CHARACTERIZATION OF CD8 T CELL RESPONSES IN *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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Declaration

I, Noella Moshi, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.
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Finally, I wish to thank my family, especially my grandmother Matesha.

Abbreviations

Activated cell autonomous death	ACAD
Activation induced cell death	AICD
Antibody	Ab
Antigen	Ag
Antigen presenting cells	APC
Bacillus Calmette-Guérin	BCG
Bone marrow derived cells	B cells
Broncho-alveolar lavage	BAL
Culture filtrate protein 10	CFP10
Cytomegalovirus	CMV
Cytotoxic T-lymphocyte	CTL
Dendritic cell	DC
Dimethylsulfoxide	DMSO
Early secretory antigenic target 6	ESAT6
Enzyme-linked immunospot assay	Elispot
Extensively drug-resistant	XDR
Fas-ligand	Fas-L
Forward scatter	FSC
Forward scatter area	FSC-A
Forward scatter height	FSC-H
Gravitational force	g
Hepatitis C virus	HCV
Human immunodeficiency virus	HIV
Immunoglobulins	Ig
Interferon	IFN
Interferon gamma release assays	IGRAs
Interleukin	IL
Intracellular cytokine staining	ICS
Latent tuberculosis infection	LTBI
Long term non-progressors	LTNP
Lymphocytic choriomeningitis virus	LCMV
Major histocompatibility complex	MHC

Median absolute deviation	MAD
Median fluorescence intensity	MFI
Mycobacterium tuberculosis	MTB
Multidrug-resistant	MDR
Natural killer cell	NK cell
Oregon Green (2', 7'-difluorofluorescein)	OG
Pathogen associated molecular patterns	PAMPs
Peripheral blood mononuclear cells	PBMC
Plasma cells	PCs
Polyvinylidene difluoride	PVDF
Restimulation induced cell death	RICD
Room temperature	RT
South African Tuberculosis Vaccine Initiative	SATVI
Side scatter	SSC
Side scatter area	SSC-A
Spot forming cells	SFCs
Staphylococcal enteretoxin B	SEB
Tuberculin skin test	TST
Tumor necrosis factor	TNF
Toll-like receptors	TLRs

Overall Summary

Vaccination with BCG has variable efficacy in protection against pulmonary tuberculosis (TB), thus highlighting the critical need for development of more effective TB vaccines. Increasing evidence indicates a role for CD8 T cells in controlling chronic infections, hence novel TB vaccines may benefit from induction of CD8 T cell responses. The aim of this project was to compare the breadth and magnitude of CFP10 and ESAT6-specific CD8 T cell responses in individuals with latent *Mycobacterium tuberculosis* (MTB) infection (LTBI) and active TB disease, and further define MTB-specific CD8 T cell phenotypes associated with latent infection and active disease. *Ex vivo* IFNγ Elispots and proliferation assays were used to identify immunodominant ESAT6 and CFP10 15mer peptides targeted by CD8 T cells in LTBI and TB donors. A multiparameter flow cytometry panel was designed and optimized to assess turnover, susceptibility to apoptosis and terminal differentiation/senescence in CD8 T cells from TB and LTBI donors. Bcl-2, Ki67, CD95, CD57, CD127 and IFNγ were thus measured in each group. A subset of TB donors were analyzed longitudinally at <1week, 2 months, and 6 months after initiation of anti-TB treatment. CD8 T cells in patients with TB exhibited poor proliferative capacity, compared with LTBI. CD8 T cells in LTBI donors recognized a significantly greater breadth of CFP10 peptides than TB donors, however there was no difference between LTBI and TB in the breadth of recognition to ESAT6 peptides. Bcl-2 and CD57 expression were increased in the total CD8 T cell population in individuals with TB disease. CD127 expression was increased on MTB-specific CD8 T cells, relative to the total CD8 T cell population, in LTBI but not TB donors. Results from longitudinal data suggest that initiation of anti-TB treatment may reverse the phenotype seen in TB disease. Together these data suggest that differential recognition of CFP10 regions by CD8 T cells may distinguish between LTBI and TB disease. Additionally, the greater proliferative capacity of MTB-specific CD8 T cells in LTBI donors suggests differential functional capacities of MTB-specific CD8 T cells in latent and active TB disease. Overall, MTB-specific CD8 T cells in TB have a phenotype indicative of reduced functional capacity and higher susceptibility to apoptosis, relative to MTB-specific cells in LTBI, suggesting MTB-specific CD8 T cells may be impaired in the context of high mycobacterial antigen loads in active TB disease.

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CHAPTER ONE

Literature Review

1.0 Overview

Mycobacterium tuberculosis (MTB) is responsible for approximately 1.7 million deaths worldwide (WHO 2010). 90% of MTB-infected individuals remain asymptomatic, providing strong evidence that the host immune response is usually successful in containing the infection. Although specific components of host cellular immunity that are responsible for successful containment of bacterial replication are not fully understood, increasing evidence from both human and non-human primate models indicate a significant role for CD8 T cells in successful immune control of MTB. However, a paucity of data remains regarding the epitope specificity, functional capacity and phenotype of MTB-specific CD8 T cells that are associated with successful immune control of MTB infection. In other chronic infection models, antigen-specific CD8 T cells have been shown to undergo a process of functional exhaustion, characterized by decreased cytokine production, cytotoxic activity, and proliferative capacity. It is not known whether MTB-specific CD8 T cells are similarly functionally exhausted in individuals with chronic MTB infection, thereby contributing to loss of immune control and progression to symptomatic, active disease. The underlying hypothesis of this study is that successful immune control of MTB infection is associated with maintenance of highly functional MTB-specific CD8 T cells. The specific aims were to identify MTB epitopes targeted by CD8 T cells, and to characterize the phenotype of MTB-specific CD8 T cell responses in individuals with controlled and uncontrolled MTB infection.

1.1 The innate immune system

The first line of defense against invading pathogens is the innate immune system. It detects highly-conserved pathogen associated molecular patterns (PAMPs), and mounts a rapid, short-lived response (Iwasaki and Medzhitov 2010). Upon pathogen clearance, cells of the innate immune system retain no memory of the pathogen hence their response to a second invasion will be similar in magnitude and duration. The first barrier by the innate immune system consists of antimicrobial compounds found in fluids such as tears, mucus and sweat. The skin also provides a physical barrier against pathogens. If these mechanisms are traversed, the pathogen will encounter inflammatory proteins in the serum that may neutralize a pathogen before it invades any cells. For example, such proteins may bind to the surface of the pathogen, making it more susceptible to phagocytosis, in a process known as opsonization. Monocytes, macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells are cells of the innate immune system.

Monocytes are phagocytic leukocytes that are present in the blood. They are precursors of differentiated tissue-specific macrophages, which along with DCs also possess phagocytic properties. DCs and macrophages reside in interstitial spaces, for example near the surface of the skin. Upon encounter with a pathogen, these cells will recognize PAMPs via toll like receptors (TLRs), which can be found on their cell surface (to recognize extracellular pathogens) or intracellularly (to recognize viruses). Signaling through TLRs leads to cell activation and maturation. Chemo-attractants secreted by activated macrophages will recruit polymorphonuclear granulocytes known as neutrophils to the site of infection. Upon ingestion of the pathogen, both macrophages and neutrophils undergo a respiratory burst, which fuels the release of bactericidal molecules such as reactive oxygen species. In addition to killing the pathogen, the release of these molecules plays a role in cell signaling (Forman and Torres 2002). The development of homing receptors occurs during DC maturation and allows DCs to travel to areas where there is a high concentration of cells of the adaptive immune system, such as T cells. Mature DCs will increase IL-12 and IL-15 production causing proliferation of naïve T cells and increased activation of DCs, respectively (Schluns and Lefrancois 2003). Mature DCs will also upregulate expression of major histocompatibility (MHC) molecules,

which are required for antigen presentation to naïve T cells. Activated DCs form a chemokine gradient that attracts other cells to the site of infection, including T cells. In this way, DCs serve as an important link between the innate and the adaptive immune system (Vivier, Raulet et al. 2011).

While DCs link the innate and adaptive immune system, NK cells are said to possess properties of both. NK cells are rapidly responding effector cells with cytolytic capacity. NK cells are biased to produce IFNγ in response to antigen encounter, however they also produce TNFα. Both of these cytokines assist in DC maturation. In the case of chronic antigen stimulation, NK cells have been known to produce IL-10 (Vivier, Raulet et al. 2011). NK cells will respond to cells displaying markers of infection by facilitating apoptosis of the target cell. NK cells are traditionally classified as cells of the innate immune system, however recent evidence has shown that they are capable of responding more rapidly to previously encountered antigen (Sun, Beilke et al. 2009), a characteristic typical of the adaptive immune system.

1.2 The adaptive immune system

The adaptive immune system evolved to counter pathogens that managed to evade the innate immune system. It is distinguished by its later response time, its ability to recognize a wide range of un-conserved pathogen-derived molecules, and its capacity to mount a more rapid response upon a second exposure to the same pathogen; i.e. immune memory. There are two arms of the adaptive immune system, including humoral and cell-mediated immunity.

1.2.1 Humoral response

The humoral immune response is mediated by antibodies (also known as immunoglobulins; Ig), which are secreted by bone marrow derived cells (B cells). Prior to antigen exposure, B cells will develop relatively moderate antigen-specificity via rearrangement of genes encoding for antibody segments. They then exit the bone marrow as immature B cells. These immature B cells will develop into naïve B cells, expressing two classes of antibodies, IgD and IgM, as they circulate through

the body. There are five known classes or isotypes of antibodies: IgG, IgM, IgE, IgA and IgD, which are composed of a heavy chain and a light chain. There are four genes encoding for the heavy chain (V_H, D, J_H, and C_H), which may produce nine different types of heavy chain protein. There are three genes encoding for the light chain region (V_L, J_L, and C_L) which may produce two types of light chain protein (Bonilla and Oettgen 2010).

The second phase of B cell development occurs during their circulation through secondary lymphoid organs such as the spleen, mucosal associated lymphoid tissue, and lymph nodes. This second phase is antigen-dependent. Most antigens require T cell help to elicit a B cell response. A sequence of two signals occurs, firstly to allow B cells to develop the capacity to interact with T cells, and secondly to allow B cells to present antigen on their cell surface to CD4 T helper cells. Once these two signals are complete, B cells will clonally expand to produce antibodies with a moderate affinity to the antigen. The dose of antigen and its binding affinity determine the magnitude of B cell expansion (Elgueta, de Vries et al. 2010), and whether cells will differentiate into memory B cells, long lived plasma cells (PCs) or short lived PCs. Sufficient antigen exposure will cause some activated B cells to travel to the follicle and establish a germinal centre. In the germinal centre, B cells producing IgM and IgD antibodies may undergo an isotype change through VDJ gene rearrangement, to produce other types of antibodies such as IgA, IgE and IgG. This process is known as class switching. In the variable regions of heavy and light chain genes (V_H and V_L), somatic hyper-mutation occurs concurrently with class switching. This leads to the generation of antibodies with a higher affinity for the antigen and is known as affinity maturation. Secreted antibodies are involved in the complement system, which is a cascade of reactions designed to lead to the clearance of an extracellular pathogen. The antibodies bind to their target and mediate its phagocytosis by innate cells. Antibodies can also cause chemotaxis of other cells of the immune system, and inflammation.

After approximately two weeks of initial exposure to an antigen, B cells will synthesize high affinity antibodies. If re-infection occurs, high affinity antibodies can be synthesized within one week of exposure, due to the memory capacity of these cells. Upon secondary exposure to antigen, long-lived PCs differentiate into short-

lived effector cells. Memory B cells are postulated to differentiate into (and hence replenish) the long lived PC population (Bonilla and Oettgen 2010).

1.2.2 Cell-mediated immunity

CD4 and CD8 T cells are responsible for cell-directed, highly specific immune responses by the adaptive immune system. The high antigen-specificity achieved by somatic recombination of T cell receptor (TCR) genes to generate TCR diversity involves a process analogous to Ig gene rearrangement. Immature T cells originate in the thymus where they undergo a selection process, which eliminates self-reactive cells. They circulate through the body where they may encounter foreign antigens in the peripheral lymphoid organs. Exposure to an MHC-peptide complex that is bound by their TCR promotes maturation into a particular effector subset, as dictated by the cytokine environment. Two signals are necessary for the activation of naïve T cells: the first signal is the interaction between the TCR on a T cell and the MHC-antigen complex on an APC. The second signal involves co-stimulatory molecules, specifically B7.1 or B7.2 on the APC, and CD28 on the T cell. Without this second signal, T cells binding to the MHC-antigen complex will become anergic. Once a T cell is activated, cytokine production by APCs provides a third signal that directs T cell differentiation into various effector subsets (Williams and Bevan 2007).

There are two main types of conventional T cells: CD8 T cells and CD4 T cells. A definitive difference between them is their recognition of antigens bound to either MHC I or MHC II, respectively. MHC I binds peptides from cytosolic antigens. These peptides have been synthesized in the cytoplasm via proteosome-mediated degradation of cytosolic proteins. MHC I peptides are usually eight to ten amino acids long, however recent research shows that CD8 T cells can recognize peptides displayed by MHC I that are up to 25 amino acids long (Bell, Burrows et al. 2009). MHC II binds peptides from extracellular antigens; the peptide length is more flexible than MHC I because of structural differences in the peptide binding groove. Extracellular pathogens or antigens are internalized within phagosomes that fuse with lytic vesicles to form phagolysosomes. This process results in the digestion of the antigen in APCs. Digested peptide fragments are bound to MHC II and displayed

on the cell surface of professional APCs. In contrast, MHC I is expressed on all somatic cells. Both CD4 and CD8 T cells can recognize super-antigens such as *Staphylococcal* enterotoxin B; these super-antigens may bind to MHC II molecules that are already bound to peptide, or even bind directly to the T cell receptor, causing T cell activation (Adachi and Davis 2011).

A similarity between CD4 and CD8 T cells is their differentiation and memory properties. In the event of a second exposure to a pathogen that causes an acute infection, memory T cells will differentiate into a rapidly expanding effector cell population and home to the site of infection (Sallusto, Geginat et al. 2004; Janossy, Barry et al. 2008). The resolution of an acute infection results in clearance of the pathogen and correlates with contraction of the effector cell population. The surviving T cells develop a central memory or effector memory phenotype, which differ in their response to re-encounter with antigen. Figure 1.1 shows effector T cell differentiation during an acute infection and generation of memory T cell responses. Central memory cells are thought to re-circulate in secondary lymphoid organs more readily than effector memory cells. They proliferate rapidly and differentiate into effector memory cells upon re-encounter with antigen. Effector memory cells have reduced proliferative capacity and can produce signaling molecules such as cytokines that activate other cells to neutralize the pathogen (Janeway 2005; Williams and Bevan 2007). The effector functions differ between CD4 and CD8 T cells, and even within subsets of each cell type. To summarize, the difference between memory and naïve T cells is that memory cells have a more rapid and robust response to antigen stimulation (Sallusto, Geginat et al. 2004). Central memory and effector memory T cell populations can be found in the blood after an infection has been cleared. They are maintained by homeostatic cytokines such as IL-7 and IL-15 that allow cells bearing their cytokine receptor to remain viable.

