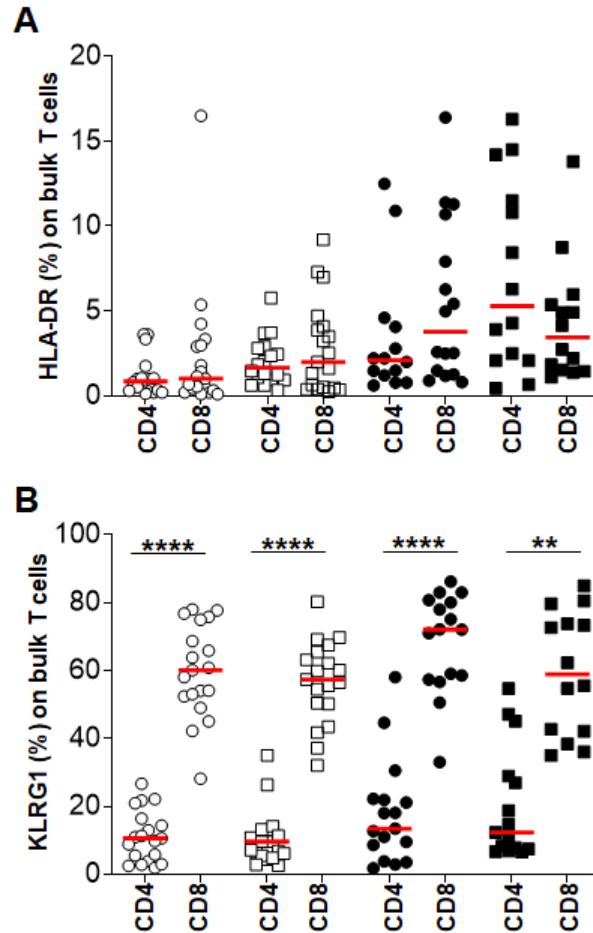


Figure 4.6: Comparison of HLA-DR and KLRG1 expression on total CD4+ and CD8+ T cells in individuals with distinct HIV and TB disease states. Medians are depicted. Statistical comparisons were performed using a one-way ANOVA Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$.

In summary, we show that the presence of replicating pathogen (Mtb) during TB disease leads to activation of Mtb peptide-specific CD4+ T cells. Further assessment of CD8+ T cell activation is needed, and the cytotoxic potential of these CD8+ T cells is required to elucidate the role of CD8+ T cells in TB immunity.