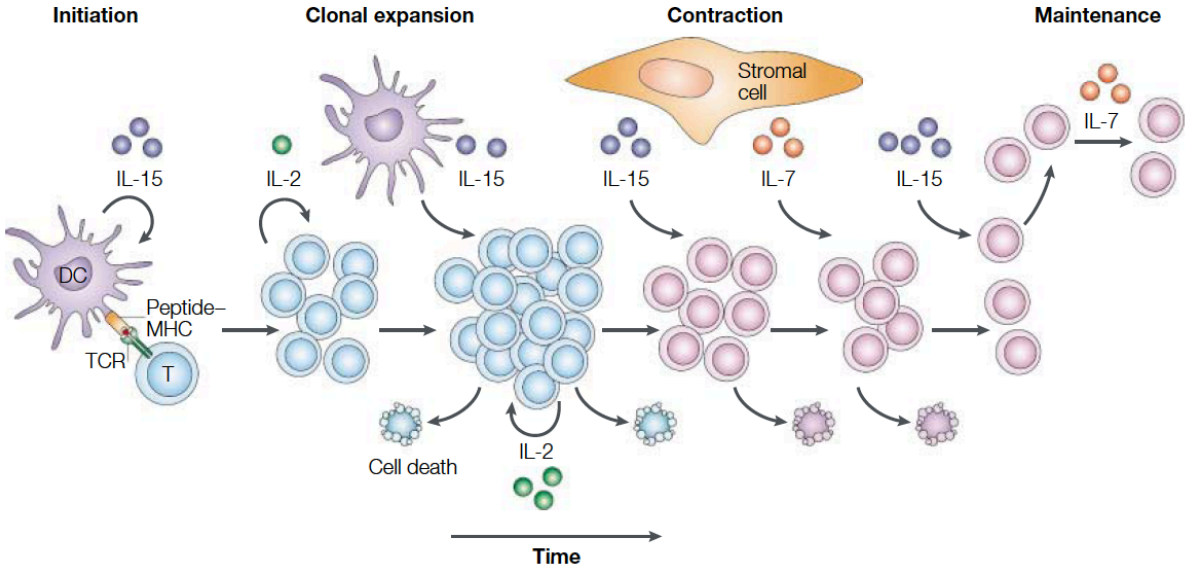


Figure 1.1. T cell differentiation in an acute model of infection. IL-15 promotes activation of DCs presenting antigen to T cells on MHC molecules. Activated T cells will clonally expand, at least partly due to signaling mediated by IL-2. IL-15 may cause further expansion and activation. With time, the infection is resolved and the T cell population contracts to become a small pool of long-lived memory cells that are maintained by IL-7. IL-15 is also responsible for memory cell turnover. Taken from (Schluns and Lefrancois 2003).

CD4 T cells are also known as helper T cells. There are various CD4 T cell effector subsets, which play different roles in modulating the immune system. T_h1 CD4 cells recognize antigens displayed on MHC II molecules on the surface of APCs and respond by producing IFNγ. This cytokine activates macrophages and enhances phagocytosis and intracellular killing of pathogens (Flynn and Chan 2001; North and Jung 2004; Grotzke and Lewinsohn 2005). T_h1 cells also assist in priming and maintenance of CD8 T cell responses as shown in viral models of infection (Flynn and Chan 2001; Ahlers and Belyakov 2010; Sharma, Hou et al. 2010). Other well characterized CD4 T cell subtypes are responsible for B cell activation (T_h2 cells), eliminating fungi and extracellular bacteria (T_h17 cells), and suppressing effector cells (regulatory T cells) (Zielinski, Corti et al. 2011). Some CD4 T cells are known to secrete granulysin and perforin, which are responsible for apoptosis of infected target cells (Flynn and Chan 2001). Upon exposure to antigen, naïve CD8 T cells differentiate into effector cells with the ability to kill target cells. They do so via the release of cytotoxic molecules such as granzymes and perforin (Seder and Ahmed 2003; Cellerai, Perreau et al. 2010) and cytokines such as IFNγ and TNFα (Seder

and Ahmed 2003), which are responsible for cell-targeted damage (Ratner and Clark 1993).

The phenotype of CD8 T cells differs between acute (where antigen is cleared) and chronic (where antigen persists) infections. Human immunodeficiency virus (HIV), lymphocytic choriomeningitis virus (LCMV) and hepatitis C virus (HCV) are examples of chronic viral infections in which persistent antigen stimulation causes antigen-specific CD8 T cells to become less functional with time in a process known as T cell exhaustion (Yi, Cox et al. ; Williams and Bevan 2007). Additionally, the long-lived memory T cell population is thought to deteriorate in the event of continual antigen exposure, and this is reflected by reduced IL-7 receptor expression on the cell surface (Singh, Gowthaman et al. 2010). In mouse models of chronic viral infections, there is a stepwise loss of T cell function as they become exhausted. T cells producing multiple cytokines such as IFNγ, IL-2 and TNFα will eventually lose their cytokine production capacity, remaining with only the capacity to produce IFNγ (Fuller, Khanolkar et al. 2004). The exhausted T cells will constitutively express inhibitory receptors, such as PD-1, Tim-3 and Lag-3, and expression of these regulatory molecules have been associated with diminished proliferative capacity and T cell dysfunction (Yi, Cox et al. 2010). As the chronic infection persists, the population of antigen-specific T cells will deteriorate due to the deletion of exhausted T cells (Yi, Cox et al. 2010). CD8 T cells in a mouse model of chronic infection display reduced target cell killing compared to an acute model of the same infection. However, the reduced cytotoxicity in chronic infection may be antigen-dependent (Fuller, Khanolkar et al. 2004).

Studies of other chronic infections have identified markers of exhaustion, apoptosis and replicative senescence, including PD-1, CTLA-4, CD95 and CD57 (Moss and Khan 2004; Sabbaj, Heath et al. 2007; Wherry, Ha et al. 2007; Blackburn, Shin et al. 2009; Wood, Twigg et al. 2009). PD-1 is upregulated in exhausted CD8 T cells, and blockade of PD-1 signaling has been found to restore proliferative capacity in a mouse model of LCMV (Barber, Wherry et al. 2006; Day, Kaufmann et al. 2006). CD57 is up-regulated on antigen-specific cells, and its expression is associated with immunosuppression (Wood, Twigg et al. 2009). CTLA-4 mRNA is over-expressed in exhausted CD8 T cells in a LCMV mouse model (Wherry, Ha et al. 2007). However, CTLA-4 blockade does not result in the restoration of function (Barber, Wherry et al.

2006). In HIV infection, antigen-specific CD8 T cells have increased susceptibility to activation induced cell death via CD95/Fas pathway (Petrovas, Casazza et al. 2006). Figure 1.2 illustrates the gradual deletion of antigen-specific T cells in a chronic viral infection model.

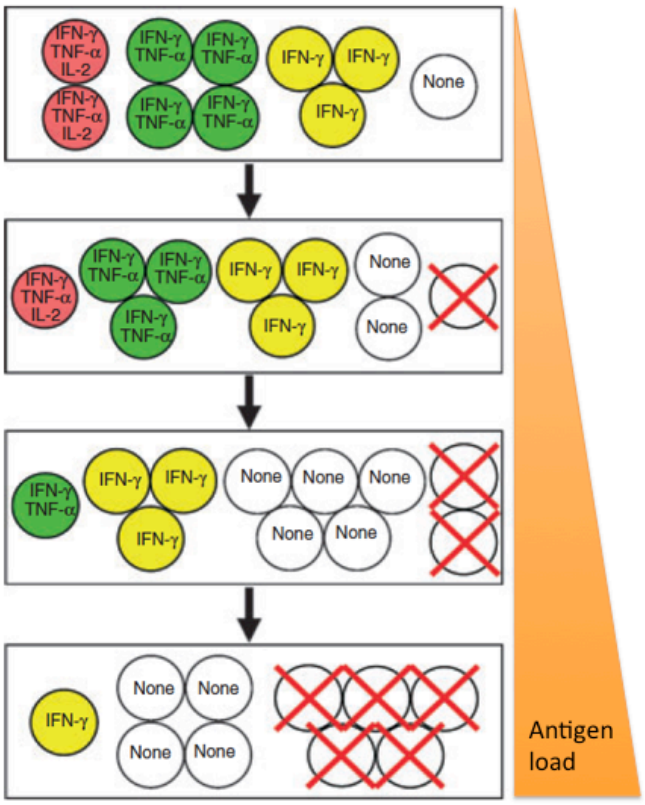


Figure 1.2. The effect of antigen load on the cytokine profile of T cells. Antigen-specific cytokine producing CD8 T cell populations decline in number with increasing antigen load in a chronic infection model. The capacity to produce multiple cytokines simultaneously (polyfunctionality) is also lost, causing the composition of the antigen-specific T cell population to change. Taken from (Yi, Cox et al. 2010).

1.3 Tuberculosis Epidemiology

Tuberculosis (TB) is a major global health problem, with over 9 million new cases of TB per year, and over 1.6 million deaths per year (WHO 2010). Upon infection with *Mycobacterium tuberculosis* (MTB), 10% of individuals will develop TB disease (Ottenhoff 2009); approximately 5% will develop TB within 2 years and 5% will develop TB later in life (WHO 2009). The majority of MTB-infected individuals will remain asymptomatic with latent infection (LTBI), characterized by containment of

the bacteria within granulomas (Algood, Chan et al. 2003). Within groups at high risk of infection, only 22% to 33% of new cases are due to recent transmission of MTB, implying that reactivation of asymptomatic latent infections are responsible for a large proportion of new TB cases (van Pinxteren, Cassidy et al. 2000). HIV coinfection further increases the rate of reactivation (WHO 2009).

TB is particularly endemic to African regions, as shown in Figure 1.3. In South Africa, the incidence of TB in 2009 was 948 per 100 000 per year, which is one of the highest incidence rates in the world and includes a rapidly increasing frequency of infections with multi-drug resistant (MDR) and extensively drug resistant (XDR) MTB strains (Johnson, Warren et al. 2010). In 2009, there were 230 deaths due to TB per 100 000 per year, making TB the most common notified natural cause of death in South Africa (Karim 2009).

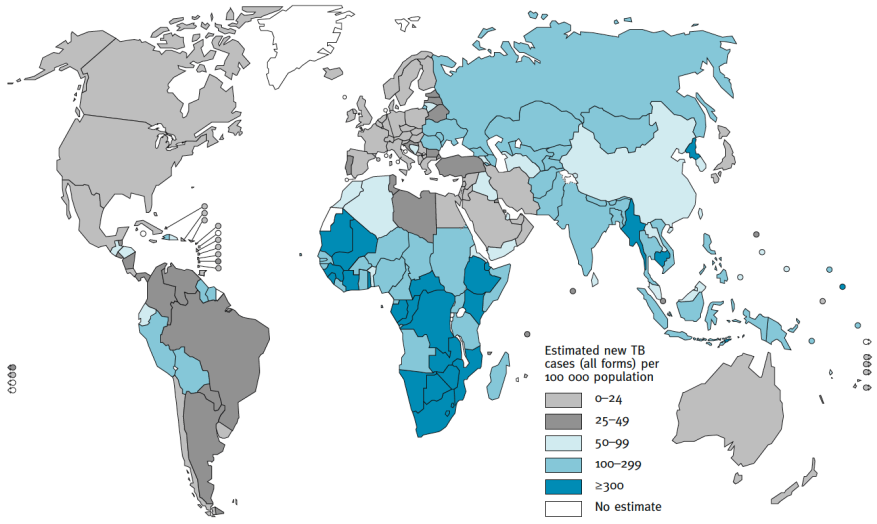


Figure 1.3. Estimated TB incidence rates by country 2009. South Africa has one of the highest TB incident rates in the world. Taken from (WHO 2010).

The failure to complete short course treatment exacerbates the TB problem, leading to the development of MDR and XDR strains. Resistance to both isoniazid and rifampicin as found in MDR TB means that first line treatment is ineffective on these isolates, and could lead to the development of other drug resistant strains such as XDR TB which, in addition to isoniazid and rifampicin resistance, is also resistant to a fluoroquinolone and a second-line injectable drug (Becerra, Appleton et al. 2011). Approximately 20% of all new incident TB cases globally are estimated to have MDR TB, but only a fraction of these are reported (WHO 2010). Better diagnosis and

treatment are urgently needed to combat the spread of MDR and XDR TB.

The global burden of TB disease is being addressed by the WHO millennium development goals, which aim to reverse the incidence of TB by 2015 (WHO 2010). In spite of this, TB case notification rates have risen nearly four times in the last twenty years (Karim 2009). One cause for this is HIV co-infection, which is known to play a role in the development of TB disease (Figure 1.4). HIV/TB co-infection is particularly endemic to non-industrialized countries (Corbett, Watt et al. 2003). The WHO African region accounted for 80% of the HIV-associated TB incident cases globally (WHO 2010).

A key area of focus in combating the TB epidemic is vaccine development. The only currently licensed TB vaccine, Bacillus Calmette-Guérin (BCG), provides inadequate protection against pulmonary TB. HIV-infected infants have an increased risk of disseminated BCG disease (Hesseling, Johnson et al. 2009). Novel TB vaccines are currently being developed and tested in phase I and II clinical trials. AERAS-402 is currently in phase II trials in Kenya (Abel, Tameris et al. 2010); MVA85A (Scriba, Tameris et al. 2010) and M72 are currently in phase II trials in South Africa.

1.4 Diagnosis of MTB infection

Exposure to MTB may lead to clearance or to persistent infection. Established MTB infection may result in development of clinical disease or asymptomatic latent infection (LTBI). LTBI has been described as “immunological sensitization to antigens synthesized by MTB in the absence of clinical symptoms” (Barry, Boshoff et al. 2009). Tuberculin skin tests (TST) have been used to identify MTB-infected individuals. Upon injection of mycobacterial proteins under the skin, memory T cell responses to the bacteria are detected in the form of redness and swelling at the site of injection. TST positivity is not always a reliable measure of MTB infection, as non-tuberculous mycobacteria, a history of cured TB disease, or even BCG vaccination could result in a false positive; immunosuppression could result in a false negative. To address this problem, several more sensitive and specific IFNγ release assays

(IGRAs) have recently been developed, including the T-SPOT.TB Elispot assay (Oxford Immunotec, Oxford, UK), and QuantiFERON®-TB Gold In-Tube (QFN; Cellestis Limited, Carnegie, Victoria, Australia) ELISA assay (Lalvani and Pareek). Both IGRA assays detect IFNγ production by T cells in response to highly immunogenic MTB proteins, namely early secretory antigenic target 6 (ESAT6) and culture filtrate protein (CFP10) (Lalvani, Brookes et al. 1998; Pathan, Wilkinson et al. 2000; van Pinxteren, Cassidy et al. 2000; Lewinsohn, Zhu et al. 2001; Caccamo, Meraviglia et al. 2006; Lalvani and Pareek 2010), as well as TB7.7 in the QFN assay.

Both ESAT6 and CFP10 are expressed by MTB, but not BCG, which allows for the distinction between immune responses induced by MTB infection versus BCG vaccination. They belong to the ESAT6 family of proteins, all of which are approximately 100 amino acids long (Brodin, Rosenkrands et al. 2004). Strong cellular immune responses to ESAT6 and CFP10 have been found in both animal and human models of infection, with a vast majority of TB diseased individuals recognizing one or both of these proteins (Grotzke and Lewinsohn 2005). ESAT6 is known to induce host membrane lysis as well as specific cytokine responses, such as IFNγ production (Grotzke and Lewinsohn 2005; de Jonge, Pehau-Arnaudet et al. 2007; Stanley, Johndrow et al. 2007). For this reason, both ESAT6 and CFP10 are relevant when measuring MTB-specific responses in humans using IGRAs.

Pulmonary TB disease is more readily detected due to the presence of clinical symptoms such as coughing for over two weeks, night sweats, weight loss, reduced appetite, fatigue and weakness. Nonetheless, clinical symptoms alone are insufficient for a TB diagnosis. The current gold standard for diagnosis is the culture of sputum from a patient in order to grow out MTB that may be present. Another popular method of TB disease detection is PCR amplification of bacterial DNA (Yang and Rothman 2004).

After diagnosis of TB disease, patients are prescribed anti-TB medication, which decreases their antigen load thus altering the dynamics of the host immune response (Barry, Boshoff et al. 2009; Russell, Barry et al. 2010). Standard short course chemotherapy guidelines for TB treatment in South Africa prescribe the administration of rifampicin, isoniazid, pyrazinamide, and ethambutol daily for two

months, followed by rifampicin and isoniazid administration daily for 4 months (Department of Health 2009).

Distinguishing between latent infection and active TB disease on an immunological basis is difficult, as current scientific opinion points to a spectrum of latent infection (Dheda, Schwander et al. 2010). This spectrum ranges anywhere from low levels of inactive bacteria effectively contained by immune cells, to the presence of replicating bacteria in the absence of clinical symptoms. Factors such as number and size of granulomas, rate of bacterial replication, and clinical symptoms vary between infection and disease states (Barry, Boshoff et al. 2009). Figure 1.4 portrays MTB infection as a spectrum of infection and disease. Little is known about factors contributing to the transition from latency to active disease (Flynn, Hanson et al. 2010), hence it is necessary to analyze in detail the host immune response to MTB infection that is either controlled (latent) or uncontrolled (active disease).

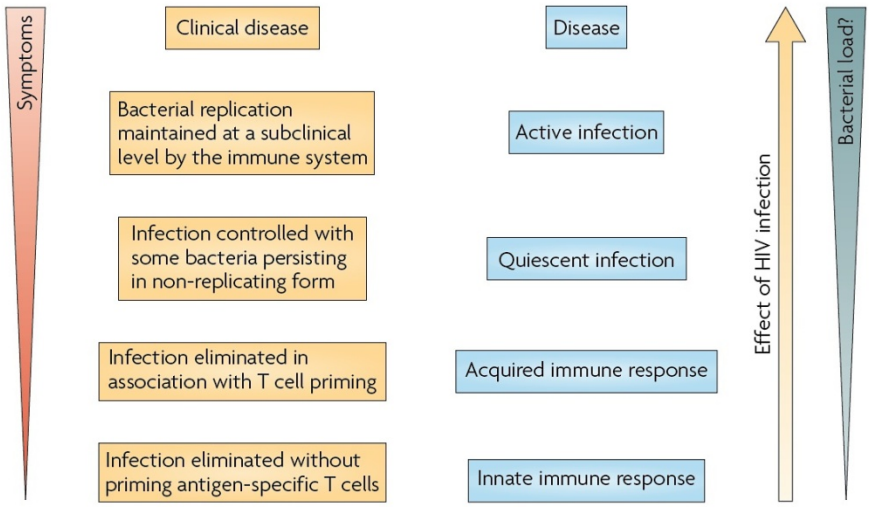


Figure 1.4. Tuberculosis is a spectrum from infection to disease. Increasing bacterial load is purported to cause the change from quiescent infection to clinical disease. HIV is also thought to adversely influence disease progression. Taken from (Barry, Boshoff et al. 2009).

1.5 Pathogenesis and the adaptive immune response to MTB

MTB is found in aerosol droplets that are transmitted when a person with TB disease coughs. Once the bacilli have entered the body they are recognized by alveolar macrophages via TLRs and phagocytosed. Phagolysosome formation will result in the destruction of most phagocytosed pathogens. However, MTB has been shown to prevent phagolysosome formation as a mechanism of immune evasion (Winau, Hegasy et al. 2005). The macrophage mounts a pro-inflammatory response, which includes recruitment of mononuclear cells and increased vascularization at the site of infection. A tightly packed group of cells is formed in order to contain bacterial replication. Cells of the adaptive immune system become part of this cell cluster; it develops a structure and is known as a granuloma. CD4 T cells within granulomas may recognize MTB antigens displayed on the macrophage cell surface and mount a T_h1 response involving IFNγ production and the priming and maintenance of CD8 T cells. Additionally, some CD4 T cells will secrete granulysin and perforin (Flynn and Chan 2001). However, due to inflammation and increased cell necrosis, the structure of the granuloma can become compromised, and rupture to free the bacteria within it (Russell, Barry et al. 2010).

Research on the role of the adaptive immune system in containing MTB has largely focused on cellular immunity, and several previous studies in humans have compared the immune response of latently infected individuals to the response in individuals with TB disease (Flynn 2004). With regard to antigen-specificity, 'latency-associated antigens' such as Rv2660 and Rv2659 have been identified, and are preferentially recognized by CD4 T cells of MTB infected individuals with no clinical symptoms of disease (Govender, Abel et al. 2010). Cytokine production has been extensively compared, and differential polyfunctional cytokine production capacity has been shown by three recent studies. One paper demonstrated an increased frequency of IL-2, IFNγ, and TNFα triple-producing CD4 T cells in TB disease compared to LTBI, when stimulated by various MTB antigens (Ag85B, 16KDa, ESAT6) in both long-term and short-term assays (Caccamo, Guggino et al. 2010). This paper also found significantly more IL-2+IFNγ+ CD4 T cells in LTBI compared to TB disease, and suggested that this was a result of lower antigen load in the former. Recently, Harari *et al* published contrasting results showing that MTB-

specific single-positive TNFα-producing CD4 T cells were the dominant population in peripheral blood of TB diseased patients, and that their increased frequency could be used to discriminate between active disease and latent infection (Harari, Rozot et al. 2011). This finding is echoed by Day *et al*, who show that patients with smear positive TB have reduced frequencies of polyfunctional CD4 T cells and increased TNFα single positive CD4 T cells compared to latently infected individuals (Day, Abrahams et al. 2011) It is possible that the contrasting results could be due to differences in the patient cohorts used. Another group has also shown increased frequencies of polyfunctional and TNFα single-positive CD4 T cells in TB cases compared to exposed household contacts (Sutherland, Adetifa et al. 2009). In contrast, there has been relatively minimal research on CD8 T cells in this disease model, primarily because the method by which CD8 T cells recognize MTB antigens was a subject of speculation for some time (Orme 1987; Flory, Hubbard et al. 1992; Seder and Ahmed 2003; Chen, Huang et al. 2009).

1.5.1 CD8 T cell responses in MTB infection

Previous research on the role of CD8 T cells in combating MTB has involved both mouse and non-human primate models of infection. Van Pinxteren *et al* showed the importance of CD8 T cells in maintaining latency in a mouse model. Latency was established by treating aerosol-infected mice with anti-TB drugs in order to maintain a low bacterial load, and reactivation was simulated by removing the drug treatment. *In vivo* CD8 T cell depletion during the latent period resulted in a 10-fold increase in bacterial numbers, whereas CD4 T cell depletion produced more modest changes (van Pinxteren, Cassidy et al. 2000). It is important to note that murine studies are limited in their applicability to MTB infection in humans, as both the disease pathology and dose of infection differ (Mestas and Hughes 2004).

Non-human primates are more relevant models of MTB infection, as approximately 50% of infected non-human primates establish latency and 50% develop active TB disease, with a disease course and pathology similar to that seen in humans (Chen, Huang et al. 2009). CD8 T cell depletion in rhesus macaques demonstrated that CD8 T cells are important in vaccine-mediated protection from TB

disease and in protecting against re-infection. BCG vaccinated macaques were depleted of CD8 T cells *in vivo*, and then challenged with MTB. Upon challenge, these macaques displayed reduced immunity to MTB compared to un-depleted controls. In a separate experiment, cured macaques were CD8-depleted and then re-infected with MTB. The CD8-depleted animals were significantly more likely to re-develop TB disease compared to the un-depleted controls (Chen, Huang et al. 2009; Weerdenberg 2009). Although animal models are relevant and informative, it is ideal to perform studies using a human model of MTB infection.

1.5.1.1 Antigen recognition and epitope specificity

By virtue of the pathogen's intracellular location, there is a clear-cut mechanism for CD4 T cell activation via MHC II antigen presentation. On the other hand, classical CD8 T cells recognize peptides presented by MHC I molecules. One of the theories that accounts for presentation of MTB antigens to CD8 T cells via MHC I (cross-presentation) is the translocation of mycobacterial proteins from the phagolysosome of an APC to the cytosol, where proteosome-mediated degradation occurs, following transport to the endoplasmic reticulum and loading onto MHC I (Weerdenberg 2009). However, non-translocating mycobacteria such as BCG are also known to induce CD8 T cell mediated immunity (Weerdenberg 2009). Another theory for explaining cross-presentation involves apoptosis of the infected innate cell (Winau, Hegasy et al. 2005). During the apoptotic process the infected cell releases vesicles containing protein and lipid components of MTB, which are taken up by uninfected by-stander APCs and processed as intracellular components via the MHC I pathway (Schaible, Winau et al. 2003).

In humans, CD8 T cells specific for MTB peptides have been identified using high throughput IFNγ release assays. In one publication, CD4-depleted cells were used to generate MTB-specific T cell lines from infected individuals (both latent and active disease). CD8 epitopes were identified using an IFNγ Elispot assay, which showed a greater frequency of responses to CFP10 peptides compared to ESAT6 peptides (Lewinsohn, Winata et al. 2007). CFP10 responses were also more variable than ESAT6 responses with reference to the amount of cytokines produced.

Because this study was not performed *ex vivo*, it was difficult to determine the *in vivo* frequency or magnitude of CD8 T cell responses to these peptides. Another study performed epitope mapping of ESAT6 in patients with active TB disease using short-term cell lines, and found that the peptides corresponding to amino acids 69-76 and 82-90 of ESAT6 were preferentially recognized (Lalvani, Brookes et al. 1998). A previous study has identified an immunodominant response in the C-terminus of CFP10 in two CD8 T cell clones from one donor (Lewinsohn, Zhu et al. 2001).

1.5.1.2 Effector functions

Many studies identify antigen-specific T cells by IFNγ production following antigen stimulation, hence its production in CD8 T cells is well characterized (Lalvani 2007). Upon encounter with antigen, both effector and central memory cells produce IFNγ and it is one of the last cytokines to stop being produced in the event of T cell exhaustion (Yi, Cox et al. 2010). Therefore IFNγ is an ideal marker of antigen specificity when studying cells that may have reduced function, such as chronically stimulated cells in TB disease. MTB-specific CD8 T cells have also been shown to produce TNFα and IL-2 (Sutherland, Adetifa et al. 2009).

Mouse models of MTB infection have indicated an important role of cytotoxic molecules in control of infection. Mice deficient in the cytotoxic molecule perforin have reduced survival time upon exposure to MTB (Grotzke and Lewinsohn 2005). It has also been found that the lytic potential of CD8 T cells waned from early infection to chronic infection in mice (Lazarevic, Nolt et al. 2005). In humans, cytotoxic CD8 T cells preferentially lyse heavily MTB-infected APCs (Lewinsohn, Heinzel et al. 2003). This may have implications when comparing cytotoxicity in LTBI and TB disease, as latently infected individuals are thought to have a lower antigen load and hence would have fewer heavily infected APCs. It has also been suggested that cytotoxic CD8 T cells may serve a role in maintaining latent infection (Pathan, Wilkinson et al. 2000). Indeed reduced perforin production was found in TB patients compared to healthy controls (Smith, Klein et al. 2000), and impaired granulysin and perforin co-expression was found at the site of infection in patients with chronic pulmonary TB (Andersson, Samarina et al. 2007).

1.5.1.3 Phenotype

Although there is a growing body of research on the epitope specificity and effector functions of CD8 T cells in MTB infection, there is still very little published research on the phenotype of chronically stimulated MTB-specific CD8 T cells in infected individuals. Mouse studies on MTB have shown that antigen-specific memory CD8 T cells produce less cytotoxic molecules and cytokines than newly primed CD8 T cells, implying that disease persistence may be due to the low quality memory response (Einarsdottir, Lockhart et al. 2009). Research on the gene expression patterns in pulmonary and lymphoid tissue of macaques with severe TB has shown over-expression of genes involved in inflammation such as chemoattractants and cytokine receptors, and molecules associated with immune dysfunction such as PD-1 and PD-L2 (Qiu, Huang et al. 2008). In human models it has been found that PD-1 is upregulated on antigen-specific lymphocytes in TB disease (Jurado, Alvarez et al. 2008). *In vitro* blockade of PD-1 and its ligands increased the frequency of antigen-specific IFNγ-producing CD8 T cells and enhanced degranulation (Jurado, Alvarez et al. 2008). Phenotypic analysis of CD8 T cells in the peripheral blood of children revealed that central memory cells were predominant in TB disease, and this population waned after therapy to give way to cytotoxic, cytokine producing effector cell populations (Caccamo, Meraviglia et al. 2006). The authors suggested that effector CD8 T cells might have been sequestered to the site of disease. Indeed, an increased frequency of effector memory and terminally differentiated CD8 T cells were found in the cerebrospinal fluid of a subject with TB meningitis (Caccamo, Meraviglia et al. 2006). Several CD8 cell markers have been identified in other chronic infection models, such as the aforementioned PD-1 molecule. CD95, Bcl-2, CD127, CD57 and Ki67 were used in this study as markers of activation, senescence and susceptibility to apoptosis of antigen-specific CD8 T cells.