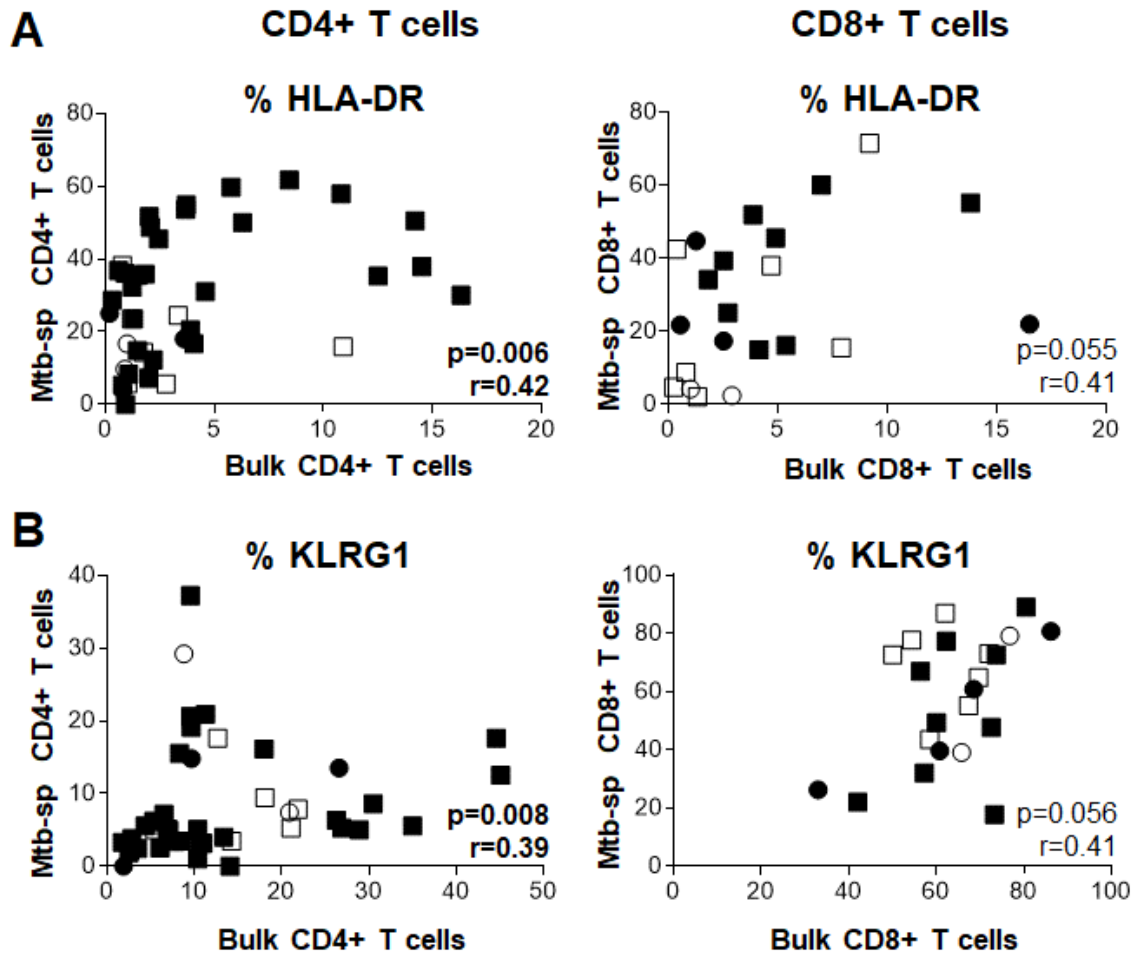


Figure 4.7: The relationship between Mtb peptide-specific CD4+ or CD8+ T cell activation with total CD4+ or CD8+ T cell activation profile. The relationship between Mtb peptide-specific CD4 or CD8 T cells with total CD4+ or CD8+ T cells expressing (A) HLA-DR and (B) KLRG1. Each dot represents an individual. Statistical analyses were performed using a non-parametric Spearman rank correlation.

4.4 Discussion

CD4⁺ T cells and CD8⁺ T cells produce Th1 cytokines (IFN- γ and TNF- α) which can activate macrophages and inhibit Mtb growth (MacMicking *et al.*, 2003). HIV infection has been shown to impair Mtb-specific CD4⁺ responses, enhancing the risk of progression from LTBI to active TB (Day *et al.*, 2014; Kalokhe *et al.*, 2015; Amelio *et al.*, 2018). However, relatively little is known about the effect of HIV on Mtb-specific CD8⁺ T cells. Therefore, we investigated the impact of HIV infection on the memory and activation profile and functional capacities of Mtb-specific CD8⁺ T cells during LTBI and TB disease and compared their phenotypic profile to Mtb-specific CD4⁺ responses.

Consistent with previous studies, we detected CD8⁺ responses following stimulation of blood with ESAT-6/CFP-10 peptide pool (Rozot *et al.*, 2013; Amelio *et al.*, 2018). Mtb peptide-specific CD8⁺ T cells were more frequently detected in individuals with active TB disease compared to latently infected individuals (60% vs 24% for HIV⁺, $p = 0.036$ and 50% vs 26% for HIV⁻; $p = 0.14$), in agreement previously reports (Rozot *et al.*, 2013; Amelio *et al.*, 2018). Reasons for this observation could be that CD8⁺ T cells preferentially recognize heavily Mtb-infected cells (Lewinsohn *et al.*, 2003), providing evidence that CD8⁺ T cells may play a role of surveillance of cells with higher numbers of intracellular bacteria. Indeed, the frequency of Mtb-specific CD8⁺ T cells is associated with Mtb bacterial burden in both humans and mice (Billeskov *et al.*, 2007; Day *et al.*, 2011). Furthermore, reduction of Mtb load by anti-TB treatment has been shown to result in a decrease in Mtb-specific CD8 responses (Nyendak *et al.*, 2013; Day *et al.*, 2014).

The CD8⁺ responses detected in this study are most likely an underestimate of the actual prevalence of Mtb-specific CD8⁺ responses in TB patients and Mtb-exposed individuals, as

we used 15 amino acid ESAT-6/CFP-10 peptides which are not entirely optimal for the detection of CD8+ responses (usually 9 amino acid peptides are used for CD8+ T cells), and we only tested two proteins, while other Mtb proteins could be more immunogenic and detect a greater range of CD8+ responses (Rakshit *et al.*, 2017). Since Mtb lysate is a mixture of all the proteins, lipids and carbohydrates present in the cell; thus, Mtb lysate stimulates innate cells, conventional and non-conventional T cells. Therefore, given that other non-conventional cells express CD8 as shown in Figure 2.8C, we would not be able differentiate between cytokines produced by CD8+ T cells and other non-conventional cells as we did not include their markers. Thus, we examined responses to Mtb peptide antigens.