CD95, also known as Fas, is expressed on effector memory cells and to a lesser extent central memory cells. Naïve cells do not express it. Upon ligation with the Fas ligand (FasL), this receptor initiates a cascade of intracellular events that culminates in apoptosis; hence increased expression of CD95 on a cell surface suggests its susceptibility to apoptosis. Upon stimulation with FasL, CD95 mediates the form of apoptosis known as activation induced cell death (AICD).

Cells may also undergo apoptosis due to an activation dependent event that is initiated within a cell by an internal signal, as opposed to an external molecule. This is known as activated cell autonomous death (ACAD). Bcl-2 is an anti-apoptotic molecule, and its decreased expression on the mitochondrial membrane in a cell has been correlated to increased cell death via ACAD. A signaling cascade within the cell occurs when the levels of pro-apoptotic molecules on the mitochondrial membrane become greater than anti-apoptotic molecules (Krammer, Arnold et al. 2007). Effector memory cells express less Bcl-2 than their naïve and central memory counterparts. This indicates that effector memory cells are more sensitive to apoptosis (Schluns and Lefrancois 2003).

Effector memory populations are sometimes defined by their increased expression of CD57 relative to central memory cells (Wood, Twigg et al. 2009). This is supportive of the use of CD57 as a marker for replicative senescence, as effector T cells have a lower proliferative capacity than central memory T cells. In a HIV disease model the expression of CD57 on CD8 T cells positively correlates with Fas and FasL expression (Wood, Twigg et al. 2009). Interestingly however, senescent cells have been shown to be resistant to apoptosis hence the accumulation of CD57+ cells in a long-term assay is hypothesized to be due to decreased death, not increased proliferation of this population (Wood, Twigg et al. 2009). Additionally, the frequency of CD57+ T cells is elevated in TB disease compared to healthy controls (Sada-Ovalle, Torre-Bouscoulet et al. 2006). The function of CD57 is yet to be determined, but it is known to bind human natural killer cell-1 (HNK-1), a glycopeptide found in cell adhesion molecules (Wood, Twigg et al. 2009). CD57 has been used to define antigen-specific IFNγ-producing cells with defects in proliferation (Brenchley, Karandikar et al. 2003).

CD127 (the IL-7 receptor α chain) is a marker of long-lived memory cells with proliferative potential, and is required for the maintenance of a central memory pool (Cellerai, Perreau et al. 2010). Terminally differentiated CD4 T cells cells do not express CD127 (Lim and Kim 2007). Previous research on CD127 shows that it is rapidly down regulated in activated cells as they proliferate (Sabbaj, Heath et al. 2007).

Ki67 is a marker for proliferation and activation that is upregulated over several days of exposure to antigen. Cells in the G_1, G_2, S and mitosis phase of the cell cycle will express Ki67, but resting cells (G_0) will not. Measurement of intracellular Ki67 directly *ex vivo* will determine the *in vivo* frequency of Ki67+ cells without inducing *de novo* expression. Therefore a short term *ex vivo* assay can used to describe the frequency of circulating Ki67+ cells, whereas a long-term *in vitro* stimulation assay is used to detect Ki67 upregulation in proliferating, antigen-specific cells (Soares, Govender et al. 2010).

1.6 Conclusion

The evidence presented here indicates an important role for CD8 T cells in the immune response to MTB, and provides rationale for further analysis of these cells for a better understanding of MTB immunopathogenesis and to facilitate design of novel TB vaccines. It also suggests that CD8 T cell dysfunction or exhaustion may play a role in the progression from latency to active TB disease, for example CD8 depletion in a mouse model of latency resulted in active TB disease (van Pinxteren, Cassidy et al. 2000), as did CD8 gene disruption (Turner, D'Souza et al. 2001). CD8 depletion in a rhesus macaque model led to increased risk of re-infection (Chen, Huang et al. 2009). In this study, we have identified ESAT6 and CFP10 epitopes that were recognized by CD8 T cells, and described the phenotype of CD8 T cells in terms of markers of activation, memory and susceptibility to apoptosis. Ultimately the results from this study contribute towards a better understanding of the role of CD8 T cells in MTB infection, and allow for development of a platform for future studies to delineate associations between MTB-specific CD8 T cell function and disease progression using prospective, longitudinal cohorts sampled prior to and following development of TB disease. Such studies will aid in identifying immune correlates of protection against development of active TB disease, and open up new avenues of research in immunotherapeutic intervention and the rational design of novel TB vaccines.

1.7 Current study: Aims and objectives

Aim 1:

Determine the breadth and specificity of MTB-specific CD8 T cell responses to ESAT6 and CFP10 peptides in individuals with latent and active TB disease.

Hypothesis: The breadth and magnitude of the MTB-specific CD8 responses is associated with infection status and antigen load. High bacterial loads in individuals with active TB disease will generate a greater magnitude and breadth of MTB-specific CD8 responses detectable in peripheral blood, compared to individuals with latent infection.

Aim 2:

Determine the phenotype of MTB-specific CD8 T cells associated with latent and active TB disease.

Hypothesis: MTB-specific CD8 T cells in individuals with active TB disease will have increased expression of both activation and pro-apoptotic markers, and markers of replicative senescence, compared with individuals with latent infection.

CHAPTER TWO

Methodology

2.0 Overview

T cell epitopes are often identified using a high-throughput method in order to screen a large sample size rapidly and efficiently. Each method may identify a different parameter of antigen-specific cells, depending on the read-out of the assay. For example, the level of IFNγ production upon antigen stimulation can be used to determine the frequency of antigen-specific T cells. Epitopes can also be identified by proliferative capacity, cytotoxic capacity, or the production of other cytokines such as TNFα and IL-2. *Ex vivo* assays can give an indication of the *in vivo* frequency of T cell responses to various epitopes within peptides. In long-term assays, the *in vivo* magnitude of responses cannot be determined, as the antigen-specific cells have been expanded. To an extent, the parameter measured in an epitope-mapping assay will narrow the identification of antigen-specific T cells to certain subtypes. For example, *ex vivo* IFNγ assays will reveal the frequency of antigen-specific effector T cells, but not of naïve or anergic T cells. In this study, experiments were performed to identify subsets of cells that are present at very low frequencies in the blood. Hence in some instances, the population of antigen-specific cells was expanded via long-term stimulation for 6 days. In *ex vivo* Elispot assays, CD4 T cell depletion of peripheral blood mononuclear cells (PBMCs) facilitates identification of CD8 T cell responses in blood. In order to analyze antigen-specific T cell populations at a single-cell level, flow cytometry with fluorochrome-conjugated antibodies was used in long term and short term assays for phenotyping cells and identifying proliferating subsets. IFNγ production was used as a marker for antigen-specificity.

2.0.1 Elispot assays

An *ex vivo* IFNγ Elispot assay is a high throughput method for identifying the presence of antigen-specific T cells. The IFNγ Elispot assay is based on the premise that T cells secrete IFNγ when stimulated with their specific antigen. Wells of an Elispot plate are coated with a polyvinylidene difluoride (PVDF) membrane. This allows for high binding of the capture anti-human IFNγ primary antibody, which is incubated on the membrane. PBMCs and specific antigens are then incubated in each well. T cells exposed to immunogenic peptides will secrete IFNγ, and this cytokine will bind to the primary antibody. The cells and antigens are then washed away leaving the bound IFNγ, which is detected in a series of steps. First, a secondary biotinylated anti-human IFNγ antibody is added and it binds wherever IFNγ is present. A streptavidin-peroxidase conjugated complex is added and binds to the biotin. Substrate is then added and is altered by the peroxidase enzyme to form a blue precipitate at every location that IFNγ has bound. An Elispot plate reader detects these precipitates as spots, with each spot representing one IFNγ-producing cell. The frequency of antigen-specific cells is then reported as the number of spot forming cells (SFC) per million PBMC.

2.0.2 CD4 T cell depletion

Because CD4 and CD8 T cells both produce IFNγ, it is not possible to determine which cell type is responding to a given antigen in an Elispot assay. In order to identify specific peptides eliciting a CD8 T cell response, CD4 T cells were depleted prior to use in the IFNγ Elispot assays. This can be done using magnetic beads conjugated to an anti-CD4 antibody. These beads will cause the attraction of bead-conjugated CD4 T cells towards a magnet in their proximity. CD4 T cells will aggregate on the side of the tube nearest to the magnet, effectively separating the T cells. The solution containing CD4-negative cells can then be extracted and used in further experiments. By removing CD4 T cells from samples, the relative proportion of CD8 T cells within the PBMC population is increased, resulting in an increased sensitivity to CD8 T cell cytokine production in CD4 T cell-depleted samples compared to non-depleted PBMCs.

2.0.3 Intracellular cytokine staining and flow cytometry

Intracellular cytokine staining (ICS) followed by flow cytometry is a popular method for characterizing antigen-specific T cells on a single cell basis. Cells are stained with fluorescently-conjugated antibodies. Flow cytometry employs a fluidics system that allows the sample to be ordered into a stream of single particles. As each particle travels, it passes through several beams of light causing the fluorescently-conjugated antibody to become excited and fluorescence. Forward scatter (FSC) and side scatter (SSC) detectors will collect scattered light from each particle to provide information about the particles size and granularity respectively. Photomultiplier tubes amplify light emitted by the antibody conjugates, thus providing information about the antibodies bound to each cell. More than one type of fluorescently-conjugated antibody can be used to stain cells in multi-color flow cytometry. An LSRII flow cytometer was used in this study, which can detect up to 12 different parameters in one sample. The wavelengths emitted by the fluorescent conjugates may overlap, which results in inaccurate measurements of the true emission of each antibody. Data analysis programs that calculate and subtract the interference of one fluorescence channel from another will compensate for spectral overlap. Some molecules such as CD3 and CD8 are found on the surface of T cells. However others such as Bcl-2 and Ki67 are found intracellularly. Certain buffers are used to fix cells and permeabilize cell walls in order to allow for fluorescently-conjugated antibodies to traverse the cell membrane and bind to their target.

2.0.4 Lymphocyte proliferation assay and flow cytometry

There are various assays to measure proliferative capacity, including incorporation of synthetic nucleosides into replicating DNA, upregulation of cell cycle molecules such as Ki67, and dye dilution assays. Ki67 is a nuclear protein expressed in cells in the G_1, S, G_2 and mitosis, but not in resting cells in the G_0 phase of the cell cycle (Gerdes 1990; Scholzen and Gerdes 2000). The use of a cell trace dye such as Oregon Green (OG: 2', 7'-difluorofluorescein; Invitrogen) is a more established method of conducting a proliferation assay. OG is initially a colorless compound that diffuses passively into cells. It is cleaved upon interaction with intracellular enzymes,

leading to the formation of fluorescent conjugates that can be detected using flow cytometry. As a cell replicates, the volume of intracellular dye is divided between the daughter cells in a step-wise dilution. Hence the number of division cycles undergone can be ascertained using flow cytometry by measuring loss of fluorescence. When excited, OG emits fluorescence over the range 475 to 700 nanometers, peaking at 518 nanometers, which is detected as a green light (Lyons 2000; Wallace, Tario et al. 2008). A proliferation assay is more sensitive than an *ex vivo* assay because cells are exposed to antigens for a longer period of time, thus expanding antigen-specific populations. Only the subsets of cells with proliferative capability will be identified as antigen-specific in this assay; i.e. central and effector memory cells. Naïve and terminally differentiated effector cells may not be identified.

2.1 Materials and Methods

2.1.1 Study participants

Mycobacterium tuberculosis (MTB)-infected male and female adults were recruited as part of a larger study of MTB immunopathogenesis at the SATVI clinical trials site in Worcester, South Africa. A subset of this group was used in this study. Individuals with active pulmonary TB disease were sputum smear positive and/or culture positive for MTB, and had been treated with first line anti-TB drugs for less than one week at the time of sample collection. A subset of participants with TB disease was followed longitudinally, with additional blood samples obtained at 2 and 6 months following initiation of anti-TB treatment. Adults with latent MTB infection (LTBI) were defined as asymptomatic healthy adults with IFNγ-positive T cell responses to CFP10 and/or ESAT6 peptides in an 8-hour whole blood ICS assay, and no previous history of diagnosis or treatment of TB disease. All study subjects had no other chronic conditions or co-infections, and were not on any steroids or immunosuppressive medication. Peripheral blood samples were obtained from all subjects and used for isolation of PBMCs as previously described (Day, Abrahams et al. 2011). All participants provided written informed consent to participate in the

study, which was approved by the Human Research Ethics Committee of the University of Cape Town, and by the Western Cape Department of Health.

2.1.2 Thawing and counting of peripheral blood mononuclear cells

PBMCs were isolated from blood and cryopreserved as previously described (Day, Abrahams et al. 2011). Before use in assays, cryopreserved PBMCs were thawed in a 37^{o}C water bath until a small ice chip was visible. 1ml of 10% FCS in RPMI (R10) containing DNase (Sigma-Aldrich; 0.01mg/ml) was immediately added to each cryovial in order to dilute the PBMCs. DNase is an enzyme that digests DNA, thus preventing live cells from becoming entangled and clumping in the DNA of lysed dead cells. The cells were transferred to 10ml of R10 containing DNase and centrifuged (456g, 10min). The supernatant was discarded and cells were washed in R10 (456g). Thawed PBMCs were counted in order to determine the number of viable cells. The cells were then counted as follows: PBS, an aliquot of PBMCs and Trypan blue were mixed together in an 8:1:1 dilution. Trypan blue is a negatively charged dye, hence it is repelled by the cell membrane of viable cells. It will enter cells with compromised cell membranes, indicating that they are not viable. 10µL of the dilution was loaded onto a Neubauer counting chamber. The number of cells present in two quadrants was averaged and divided by 10 to account for the dilution with PBS and Trypan blue. It was then multiplied by 1x10^{6} to account for the original PBMC suspension in 1000µL of RPMI.