In LTBI, both bulk CD8+ memory cells targeting Mtb reflect total memory CD8+ T cells in that they are maintained in a more differentiated memory profile (effector memory and effector cells) and express elevated KLRG1 compare to CD4+ cells. These observations are consistent with studies that have shown that KLRG1 expression on T cells is associated with an increased effector function in Mtb infection (Chattopadhyay *et al.*, 2009; Hu *et al.*, 2018). It is also consistent with the hypothesis that KLRG1 expression is dependent on cell division, as CD8+ T cells proliferate more vigorously after antigen challenge than CD4+ T cells (Foulds *et al.*, 2002; Voehringer *et al.*, 2002). While the expression of KLRG1 and having a specific effector memory (EM) phenotype for CD4+ T cells is considered detrimental for immunity against TB (Wang *et al.*, 2010; Lindenstrom *et al.*, 2013; Sakai *et al.*, 2014), effector memory subsets of CD8+ T cells were reported to have a high cytolytic potential which correlates with another senescence maker CD57 (Sallusto *et al.*, 1999; Takata and Takiguchi, 2006; Chattopadhyay *et al.*, 2009). Thus, our results highlight differences in Mtb-specific CD4+ and CD8+ T cells consistent with their distinct functions in TB immunology.

HIV infection has been shown to impair degranulation and proliferative capacity of Mtb-specific CD8⁺ T cells (Kalokhe *et al.*, 2015). Due to low number of individuals in our cohort and a low proportion of Mtb-specific CD8⁺ T cells in individuals with LTBI, we were unable to identify statistically significant defects caused by HIV infection on Mtb peptide-specific CD8⁺ T cells. However, we observed a trend towards an increase in proportion of Mtb-specific CD8⁺ T cells displaying a transitional memory profile (TM) (CD45RA⁻CD27⁺CCR7⁺) during aTB compared to LTBI, in HIV-infected individuals. This supports the hypothesis that TB co-infection alters CD8⁺ T-cell differentiation. Indeed, TB disease has been shown to impair differentiation of Mtb-specific CD8⁺ T cells, as individuals with active TB are characterized by a higher proportion of CD45RA⁻CCR7⁻ compared to LTBI individuals which display a fully differentiated memory profile (CD45RA⁺CCR7⁻) (Caccamo *et al.*, 2009; Rozot *et al.*, 2013; Suarez *et al.*, 2015). This deficient CD8⁺ T-cell memory differentiation may result from premature exhaustion of CD8⁺ T cells caused by chronic activation during HIV infection and TB disease (Breton *et al.*, 2013; Amelio *et al.*, 2018). It is not clear whether HIV infection directly influences memory maturation of Mtb-specific CD8⁺ T cells or indirectly by impairing CD4⁺ T cells, which have been implicated in CD8⁺ T cell development and maturity (Matloubian *et al.*, 1994; Serbina *et al.*, 2000; Sun *et al.*, 2004).

While CD8⁺ T cells are thought to be less important than CD4⁺ T cells in the control of Mtb, multiple lines of evidence do indeed support a role for CD8⁺ T cells in protective immunity. Like CD4⁺ T cells, CD8⁺ T cells have been shown to produce cytokines (IFN- γ , TNF- α and IL-2) that play vital roles during Mtb infection (Kamath *et al.*, 2006; Gideon *et al.*, 2015). Furthermore, CD8⁺ T cells have the potential to kill target cells through the release of perforin, granzymes, and granulysin (Serbina *et al.*, 2000; Andersson *et al.*, 2007). Indeed, *in vitro* studies showed that cytotoxic CD8⁺ T cells can directly kill intracellular Mtb in human

macrophages through the release of cytotoxic granules (granulysin and perforin) (Stenger *et al.*, 1997, 1998). Moreover, peripheral CD8⁺ T cells cell lines specific for Mtb peptides were able to recognize and lyse Mtb-infected macrophages *in vitro* (Cho *et al.*, 2000). In mouse models, CD8⁺ T cells were shown to be essential during the chronic stage of Mtb infection, as CD8-depleted mice had higher bacterial burdens (van Pinxteren *et al.*, 2000; Moguees *et al.*, 2001). Mtb-specific CD8⁺ T cells were detected in the lungs and lung-draining lymph nodes of mice infected with Mtb, and these cells expressed perforin and were able to recognize and lyse Mtb-infected macrophages (Serbina *et al.*, 2000). Moreover, challenging of CD8-depleted rhesus macaques with high dose Mtb led to loss of BCG vaccine-induced protection (Chen *et al.*, 2009). Additionally, CD8⁺ T cells expressing granulysin, perforin and granzymes have been detected in granulomas of humans, macaques and cattle (Andersson *et al.*, 2007; Diedrich *et al.*, 2018; Palmer *et al.*, 2019). Conversely, it has also been reported that Mtb-specific CD8⁺ T cells could be endowed with detrimental functions, as illustrated by the accumulation of IL-10-secreting CD8⁺ T cells in mice that was associated with elevated susceptibility to Mtb infection (Cyktor *et al.*, 2013). Moreover, expression of regulatory cytokines such as IL-10 and TGF- β by CD8⁺ T cells was related to higher sputum bacterial load in individuals with pulmonary TB (Silva *et al.*, 2014).

In conclusion, our results add on to the growing body of evidence that shows the involvement of CD8⁺ T cells in TB immune responses. Our study limitations include small cohort size, we only investigated ESAT-6/CFP-10 responses, other Mtb antigen could be more immunogenic to induce CD8⁺ responses, and our samples were limited to studying peripheral blood. Additional studies with larger number of participants and focusing on the site of TB disease are warranted to further elucidate on the role of CD8⁺ T cells in Mtb control.

CHAPTER 5

Discussion

The only licensed TB vaccine (BCG) is protective against severe, childhood forms of the TB but is not effective against pulmonary TB, the most common form of the disease (Fine, 1995; Bo *et al.*, 2008). Thus, there is an urgent need for a new and efficacious TB vaccine. Recently, there has been much excitement regarding a new promising TB vaccine candidate (M72/AS01E) that has been shown to provide 50% efficacy against pulmonary tuberculosis (Tait *et al.*, 2019). Since there is a consensus regarding the critical role of Th1 cells in orchestrating an effective immune control against *M. tuberculosis*, Th1 cytokines (particularly IFN- γ) are the traditional measure of vaccine-induced immunogenicity (Soares *et al.*, 2008; Norrby *et al.*, 2017). However, IFN- γ secretion has proven to be a poor correlate of TB protection in BCG-vaccinated mice (Majlessi *et al.*, 2006; Mittrücker *et al.*, 2007).

In this study, we sought to investigate immune responses to *Mycobacterium tuberculosis* (Mtb) beyond IFN- γ producing CD4⁺ T cells. We focused our analysis on a subset of CD4⁺ T cells named 'Th22' cells. We used healthy Mtb-exposed individuals to optimize IL-22 detection and investigate factors that influence Mtb-induced IL-22 production in whole blood and PBMC. Furthermore, we examined the impact of TB disease and HIV co-infection on Mtb-specific Th22 responses, in the context of better-characterized Th1 and Th17 responses. Blood samples from HIV-infected and uninfected individuals with Mtb exposure (latent TB infection) as well as TB disease were investigated, to assess the functional characteristics, memory, activation and homing profiles of Mtb-specific Th22 cells compared to Th1 and Th17 cells.

In Chapter 2, we showed that optimal detection of CD4⁺ IL-22 T cell responses was dependent on the type of assay and antigen formulations used for stimulation. Whole blood stimulation enabled robust detection of IL-22-producing CD4⁺ T cells, while IL-22 responses were poorly detectable in peripheral blood mononuclear cells (PBMC). We also observed that IL-22 responses were induced by more complex antigens such as Mtb lysate, gamma irradiated Mtb whole cells and BCG, but not ESAT-6 and CFP-10 peptides. These data suggest that examining Th22 responses to Mtb may be hindered when only PBMC samples are available for study. Supplementing PBMC with soluble fractions from blood only partially restored IL-22 responses. IL-22 responses in PBMCs were restored when labelled PBMC were added to autologous whole blood (Bunjun *et al.*, submitted), indicating that IL-22-producing cells are indeed present in PBMC but lack physiological environment for optimal detection. Further studies could investigate whether cell subsets present in whole blood but not PBMC (neutrophils and possibly DCs) play a role in the production of IL-22 from CD4⁺ T cells, by depleting such cells in whole blood before stimulation or supplementing PBMC with them.

Given that IL-22 induction was optimally detected following stimulation with complex Mtb antigen preparations, there was the possibility that it resulted from bystander activation from innate cytokines stimulated by pattern-associated molecular pattern molecules (PAMPs, such as mycobacterial cell wall constituents) stimulating pattern recognition receptors (PRRs, like Toll-like receptors) present in the antigen preparations. However, we determined that IL-22 production was dependent on TCR signaling, and was not the result of bystander activation. Determining what antigen is recognized is important for inducing Th22 responses with a TB vaccine, in order to include that antigen in a vaccine; in this respect, whole cell vaccines may be better at inducing IL-22 responses due to their antigen composition. Further studies could

test different components and fractions of Mtb such as cell wall, cell membrane, cytosol, lipids or lipoproteins (Seshadri *et al.*, 2013), to determine if they induce IL-22 responses.

IL-22 was mainly produced by CD4⁺ T cells that appear to be conventional, rather than unconventional MAIT, $\gamma\delta$ or iNKT cells. In addition, IL-22 producing CD4⁺ T cells displayed a similar TCR V β repertoire as IFN- γ -producing CD4⁺ T cells. However, Mtb-specific Th22 cells did display some unique phenotypic characteristics, namely differences in memory, activation and homing profiles compared to Th1 and Th17 cells. These data thus reveal interesting and novel features of IL-22-producing CD4⁺ T cells. Ongoing experiments in our laboratory aim to shed more light on the features of Th22 cells by isolating these cells using cytokine capture assays, and performing RNAseq to examine, among others, their lineage defining transcription factors, cytokine co-expression and receptor expression.