2.1.3 Antigens

15-mer peptides overlapping by 10 amino acids (aa) were synthesized corresponding to the sequence of ESAT6 and CFP10; peptides were either used individually or pooled by protein. For the *ex vivo* IFNγ Elispot assays they were used at a final concentration of 2 µg/ml/peptide. For the ICS assays they were used at a final concentration of 1.25µg/ml/peptide and for the proliferation assay they were used at a final concentration of 0.1µg/ml/peptide. A CMV peptide pool (15-mers overlapping by 11 aa spanning the entire CMV phosphoprotein 65 protein sequence)

was used at the same concentrations as ESAT6 and CFP10 peptide pools for each assay. *Staphylococcal* enterotoxin B (SEB) was used as a positive control in the *ex vivo* IFNγ Elispot, the ICS and the proliferation assays at 1µg/ml, 1µg/ml and 0.1µg/ml respectively. Individual peptides from ESAT6 and CFP10 were used at the same concentration as they were in the peptide pool for each assay.

2.1.4 CD4 T cell depletion of PBMCs

Cryopreserved PBMCs were thawed and counted as described in section 2.2.2. They were then washed in PBS (931g) and the supernatant discarded. They were suspended in PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) and 2mM EDTA (Sigma Aldrich) at a concentration of $1x10^7$ cells/ml. 50µL/ml of anti-human CD4 antibodies conjugated to magnetic beads (IMag; BD Biosciences) were added to the tubes and incubated at room temperature (RT) for 20-30 min. The tube was placed on a magnet and incubated for 8min at RT. CD4+ cells aggregated on the side of the tube nearest to the magnet, whereas CD4− cells remained in suspension. Without removing the tube from the magnet, supernatant was aspirated and transferred to a second tube. The tube on the magnet was replaced with the second tube, and incubated for 3min after which the aspiration and transfer of CD4− cells was repeated. After this process, the CD4-depleted PBMCs were counted using the method described in section 2.2.2.

In order to assess the efficiency of the magnetic bead depletion, CD4-depleted samples were centrifuged (931g), and the supernatant discarded. The pellet was vortexed and the cells stained with fluorescently-conjugated anti-CD3 PE (SK7), anti-CD4 FITC (SK3), and anti-CD8 APC (SK1) antibodies (all from BD Biosciences). The cells were incubated with antibodies for 1 hour at 4^{o}C before they were washed in PBS (931g) and re-suspended in PBS prior to acquisition on a FACSCalibur flow cytometer (BD Biosciences).

2.1.5 Ex vivo IFNγ Elispot

An IFNγ Elispot was performed in order to identify individual 15-mer peptides within ESAT6 and CFP10 that elicit a CD8 T cell response. 100µl/ well of 0.5 µg/ml anti-human IFNγ antibody (MABTECH; 1-D1K) was added to a 96-well filtration plate (MILLIPORE) and incubated overnight at 4°C. The plate was then washed with 1%FCS in PBS, before adding 90µl of R10 to each well and incubating for 2-4hours at 37°C in order to block non-specific binding to the primary antibody. Cryopreserved PBMCs were thawed and counted as described in section 2.2.2. CD4 cells were depleted as described in section 2.2.4. Depleted samples were counted and diluted in R10 at a concentration of $1x10^6$ cells/ml before being rested for 6 hours at 37°C. 100,000 cells were added to each well of the Elispot plate, followed immediately by the addition of antigens to the appropriate wells. The plate was incubated overnight at 37°C (15-18 hours) before being washed with PBS. 0.5µg/ml of biotinylated anti-human-IFNγ antibody (MABTECH; 7-B6-1) was incubated in the wells for 90min at RT before being washed off with PBS. 0.5 µg/ml of streptavidin-conjugated alkaline-phosphatase (MABTECH) was incubated in the wells for 40min at RT before being washed off. A substrate for alkaline phosphatase was added in the following pre-mixed solution (MABTECH): 4% AP color buffer, 1% Buffer A, 1% Buffer B, in water and incubated on the plate for 15min at RT in the dark. Tap water was poured over the plate in order to terminate the reaction, and the plate was left to dry in the dark. The number of spots per well was determined using an Immunospot Elispot plate reader (AID).

2.1.6 PBMC proliferation assay

In order to identify which peptides elicited an immune response by T cells, cryopreserved PBMCs were stimulated with individual ESAT6 and CFP10 peptides in a 6-day proliferation assay as follows: thawed PBMCs were washed in PBS (931g). PBMCs were labeled with 0.5µg/ml of CellTrace™ Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (OG; Invitrogen) in PBS. The tube was mixed by flicking, and left to incubate at RT for 3min. The tube was then vortexed for 10 seconds and left to incubate for a further 4min. 2ml of PBS was then added to the

tube. After another 4min incubation, the tube was topped up to 10ml with PBS and centrifuged (456g); the supernatant was discarded. The cells were diluted in R10 to a concentration of $1.5x10^6$ PBMCs/ml and plated onto a 96-well culture plate (300,000 cells/well). The plate was incubated at 37°C overnight. The next day, antigen was added to each well and the plate was placed in a 37°C 5% CO_2 incubator for 6 days. The 96-well plate was removed on day 6 and the contents of each well were transferred to FACS tubes and washed with 1ml PBS. The cells were centrifuged (931g), the supernatant was discarded and the cells incubated with violet viability dye (VIVID; LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Molecular Probes, Invitrogen) for 20min. The cells were washed with PBS (931g) before an antibody cocktail containing anti-CD3 PerCP.Cy5.5 (SK7), anti-CD4 APC (SK3), and anti-CD8 PE (SK1), all from BD Biosciences, were added. The cells were incubated with antibodies for 1hr at 4°C in the dark. Unbound antibodies were washed off using PBS (931g). The supernatant was discarded and the cells were re-suspended in PBS for acquisition using an LSR II flow cytometer (BD Biosciences).

2.1.7 Intracellular cytokine staining assay

An intracellular cytokine staining (ICS) assay was used to phenotype CD8 T cells *ex vivo*. PBMCs were thawed using the protocol described in section 2.2.2. They were washed in R10 (931g), the supernatant was discarded, and the cells were rested for approximately 6hrs in R10 at 37°C. After resting, antigens were added to the tubes and they were incubated at 37°C for a total of 7hrs. Brefeldin A (10µg/ml; Sigma-Aldrich) was added after the first 2hrs of the 7hr incubation in order to inhibit cytokine secretion. Cells were washed in PBS (931g) and the supernatant was discarded. They were incubated with VIVID for 15min at RT, followed by washing in PBS (931g). The supernatant was discarded and the cells were fixed (FACS Lysing solution; BD Biosciences), washed with PBS (931g), and permeabilized with Perm/Wash buffer (BD Biosciences) for 15min at RT. The cells were pelleted by centrifugation (931g) and the supernatant was discarded. Cells were stained at RT with the following fluorescently-conjugated monoclonal antibodies: anti-CD3 APC-H7 (SK7), anti-CD8 QDot605 (3B5), anti-IFNγ Alexa Fluor 700 (B27), anti-Bcl2 FITC (bcl-2/100), anti-CD57 APC (NK-1), anti-Ki67 PE (B56), anti-CD127 PerCP Cy5.5

(HLR-7r-M21), and anti-CD95 PE Cy7 (DX2). After 1hr, unbound antibodies were washed off with Perm/Wash Buffer (931g), the supernatant was discarded and cells were suspended in PBS for acquisition on a flow cytometer. In order to optimize this assay, antibodies were titrated, and a fluorescence-minus-one experiment was performed using an LTBI PBMC sample. All antibodies were obtained from BD Biosciences except anti-CD8 QDot605 antibody from Invitrogen, and anti-CD95 PE Cy7 and anti-CD57 APC antibodies from BioLegend.

2.1.8 Flow cytometry analysis

An LSRII (BD Biosciences) flow cytometer was used to acquire the ICS and proliferation assay data. Flow cytometric data was analyzed using FlowJo v9.2 (Tree Star, Ashland, OR). Single-stained anti-mouse Ig κ beads (BD Biosciences) were used to calculate compensation. Anti-CD3 Pacific Blue antibody was used to compensate for VIVID. In the proliferation assay, anti-CD4 FITC antibody was used to compensate for OG. A compensation matrix was generated using FlowJo.

The gating strategy involved first gating out morphologically abnormal cells that were identified by their FSC-height vs. FSC-area properties in a singlet gate. Lymphocytes were then gated on by plotting FSC-height versus SSC-area. After these initial gates, the fluorescently-conjugated antibodies were used to further discriminate specific populations as shown in later chapters. For continuous markers, or markers in which gates could not be accurately placed, median fluorescence intensity (MFI) was used to gauge expression levels. Gate placement for various markers and for proliferated cells was determined using the unstimulated samples. For the ICS assay, IFNγ positive cells were gated on to define antigen-specific subpopulations. In longitudinal analyses, PBMCs from the same donor at different time points were processed concurrently.

2.1.9 Data analysis

For the IFNγ Elispot assays, the average number of spots in the unstimulated wells was subtracted from the number of spots in the stimulated wells in order to control

for background IFNγ production. A response was considered positive if it was greater than the median plus 3 times the median absolute deviation (MAD) of the unstimulated samples after background subtraction (53.3 SFU/million PBMCs).

In the 6-day proliferation assay, the frequency of proliferating (OG^{low}) cells in the unstimulated sample was subtracted from the stimulated samples for each donor. In addition, acquisition of a minimum of 10,000 live T cells ($CD3^{+}Vivid^{low}$) in the unstimulated sample was required for analysis of flow cytometry data as this number accounted for over 97% of data from unstimulated samples acquired. Any samples that failed to elicit a positive response to SEB stimulation were excluded. A response was considered positive if it was greater than the median plus 2 times the MAD of the unstimulated samples after background subtraction (0.757%).

When analyzing results from the *ex vivo* phenotyping assay performed in Chapter four, three criteria were employed to identify a positive antigen-specific response preceding phenotypic analysis of antigen-specific CD8 T cell populations: (1) The frequency of CD8+IFNγ+ cells had to be more than double the frequency of CD8+IFNγ+ cells in that individual's unstimulated condition, (2) the IFNγ+ population had to be greater than the median plus 2 times the MAD of CD8+IFNγ+ cells from the unstimulated samples, (3) the cell count of CD8+IFNγ+ cells had to be >20. These criteria were employed to ensure that each response was real. In stimulation conditions that did not meet all 3 criteria, information on the CD8+IFNγ+ subset was excluded.

2.1.10 Statistical analysis

When comparing responses between LTBI and TB donors, a two-tailed non-parametric Mann-Whitney test was used. In the phenotypic analysis, a non-parametric Wilcoxon matched pairs test was used to compare expression of phenotypic markers between different cell populations within the same donor. P values of $p<0.05$ were considered significant. Prism (v5.0) and Intercooled STATA v8.0 were used for statistical analysis.

CHAPTER THREE

Identification of immunodominant CFP10 and ESAT6-specific CD8 T cell responses in individuals with latent and active TB disease

3.0 Overview

BCG has variable efficacy against pulmonary TB, thus the development of a more effective vaccine against TB is a matter of urgency. Increasing evidence indicates a role for CD8 T cells in controlling chronic infections, hence novel TB vaccines may benefit from induction of CD8 T cell responses. A first step is the identification of immunodominant CD8 T cell epitopes from antigens that are associated with successful immune control of *Mycobacterium tuberculosis* (MTB) infection. The aim of this chapter was to identify peptides within CFP10 and ESAT6 that elicit a CD8 T cell response in MTB-infected individuals. The secondary aims were to compare the breadth and magnitude of MTB-specific CD8 T cell responses within these proteins between individuals with LTBI and TB disease, and also to identify specific CFP10 and ESAT6 peptides that elicit proliferative CD4 T cell responses. Proliferation assays and *ex vivo* IFNγ Elispot assays were used to identify CD8 T cell responses to immunodominant peptides in CFP10 and ESAT6. Approximately 20% of LTBI donors responded to peptides C4 and C16 in CFP10, and to peptide E12 in ESAT6. CD8 T cells in individuals with TB exhibited poor proliferative capacity, compared with LTBI. CD8 T cells in LTBI donors recognized a significantly greater breadth of CFP10 peptides than TB donors, however there was no difference between LTBI and TB in the breadth of recognition to ESAT6 peptides. MTB-specific CD4 T cells from LTBI donors exhibited greater proliferative capacity than TB donors, and recognized a greater breadth of peptides. Peptide mapping using an *ex vivo* Elispot assay identified IFNγ-producing CD8 T cell responses in 9/18 TB donors and 2/7 LTBI donors. Together these data suggest that differential recognition of CFP10 regions by CD8 T cells may distinguish between LTBI and TB disease, as CD8 T cells from LTBI donors responded to peptides in the N and C termini whereas TB donors responded to the C terminus. In addition, these results suggest differential functional capacities of MTB-specific CD8 T cells in latent and active TB disease. This is relevant for vaccine design as the response present in latently infected individuals may be associated with protection from the development of TB disease.

3.1 Introduction

The currently licensed vaccine against TB, BCG, has a varying efficacy and a low level of protection against pulmonary TB (Hussey, Hawkridge et al. 2007). BCG is a live attenuated bacterium, hence its administration as a vaccine poses a risk of disseminated BCG in immune-compromised individuals (Hesseling, Cotton et al. 2007). BCG was discovered and developed before 1920 and it has been in use since then. However, TB remains a global health problem, hence it is important to design a more effective vaccine.

There must be a scientific basis behind the design and implementation of a new TB vaccine. According to Hussey *et al*, the ideal TB vaccine should be affordable, cost-effective and suitable for all ages. It should be safe and immunogenic in individuals that have been vaccinated with BCG or previously exposed to TB, as well as in immune-compromised individuals (Hussey, Hawkridge et al. 2007). From an immunological perspective, the vaccine should elicit a response that is associated with protection. This is challenging because the immune correlates of protection against TB have not yet been identified. A T cell response that is capable of multiple effector functions ('polyfunctional') may or may not be important, as the link between polyfunctionality and protection is unclear (Abel, Tameris et al. 2010). TB vaccines in development incorporate specific proteins that are known to be immunogenic to T cells, for example the novel TB vaccine candidate AERAS 402 contains Ag85A, Ag85B, and TB10.4 and elicits robust responses in both CD4 and CD8 T cells (Abel, Tameris et al. 2010). In order to identify immunodominant epitopes within a protein, fine mapping of its sequence is performed and the minimal sequence to elicit an immunodominant response may be incorporated into novel vaccines. ESAT6 and CFP10 are highly immunodominant MTB proteins (Millington, Fortune et al. 2011) for which T cell epitope mapping has been performed.