Since its discovery in 2000, the role of IL-22 has been described in several diseases caused by bacteria, viruses, fungi, and parasitic infections (Dumoutier *et al.*, 2000; Dudakov *et al.*, 2015; Brias *et al.*, 2016; Valeri and Raffatellu, 2016). IL-22 mainly acts on non-hematopoietic cells, to preserve mucosal barriers by promoting tissue proliferation, regeneration, and healing (Witte *et al.*, 2010; Sonnenberg *et al.*, 2011; Sabat *et al.*, 2014). IL-22 also induces epithelial cells to produce antimicrobial peptides and proteins such as β -defensins, the S100 family of peptides, Reg3 γ , calprotectin and calgranulin A (Lin *et al.*, 2017; Moyat *et al.*, 2017; Tyler *et al.*, 2017). Furthermore, IL-22 is involved in inducing chemokines from epithelial cells, which leads to recruitment of innate cells to the site of infection (Whittington *et al.*, 2004; Aujla *et al.*, 2008; Nograles *et al.*, 2008). During infection with *Citrobacter rodentium*, an extracellular bacterium that infects the intestine, mice lacking IL-22 displayed more intestinal epithelial damage, systemic bacterial burden and mortality (Zheng *et al.*, 2008). IL-22 was also shown to be

critical against during lung infections in mice, where IL-22 neutralization aggravated dissemination of *Klebsiella pneumoniae*, but only in the absence of IL-17 (Aujla *et al.*, 2008). Moreover, IL-22 deficient mice displayed severe lung injury and were impaired in the regeneration of tracheal epithelial cells during influenza infection (Kumar *et al.*, 2013; Pociask *et al.*, 2013). In the context of TB, IL-22 was reported to induce antimicrobial proteins, causing chemokine secretion (CCL2) that recruited macrophages to the site of disease (Treerat *et al.*, 2017). There is also possibly a direct effector function for IL-22, since IL-22 receptor has been detected on Mtb-infected macrophages, as well as macrophages in TB granulomas in humans and non-human primates (Dhiman *et al.*, 2009; Zeng *et al.*, 2011; Treerat *et al.*, 2017). Indeed, IL-22 was shown to inhibit the growth of Mtb in mice by enhancing phagolysosomal fusion (Dhiman *et al.*, 2009). Further studies are required to demonstrate whether IL-22 is indeed involved in protective TB immunity. These could include adoptive transfer of Th22 cells in mice, IL-22 neutralization in NHPs and examining vaccine-induced Th22 responses using current TB vaccine candidates in humans and NHPs. Longitudinal human studies involving LTBI individuals or household contact of TB patients could also be used to investigate Th22 responses in individuals who progress to TB disease. A longitudinal study analyzing whole blood transcriptomic signatures in Mtb-infected adolescents reported on the low expression of Th17-associated genes IL-17F, IL-23R, RORC and CCR2 in individuals who progressed to TB (Chen *et al.*, 2016; Scriba *et al.*, 2017). Given that some of these genes are also associated with Th22 cells, further studies of Th22 cells are indeed warranted.

IL-22 was reported to be essential for control of Mtb infection using the hyper-virulent clinical Mtb strain HN878 but dispensable when using laboratory adapted H37Rv and Erdman strains (Wilson *et al.*, 2010; Khader *et al.*, 2011; Behrends *et al.*, 2013; Segueni *et al.*, 2016; Treerat *et al.*, 2017). Interestingly, HN878 has been associated with reduced BCG vaccine-induced

protection (Tsenova *et al.*, 2007; Ordway *et al.*, 2011). However, this observation is not consistent for all W-Beijing lineage strains (Bo *et al.*, 2008). The high virulence of Mtb strain HN878 has also been shown to be associated with lower Th1 responses (Manca *et al.*, 1999). Other studies propose that both Mtb and host genotype influence the disease outcome (Caws *et al.*, 2008; van Crevel *et al.*, 2009). It is important to determine whether a protective role for IL-22 is limited to infection with hyper-virulent Mtb strains. Further studies could investigate the role of IL-22 in TB during infection with clinal strains of Mtb that are not hypervirulent.

Detrimental roles of IL-22 have also been reported, where uncontrolled expression caused tissue damage and chronic inflammation, leading to inflammatory skin diseases such as psoriasis through regulation of cellular differentiation, or development of several types of cancer by promoting the survival, proliferation and migration of cancerous cells (Wolk *et al.*, 2006; Schmechel *et al.*, 2008; Akil *et al.*, 2015; Lullo *et al.*, 2015; Nardinocchi *et al.*, 2015). Furthermore, induction of antimicrobial proteins by IL-22 was shown to favor *Salmonella typhimurium* colonization in the gut (Behnsen *et al.*, 2014). Moreover, IL-22 contributes to *Helicobacter pylori*-associated gastritis by stimulating epithelial cells to secrete CXCL2, which leads to recruitment of myeloid-derived suppressor cells and inhibition Th1 cell responses (Zhuang *et al.*, 2015). Further studies are required to determine which environmental, cellular, and molecular factors contribute to regulation and dysregulation of IL-22 production and function. Since IL-22 has been implicated in both pathogenic and protective roles (Sonnenberg *et al.*, 2010; Zenewicz and Flavell, 2011), further studies are required to determine factors that could contribute to IL-22 having protective versus inflammatory pathogenic roles, including levels of IL-22 in the microenvironment, other cytokines present, IL-22 inhibitors (such as IL-22 binding protein), responding cells, tissues and disease (Zenewicz *et al.*, 2018).