CFP10 (culture filtrate protein-10) and ESAT6 (early secretory antigenic target-6) are highly conserved pathogenic factors secreted by MTB. They are 100 and 95 amino acids long, respectively. Their expression is co-regulated within the bacteria (Renshaw, Lightbody et al. 2005), and upon expression they form a complex that is essential for bacterial pathogenesis. This complex binds to the

surface of macrophages and is thought to modulate host behavior (Renshaw, Lightbody et al. 2005). The RD1 locus containing ESAT6 and CFP10 has been deleted from BCG; therefore, they can be used to distinguish between an immune response induced by BCG vaccination versus MTB infection.

Previous studies identifying CD8 T cell epitopes within CFP10 have employed the generation of antigen-specific T cell lines, in some cases through clonal expansion *in vitro*. Lewinsohn *et al* generated CD8 T cell clones from an asymptomatic LTBI donor. These T cell clones were stimulated with various peptides from ESAT6 or CFP10. IFNγ was produced in response to an epitope within the carboxyl (C) terminus of CFP10, accounting for the majority of IFNγ production upon stimulation with the full protein (Lewinsohn, Zhu et al. 2001). In another study, Shams *et al* identified two epitopes within a 15-mer peptide corresponding to aa 71 - 85 of CFP10. One epitope elicited a CD4 T cell response and the other epitope elicited a CD8 T cell response in asymptomatic TST-positive TB contacts. Stimulation with these epitopes caused IFNγ production as measured by a cultured Elispot, and cytotoxic activity in a chromium release assay (Shams, Klucar et al. 2004). The epitopes detailed in the literature correspond to the amino (N) and C terminus of CFP10.

Epitopes within ESAT6 have been identified using similar methods. For example, in a study by Lalvani *et al*, ESAT6 peptides that were predicted to bind to HLA class I molecules and be recognized by CD8 T cells were synthesized. When used to stimulate CD8 T cell lines from one donor with TB disease, two immunodominant peptides were found, each restricted by different HLA types. As well as IFNγ production, these cells showed cytolytic activity (Lalvani, Brookes et al. 1998). Another ESAT6 immunodominant peptide was identified in an asymptomatic MTB-infected donor using an *ex vivo* Elispot assay. This 9 aa sequence was HLA-A68.02-restricted, and elicited a highly focused cytotoxic response (Pathan, Wilkinson et al. 2000).

The information above is pertinent to CD8 T cell responses, however the majority of studies identifying MTB-specific T cell responses have not distinguished between CD4 and CD8 T cell responses. For example, CFP10 and ESAT6 peptide mapping has been described in a human immunodeficiency virus (HIV)-positive

cohort of TB diseased and asymptomatic MTB-infected Zambian adults using an *ex vivo* IFNγ Elispot assay. It was found that every peptide was recognized by the T cells of at least one TB patient and one asymptomatic adult, from both CFP10 and ESAT6. A response in the C terminus of CFP10 was emphasized (Chapman, Munkanta et al. 2002). Another study using an *ex vivo* IFNγ Elispot assay by Lalvani *et al* found broad T cell recognition to ESAT6 in patients with TB and their household contacts (Lalvani, Nagvenkar et al. 2001).

It has been shown in mice and cattle studies that the activation of T cells that produce IFNγ only does not correlate with protection (Mittrucker, Steinhoff et al. 2007). Therefore, *ex vivo* Elispot assays alone may be insufficient to identify peptide responses that correlate with protection. Although it has not been proven in a TB disease model, memory T cells are hypothesized to be important in vaccine design. A hallmark of memory subsets is proliferative capacity. In previous research T cells have been expanded by stimulation with ESAT6 in a proliferation assay. After incubation with one of eight peptides spanning ESAT6, varied responses to sequences along the protein were found, implying that there are indeed differences in immunogenicity along the protein. The assay employed thymidine incorporation to determine the level of proliferation (Mustafa, Shaban et al. 2003), however another effective method involves cytosolic fluorescent dye dilution to identify cells that have proliferated. Together this information suggests that proliferative capacity provides another important measurement of immunogenicity in MTB infection.

In summary, previous research identifying MTB-specific CD8 T cell responses generally employed small sample sizes and were highly specific to other countries and populations due to HLA restriction. In the South African population it is possible that there is differential peptide recognition compared with previously reported studies, due to different HLA types predominant in this population. This chapter compares T cell responses within individuals with newly diagnosed TB to latently infected individuals in a TB endemic area, using relevant measurements of immune function; i.e. *ex vivo* IFNγ production and proliferation.

3.2 Results

3.2.1 Participant characteristics

A total of 54 MTB-infected male and female adults were recruited for these studies at the SATVI clinical trials site in Worcester, South Africa. Individuals with active pulmonary TB disease were sputum smear positive and/or culture positive for MTB, and had been treated with first line anti-TB drugs for less than one week. Individuals with latent MTB infection (LTBI) were defined as asymptomatic healthy adults with IFNγ-positive responses to CFP10 and/or ESAT6 antigens in an 8hr whole blood intracellular cytokine staining (ICS) assay (data not shown), and no previous history of diagnosis or treatment of TB disease. Participant characteristics are shown in Table 3.1.

Table 3.1. Participant characteristics.

Characteristics	TB	LTBI
Subjects (n)	30	24
Female, n (%)	7 (23%)[a]	21 (88%)
Smear positive (%)	24 (80%)	N/A
Smear negative (%)	6 (20%)	N/A
Age[b], (range)	39.9 (18-60)[c]	29.2 (18-50)

[a] p <0.0001 compared with LTBI

[b] Values denote mean (range)

[c] p =0.0011, compared with LTBI

N/A: not applicable.

3.2.2 Flow cytometric analysis of a PBMC-based dye dilution proliferation assay

An Oregon Green (OG) dye dilution assay is a means to identify the proliferation of lymphocytes after stimulation with antigen for several days *in vitro*. In order to identify immunodominant CD8 T cell epitopes targeted in MTB infection, a 6-day OG proliferation assay was used to identify individual peptides in CFP10 and ESAT6 that elicit a proliferative CD8 T cell response in 19 TB diseased and 20 LTBI donors. The sequential gating strategy used is shown in Figure 3.1A. PBMCs were stimulated with CFP10, ESAT6, and CMV pp65 peptide pools, as well as individual overlapping 15-mer peptides spanning the entire length of CFP10 and ESAT6. All donors had a positive response to SEB (data not shown).

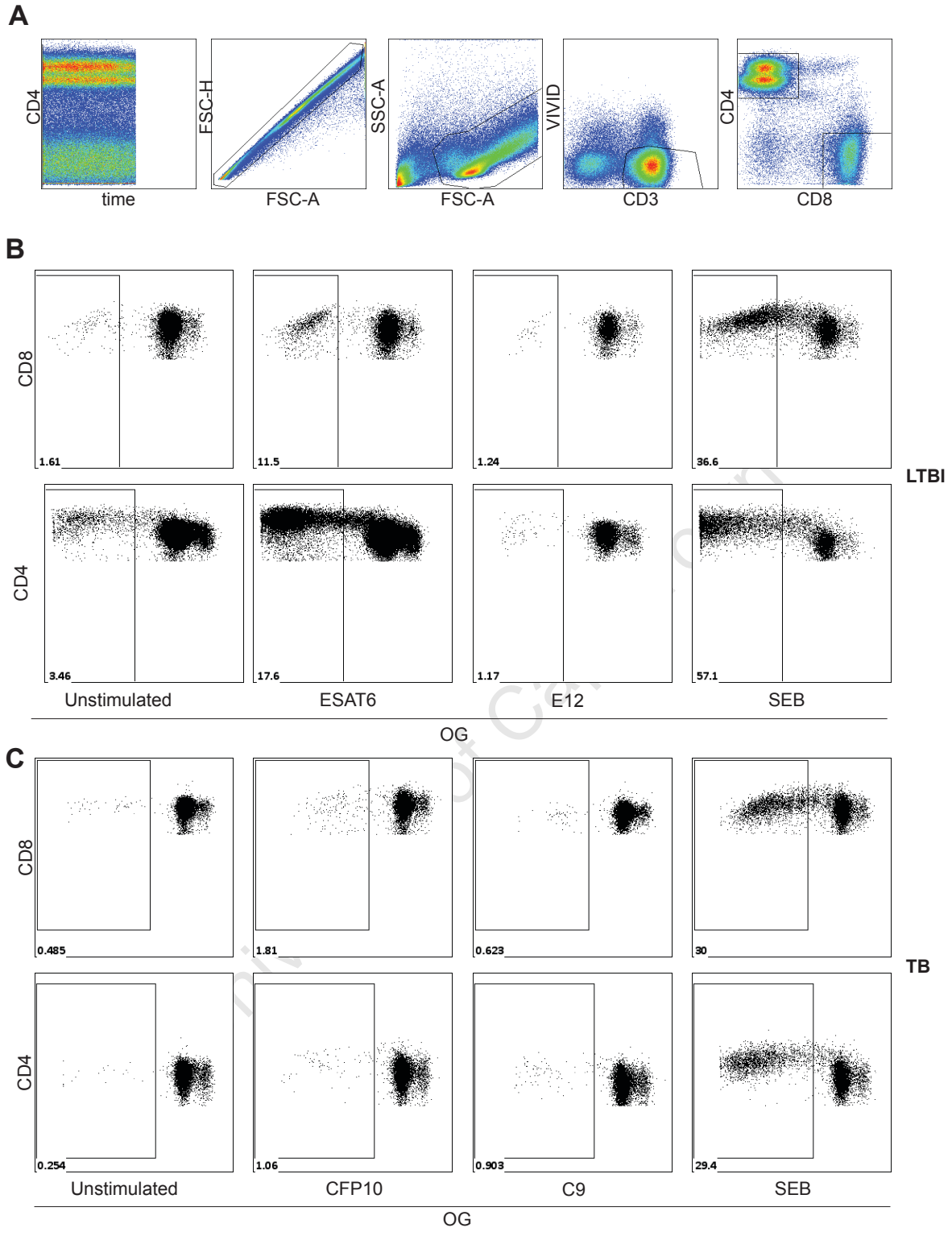


Figure 3.1. Flow cytometry gating strategy and representative data from the PBMC-based dye dilution proliferation assay. (A) A time gate was used to identify any abnormalities in fluorescence. Cells are gated on singlets and lymphocytes based on FSC-H and SSC-H characteristics. Live T cells (Vivid^{low}CD3+) were then gated on, followed by gating on CD4+ and CD8+ populations. (B) Representative flow plots of proliferating CD8 and CD4 T cell responses from an LTBI donor. No stimulation, stimulation with ESAT6 peptide pool, peptide E12, and SEB respectively are shown. (C) Representative flow plots of proliferating CD8 and CD4 T cell responses from a TB donor. No stimulation, stimulation with CFP10 peptide pool, peptide C9, and SEB respectively are shown.

3.2.3 Individuals with LTBI display greater breadth and magnitude of MTB-specific proliferative CD8 T cell responses, compared to patients with TB

The magnitude of CD8 T cell proliferative responses was compared between individuals with LTBI and TB. Representative flow cytometry data of T cell proliferation by LTBI and TB donors are shown in Figure 3.1B, C. LTBI individuals had higher frequencies of proliferating CD8 T cells to CFP10 and ESAT6 peptide pools and SEB, compared to TB diseased donors (Figure 3.2). We next mapped immunodominant peptides eliciting CD8 T cell proliferative responses in these two groups. We found that CD8 T cells from LTBI donors recognized a greater breadth of CFP10 peptides than TB donors; however, CD8 T cell proliferative responses to ESAT6 peptides were similar in breadth between the two groups (Figure 3.3).

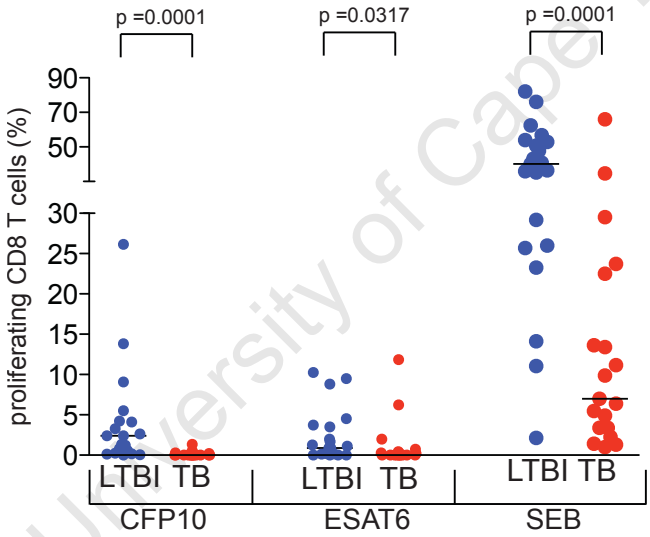


Figure 3.2. Reduced proliferative capacity of CD8 T cells in individuals with TB, compared with LTBI. The frequency of proliferating (OG^{low}) CD8 T cells upon stimulation for 6 days with CFP10 or ESAT6 peptide pools, or SEB is shown for LTBI (n=21) and TB (n=19) donors. Results are shown after subtraction of background proliferation in the negative control sample. Horizontal lines represent medians. Statistical differences were assessed by the Mann Whitney test.

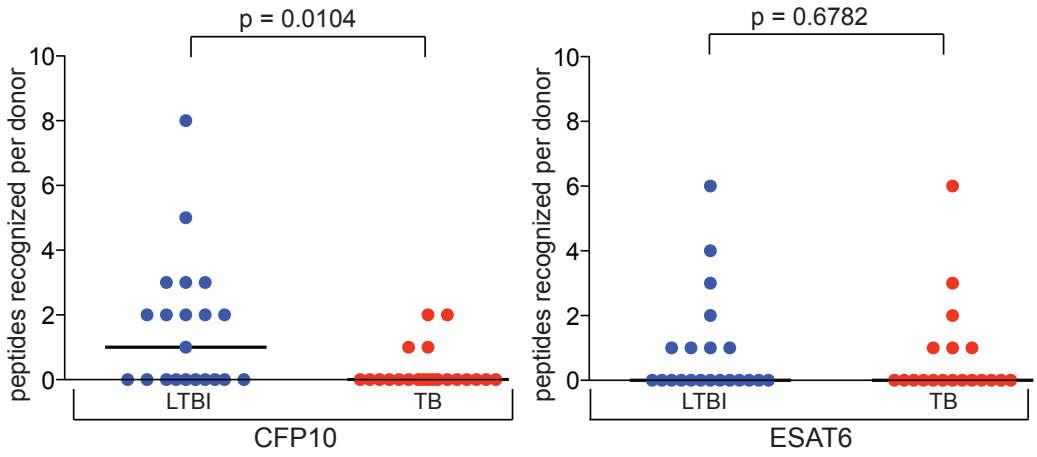


Figure 3.3. Greater breadth of proliferative CD8 T cell responses to CFP10 peptides in individuals with LTBI, compared to patients with TB. Individual 15-mer overlapping peptides spanning the sequence of CFP10 (left panel) and ESAT6 (right panel) were used to stimulate PBMCs from donors with LTBI (n=21) and TB (n=19) in a 6-day proliferation assay. The number of peptides eliciting a positive proliferative CD8 T cell response for each donor is shown. Horizontal lines represent the median number of peptides recognized by each individual. Statistical differences were assessed by the Mann Whitney test.