Extending previous work from our laboratory describing mycobacterial IL-22 responses (Bunjun *et al.*, submitted), we detected substantial IL-22 production in response to stimulation with Mtb lysate, comparable to the IFN- γ response. Interestingly, Mtb-specific Th22 cells were reduced by 50% in TB patients. It is likely that Mtb replication could trigger recruitment of Th22 cells to the site of disease. Indeed, soluble IL-22 has been shown to be elevated at the site of disease during pulmonary as well as extra-pulmonary TB (Scriba *et al.*, 2008; Matthews *et al.*, 2011b). Furthermore, IL-22 producing cells have been detected in the lungs and granulomas of rhesus monkeys with TB (Qiu *et al.*, 2008; Yao *et al.*, 2010). The majority of IL-22 producing CD4⁺ T cells express CCR6, a chemokine receptor that binds chemokine ligand MIP-3 α , mediating T cell homing to mucosal tissues during inflammation (Liao *et al.*, 1999; Acosta-Rodriguez *et al.*, 2007; Bunjun *et al.*, submitted). Interestingly, IL-22 concentrations were increased in the blood after anti-TB treatment, indicating that the absence of Mtb replication may result in rehoming of Th22 cells to the circulation (Zhang *et al.*, 2014). If indeed IL-22 is a sensitive marker for clearance of Mtb at the site of infection, the recirculation of IL-22 to blood could potentially be a biomarker for monitoring treatment response. This could be tested using longitudinal studies involving TB patients receiving anti-TB treatment, where IL-22 production from both the blood and the site of Mtb infection is measured throughout treatment.

HIV infection is the greatest risk factor for TB mortality (WHO, 2019). The hallmark of HIV infection is the progressive decrease in CD4⁺ T cells which is associated with an increased risk of TB (Geldmacher *et al.*, 2012). HIV is thought to preferentially infect and deplete Mtb-specific CD4 T cells (Geldmacher *et al.*, 2010), cause a decrease in polyfunctional CD4⁺ responses (Day *et al.*, 2008) and alter the equilibrium of Mtb-specific CD4⁺ subsets (Riou *et al.*, 2016). Studying HIV-induced defects in the immune response to TB could elucidate some aspects of the protective immune response to Mtb, which can be used for development of

effective TB vaccine. In this study, HIV infection resulted in reduced frequency and absolute Mtb-specific Th22 cells, especially in patients with TB. This is consistent with our recent findings (Bunjun *et al.*, submitted). This is not surprising as Th22 cells are thought to be highly permissive to HIV based on expression of HIV co-receptors and integrin $\alpha 4\beta 7$, and the lack of CCR5 ligands that induce autocrine protection against HIV infection (El Hed *et al.*, 2010; Kim *et al.*, 2012; Alvarez *et al.*, 2013; Sivro *et al.*, 2018). Preferential infection of Th22 cells can further be tested in longitudinal studies, by determining Th22 responses before HIV infection and at different time points after HIV infection. Other studies could infect Th22 cells with HIV *in vitro* or isolate Th22 cells from HIV-infected individuals/SIV-infected macaques to quantify integrated HIV DNA compared to other Th subsets in blood and tissues.

In Chapter 4, we investigated Mtb peptide-specific CD4⁺ and CD8⁺ T cell responses in LTBI, active TB and HIV infection. Consistent with previous studies, we show that CD8⁺ T cells are able to produce Th1 cytokines (IFN- γ and TNF- α), which have been shown to play an important role in effective control of Mtb infection (Rozot *et al.*, 2013; Amelio *et al.*, 2019). Again, consistent with published studies, these Mtb peptide-specific CD8⁺ responses were mostly detected in patients with TB, with fewer proportion in individuals with LTBI (Rozot *et al.*, 2013; Amelio *et al.*, 2019). We further report that HIV infection can impair memory differentiation of Mtb-specific CD8⁺ T cells, which could result in loss of CD8⁺ cytolytic activity (Appay *et al.*, 2000; Andersson *et al.*, 2007). Our results largely confirm data from previous studies and highlight the contribution of CD8⁺ T cells in TB immune responses. This compels the question of whether a TB vaccine will need to induce CD8⁺ responses to be protective against TB. Notably, several TB vaccines including BCG vaccine and candidate TB vaccines M72/AS01_E, VPM1002 (rBCG), AERAS-402 have been shown to elicit robust CD8⁺ T cell responses (Abel *et al.*, 2010; Penn-Nicholson *et al.*, 2015; Loxton *et al.*, 2017; Rodo *et*

al., 2019). Furthermore, CD8⁺ T cells were shown to be important in BCG vaccine-induced protection in rhesus macaques (Chen *et al.*, 2009). Therefore, is it likely that an effective TB vaccine may need to induce CD8⁺ responses.

This study had several limitations. We evaluated Mtb-specific responses in blood, and TB primarily affects the lungs. Therefore, it is important to determine whether the responses we detected in blood are consistent with those at the site of Mtb infection. Moreover, this was a cross-sectional study, and longitudinal studies investigating Mtb-specific responses in individuals who proceed to develop TB are warranted, to shed more light on Mtb immune responses. Future studies could also examine vaccine-induced Th22 cells as correlates of protection. Much remains to be determined regarding the role of Th22 cells in TB. Further studies are required to determine whether IL-22 is involved in direct killing of Mtb, or plays an indirect role in TB immunity; and conditions under which IL-22 may possibly play a protective or pathogenic role during Mtb infection.

In conclusion, we confirm that Th22 cells contribute substantially to the CD4⁺ T cell response to Mtb. IL-22 appears to be produced by conventional CD4⁺ T cells but IL-22 induction may have specific antigen presentation requirements. Interestingly, HIV infection during TB disease led to a near absence of Th22 cells in blood, suggesting that increased risk of TB during HIV infection could be in part due to depleted Th22 cells. Our results warrant further study of the role of Th22 cells in TB immunity, which may lead to insights that could assist with the further development of an effective vaccine against TB.

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APPENDIX

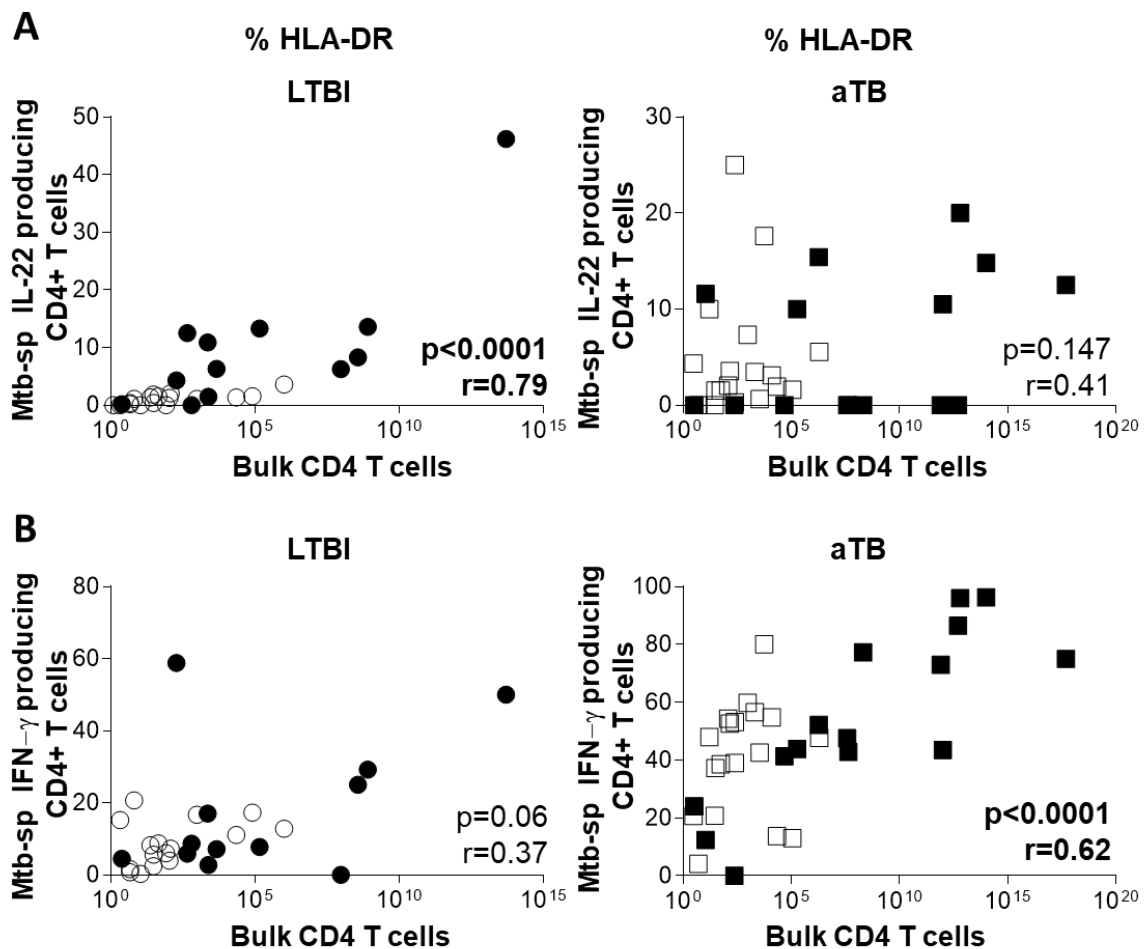


Figure S1: The relationship between Mtb-specific CD4+ T cell activation with total CD4+ T cell activation profile. The relationship between total CD4+ T cells expressing HLA-DR with **(A)** IL-22 producing CD4+ T cells and **(B)** IFN- γ producing CD4+ T cells expressing HLA-DR. Each dot represents an individual. Statistical analyses were performed using a non-parametric Spearman rank correlation.