Individual 15-mer peptides from ESAT6 and CFP10 elicited CD8 T cell proliferation in approximately half of MTB-infected donors. Peptides C4 and C16 in CFP10, and peptide E12 in ESAT6, elicited a response in over 20% of LTBI donors. Interestingly, we were unable to detect proliferative CD8 T cell responses to E12 in any of the TB donors. It is noteworthy that specific peptides eliciting a proliferative response in TB donors were not concentrated in any particular region of CFP10 or ESAT6 in this assay (Figure 3.4). Overall these results show that LTBI donors have an overall increased proliferative capacity, and a greater breadth of recognition of CFP10 epitopes, compared with TB diseased donors, using a 6-day proliferation assay.

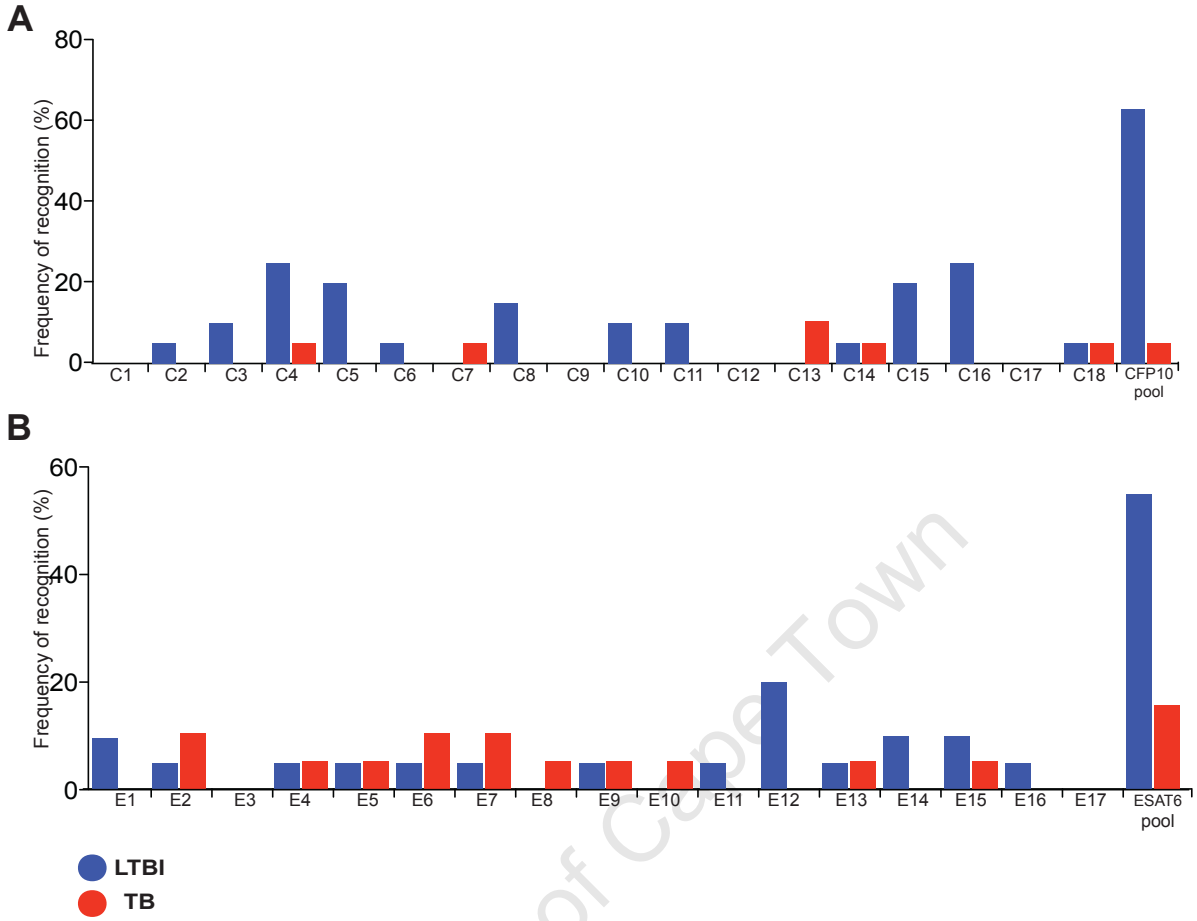


Figure 3.4. Frequency of recognition of individual 15-mer peptides in CFP10 and ESAT6 by CD8 T cells. PBMCs were incubated with individual overlapping 15-mer peptides from CFP10 (A) or ESAT6 (B) in a 6-day proliferation assay as described in Figure 3.3. The percentage of TB (n=19) and LTBI (n=21) donors who had a positive proliferative CD8 T cell response to each peptide is shown.

3.2.4 Identification of CFP10 and ESAT6-specific CD8 T cell responses by an ex vivo IFNγ Elispot assay

Individuals with TB generally displayed poor proliferation in the 6-day proliferation assay, compared with LTBI individuals. Hence assessment of proliferative capacity alone as a marker for antigen specificity may preclude the identification of antigen-specific T cells with effector functions other than proliferation. An *ex vivo* IFNγ Elispot assay was therefore used to identify CD8 T cell responses in TB as well as LTBI donors. In order to identify MTB-specific CD8 T cell responses, CD4 T cells were depleted from PBMCs using magnetic beads conjugated to anti-CD4 antibodies prior to use in the Elispot assay. The level of depletion was assessed in 15 donors by

comparing pre-depletion and post-depletion CD4 T cell frequencies by flow cytometry. Representative flow cytometry data pre and post CD4 T cell depletion are shown in Figure 3.5A. Over 99% of CD4 cells present in PBMCs were depleted, as shown in Figure 3.5B, C.

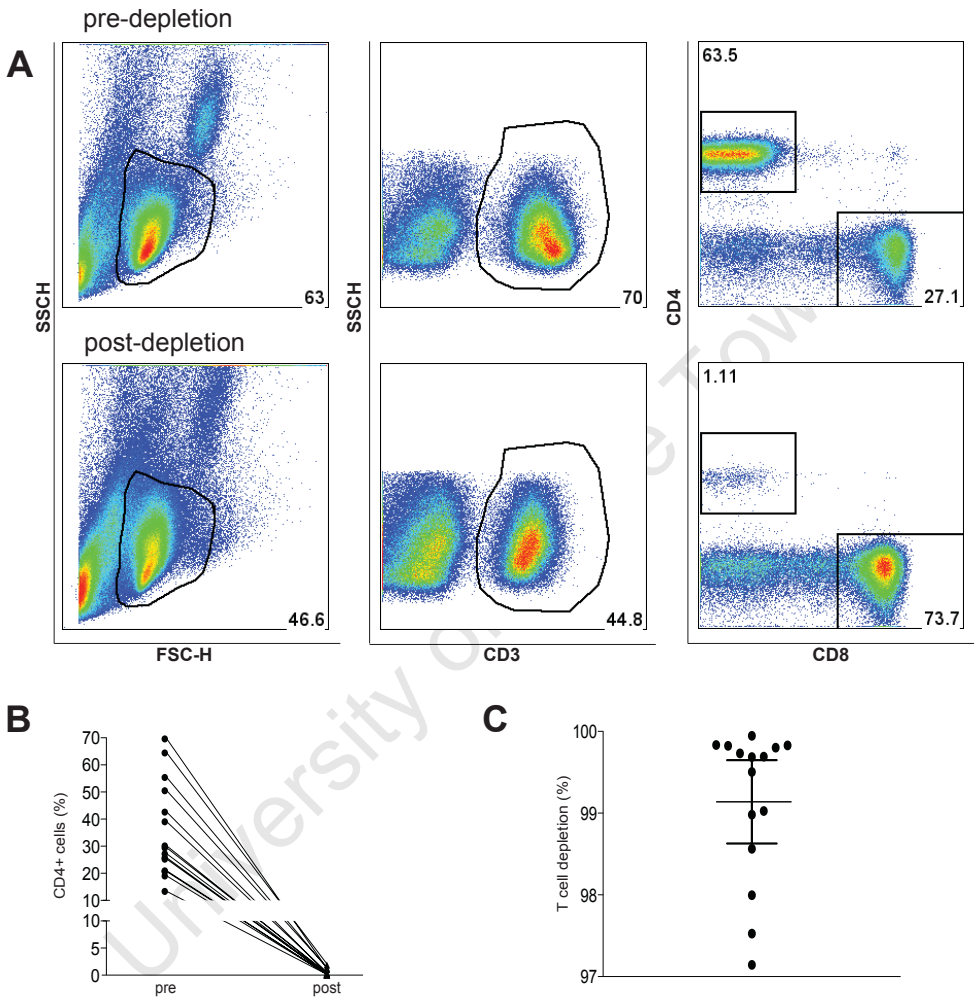


Figure 3.5. Flow cytometric analysis of CD4 T cell depletion efficiency with magnetic bead-conjugated antibodies. Cryopreserved PBMCs were thawed and incubated with magnetic bead-conjugated anti-CD4 antibodies. (A) Representative flow plots of a PBMC sample prior to CD4 T cell depletion (top row), and the same sample post-depletion (bottom row). Cells were gated on lymphocytes, followed by CD3 T cells, and then CD4 and CD8 cells. (B) The frequency of CD4 T cells pre- and post-depletion. (C) Summary data of efficiency of CD4 T cell depletion. Bars correspond to the median with interquartile range (n=15).

Following CD4 T cell depletion, PBMCs from 18 TB donors were stimulated with either CFP10 or ESAT6 individual peptides in an overnight IFNγ Elispot assay. All 18 donors were also stimulated with CFP10 and ESAT6 peptide pools in this assay. 11 donors responded to ESAT6 or CFP10 peptide pools, and 9 of the 11 responders had a detectable CD8 T cell response to one or more individual peptides in the Elispot assay. Only 2/7 LTBI donors had *ex vivo* CD8 T cell responses above the cutoff to CFP10 or ESAT6 peptide pools, indicating MTB-specific CD8 T cells are present in very low frequencies in peripheral blood during latent infection. Representative Elispot assay data from a TB diseased individual with a CD8 T cell response to CFP10 are shown in Figure 3.6A; representative data from an LTBI donor are shown in Figure 3.6B. Figure 3.6C shows the SFU/million CD4-depleted PBMCs after background subtraction in all the donors used in this assay.