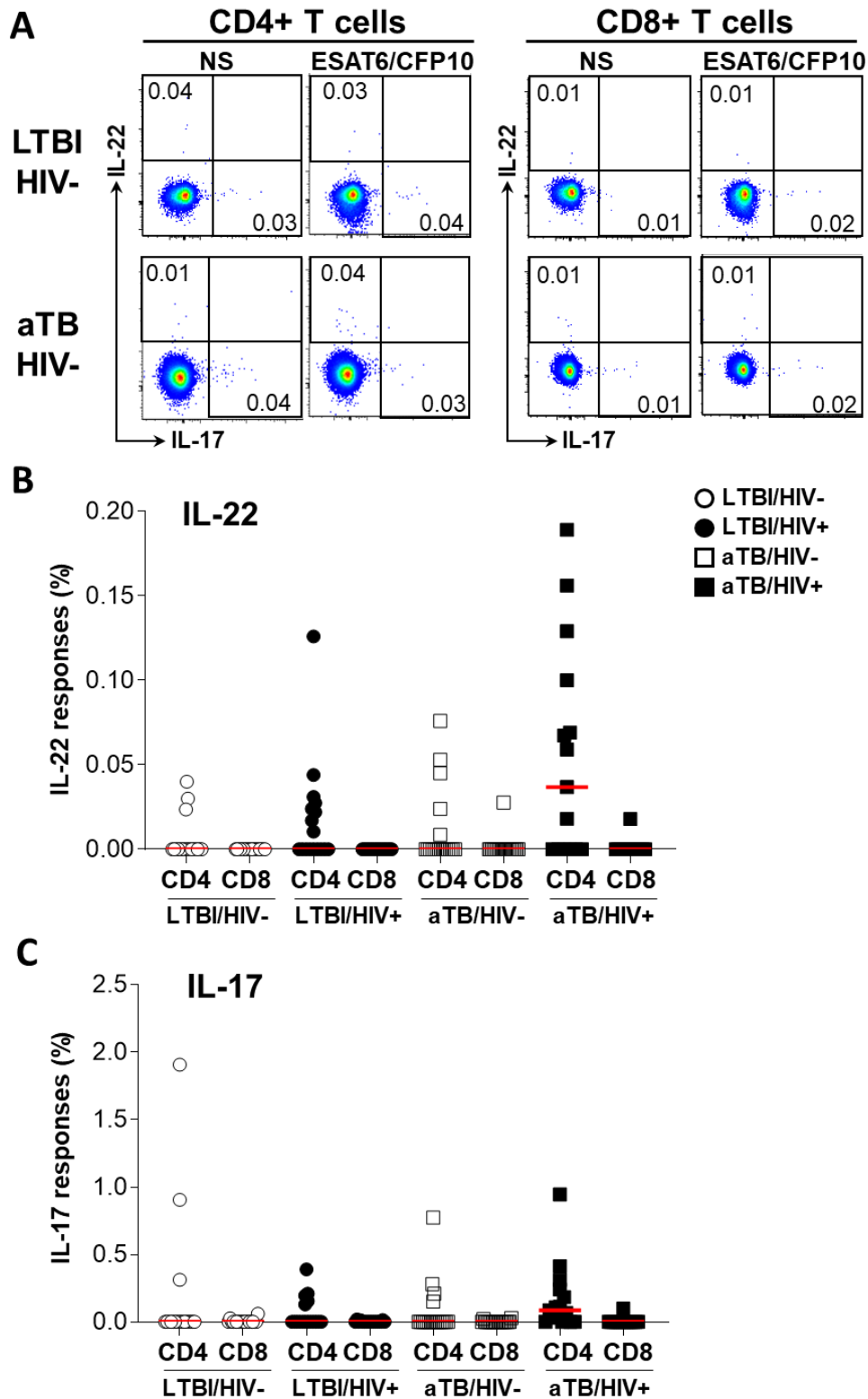


Figure S2: Comparison of the frequency of Mtb-specific CD4+ and CD8+ T cells in LTBI/HIV- (n=19), aTB/HIV- (n=18), LTBI/HIV+ (n=19), and aTB/HIV+ (n=15). (A) Representative flow cytometry plots showing IL-22 and IL-17 production by CD4+ and CD8+ T cells in response to stimulation with ESAT-6/CFP-10 peptide pool. Frequency of Mtb-specific CD4+ and CD8+ T cells producing (B) IL-22 and (C) IL-17 in four clinical groups. The bars represent the median.