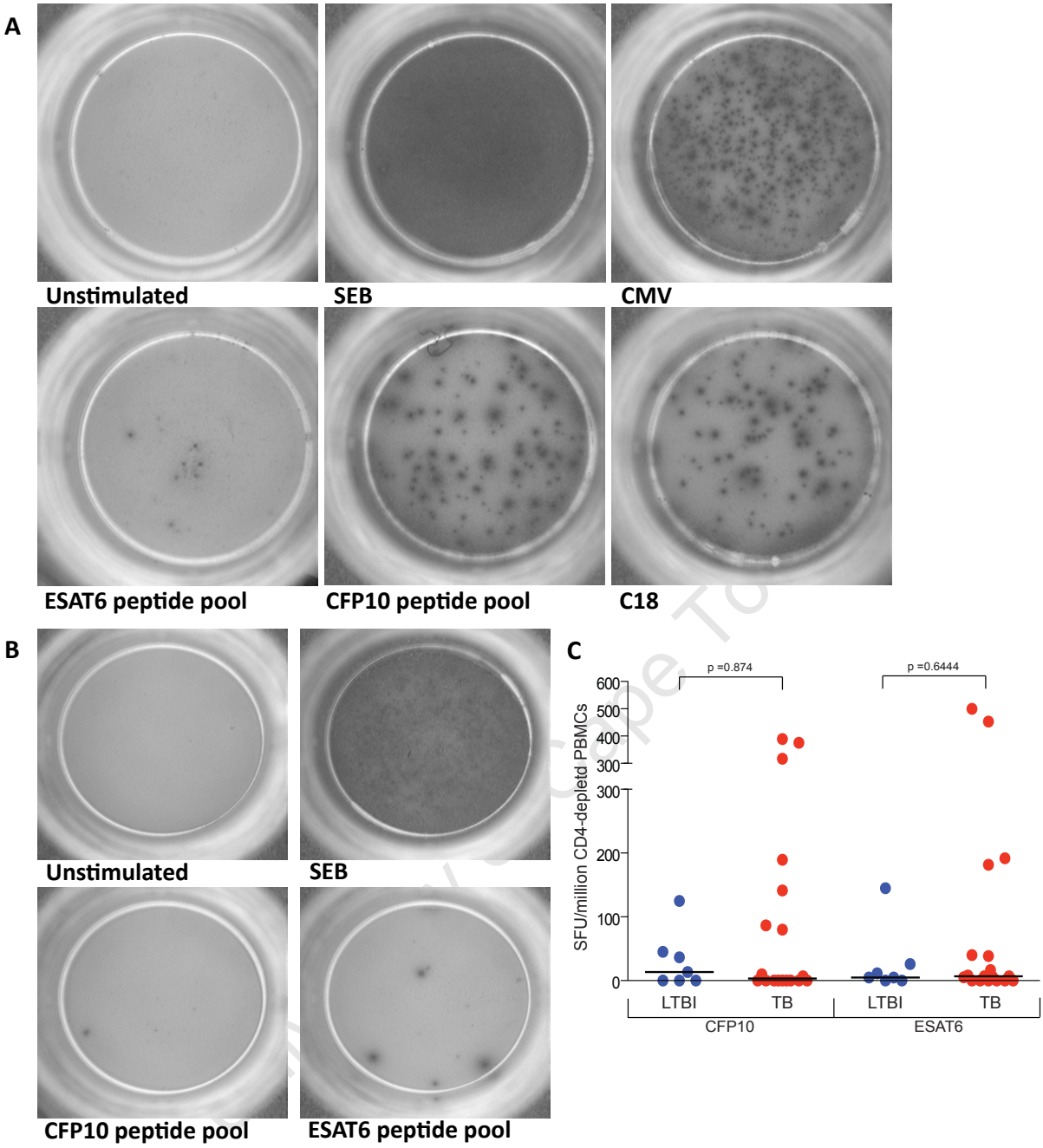
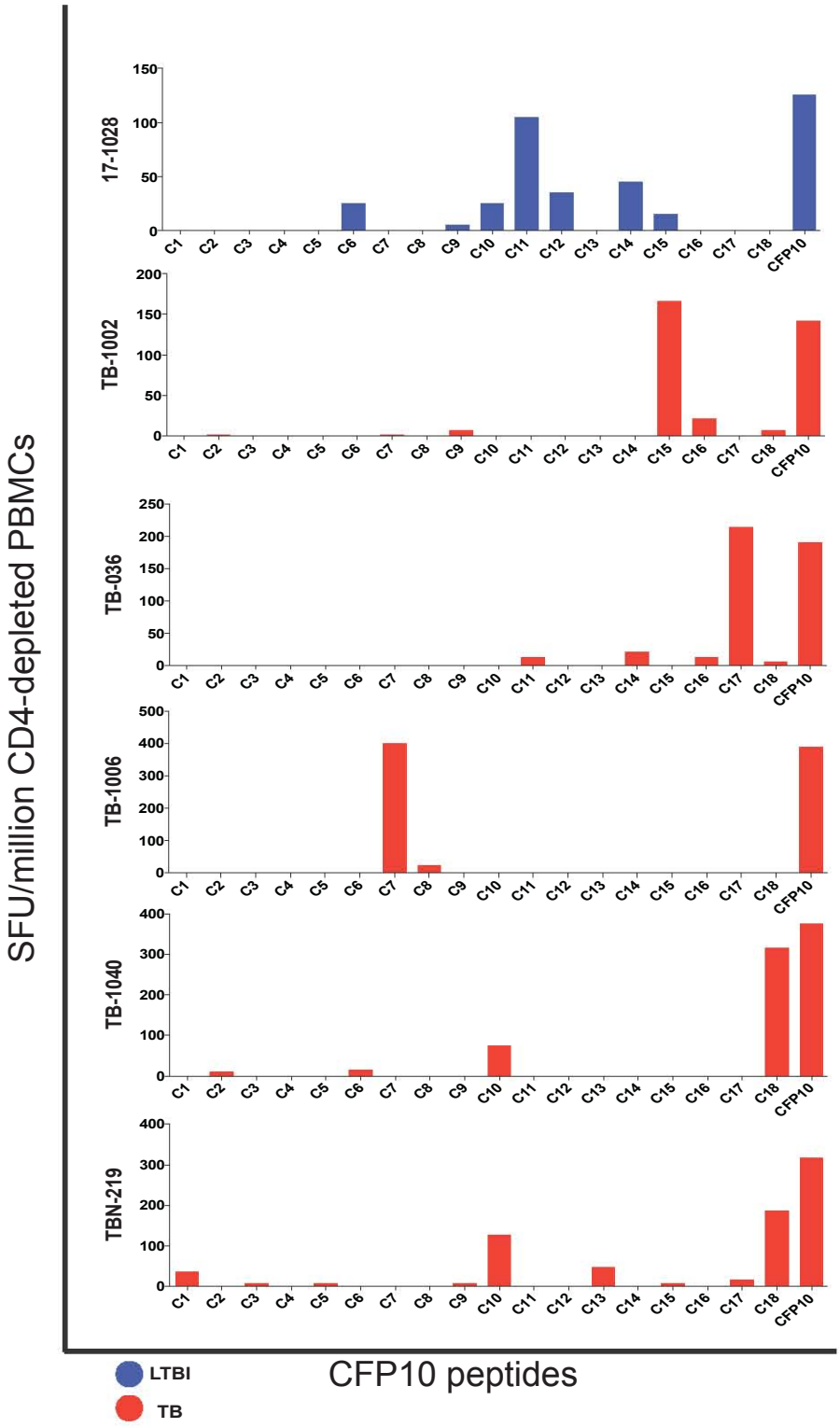


Figure 3.6 Detection of CFP10 and ESAT6-specific CD8 T cell responses in individuals with TB using an IFNγ Elispot assay with CD4-depleted PBMCs. CD4-depleted PBMC from donors with TB disease (A) or LTBI (B) was stimulated with individual overlapping 15-mer peptides or CFP10 and ESAT6 peptide pools in an overnight IFNγ Elispot assay; representative images of individual wells are shown. Unstimulated and SEB wells were done in duplicate or triplicate. 200,000 CD4-depleted PBMC were added to each well. (C) Background subtracted numbers of spot forming units (SFU) per million CD4-depleted PBMCs to CFP10 and ESAT6 peptide pools in LTBI (n = 7) and TB (n = 18) donors.


SFU/million CD4-depleted PBMCs
17-1028
TB-1002
TB-036
TB-1006
TB-1040
TBN-219
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18 CFP10
CFP10 peptides
LTBI
TB

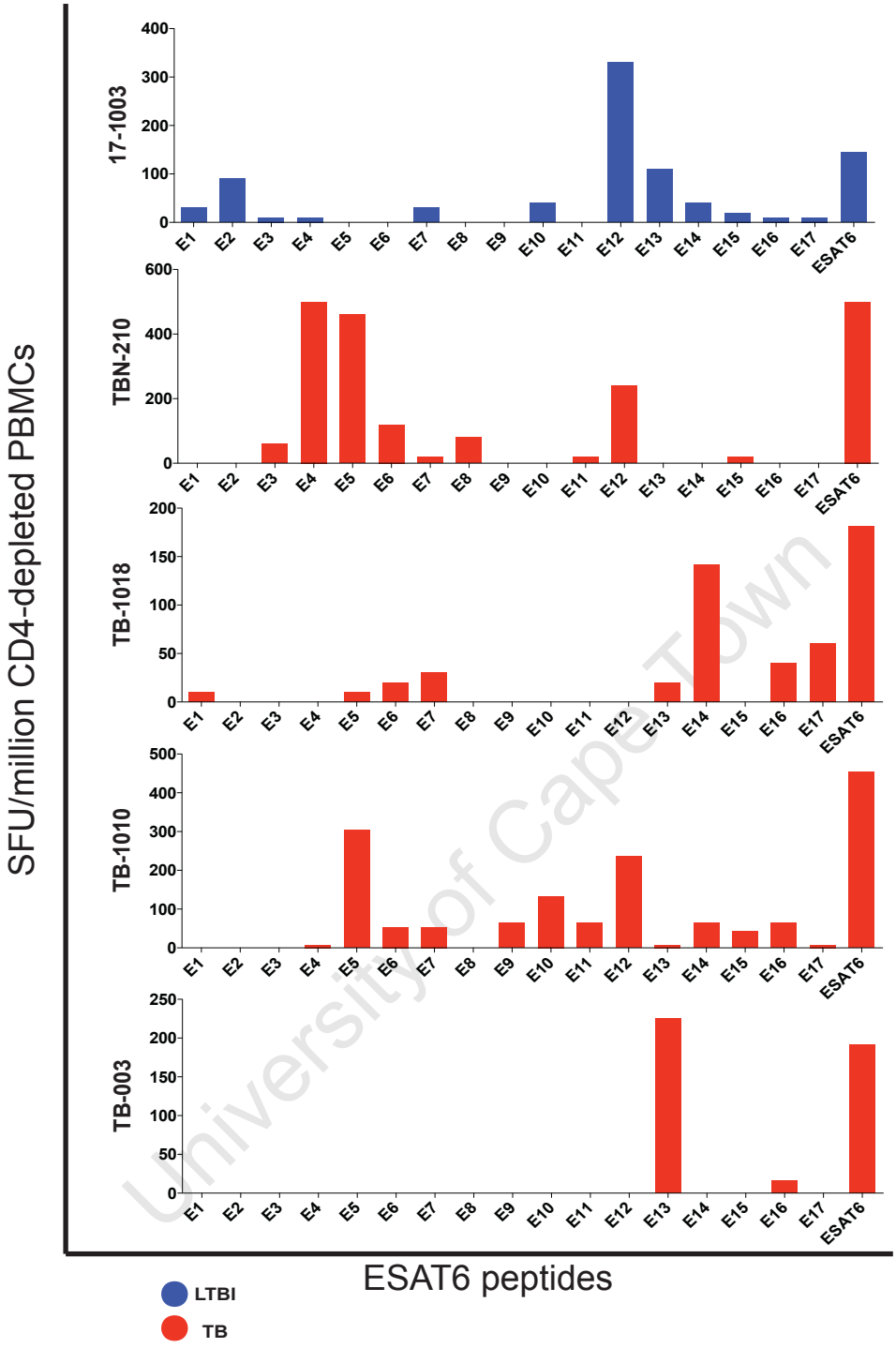


Figure 3.7. Identification of CD8 T cell responses to individual CFP10 and ESAT6 15-mer peptides in individuals with LTBI and TB by *ex vivo* IFNγ production. CD4-depleted PBMCs were used in an Elispot assay as described in Figure 3.6. The number of spot forming units (SFU) per million CD4-depleted PBMCs is shown for each peptide. Results are shown after subtraction of background cytokine production in the negative control wells. Each graph represents one donor.

Nine TB disease donors were tested with individual CFP10 15-mer peptides using an Elispot assay with CD4-depleted PBMCs, and 5/9 donors had a positive response. Out of these responders, 4/5 produced IFNγ when stimulated with peptides from the C-terminus of CFP10 (i.e. C15 to C18), whereas one responder only responded to peptide C7. The immunodominant response in the only LTBI responder to CFP10 in an Elispot assay was to peptide C11. There were no positive CD8 T cell responses detected to peptides in the N-terminus of CFP10 (i.e. C1 to C5). Although every responder displayed immunodominant recognition of one peptide, the peptide recognized differed across donors (Figure 3.7). Four out of nine TB donors had a CD8 T cell response to ESAT6 peptides in an ex vivo CD4 depleted Elispot. The differences in magnitude of response within these donors can be attributed to inter-individual variation in immune activity. The differences in peptide recognition within these donors may be due to varied HLA types present in this population. However this is speculative, as the donors were not HLA-typed.

Tables 3.2 and 3.3 summarize the immunogenic CFP10 and ESAT6 peptides eliciting a CD8 T cell response in either the 6-day proliferation assay or the *ex vivo* CD4-depleted IFNγ Elispot assay. In Table 3.2, peptides that elicited a response in CFP10 (top section) or ESAT6 (bottom section) in more than two of the 40 MTB-infected donors that were tested are shown. Table 3.3 shows any peptide eliciting a response above the cutoff after background subtraction in the TB (n = 18) and LTBI (n = 7) donors tested.

Table 3.2. Immunodominant 15-mer peptides in CFP10 and ESAT6 eliciting proliferative CD8 T cell responses in MTB infected donors.

:

Peptide	amino acid sequence	Amino acid position	frequency of recognition, n (%)	median response[a]
C4	GNFERISGDLKTQID	16 - 30	6 (15%)	1.03%
C5	ISGDLKTQIDQVEST	21 - 35	4 (10%)	1.00%
C8	AGSLQGQWRGAAGTA	36 - 50	3 (7.5%)	1.29%
C15	EISTNIRQAGVQYSR	71 - 85	4 (10%)	1.63%
C16	IRQAGVQYSR ADEEQ	76 - 90	5 (12.5%)	3.63%
E2	WNFAGIEAAASAIQG	6 - 20	3 (7.5%)	1.21%
E6	HSLLDEGKQSLTKLA	26 - 40	3 (7.5%)	2.16%
E7	EGKQSLTKLAAAWGG	31 – 45	3 (7.5%)	3.68%
E12	QKWDATATELNNALQ	56 - 70	4 (10%)	1.93%
E15	NLARTISEAGQAMAS	76 - 90	3 (7.5%)	4.56%

[a] The median percentage of proliferating (OG^{low}) CD8 T cells after background subtraction.

Table 3.3. Immunogenic CFP10 and ESAT6 peptides eliciting CD8 T cell responses in MTB infected donors in the *ex vivo* IFNγ Elispot assay.

:

Peptide	amino acid sequence	Amino acid position	frequency of recognition[a], n (%)	Donors responding	Magnitude of response[b]
C7	QVESTAGSLQGQWRG	31 - 45	1 (9.1%)	TB-1006	400
C10	AAGTAAQAAVVRFQE	46 - 60	2 (18.2%)	TB-1040, TBN-219	75 127
C11	AQAAV VRFQE AANKQ	51 - 65	1 (9.1%)	17-1028	105
C15	EISTNIRQAGVQYSR	71 - 85	1 (9.1%)	TB-1002	166
C17	VQYSRADEEQQQALS	81 - 95	1 (9.1%)	TB-036	213
C18	ADEEQQQALSSQMGF	86 - 100	2 (18.2%)	TB-1040, TBN-219	315 187
E2	TDAAT LAQEA GNFER	6 - 20	1 (7.1%)	17-1003	90
E4	SAIQGNVTSIHSLLD	16 - 30	1 (7.1%)	TBN-210	500
E5	NVTSIHSLLDEGKQS	21 - 35	2 (14.3%)	TB-1010, TBN-210	305 460
E6	HSLLDEGKQSLTKLA	26 - 40	1 (7.1%)	TBN-210	120
E8	LTKLAAAWGGSGSEA	36 - 50	1 (14.3%)	TBN-210	80
E10	SGSEAYQGVQQKWDA	46 - 60	1 (7.1%)	TB-1010	133
E12	QKWDATATELNNALQ	56 - 70	3 (21.4%)	TB-1010, TBN-210 17-1003	236 240 330
E13	TATELNNALQNLART	61 - 75	2 (14.3%)	TB-003 17-1003	225 110
E14	NNALQNLARTISEAG	66 - 80	1 (7.1%)	TB-1018	141

[a] The number of donors responding a given peptide divided by the number of donors tested for that specified peptide.

[b] Background subtracted number of spot forming units per million CD4-depleted PBMCs.

We were able to perform both the proliferation and Elispot assays on 7 TB donors and 3 LTBI donors (Table 3.4). The same peptide specificities were not identified in both assays in any of the donors, suggesting populations of MTB-specific CD8 T cells with different epitope specificities may be associated with different effector functions. Taken together, these data indicate that MTB-specific IFNγ-producing CD8 T cells that lack proliferative capacity are present in individuals with TB, and that CFP10 and ESAT6-specific CD8 T cells may be present at very low frequencies in peripheral blood of LTBI individuals, but maintain robust proliferative capacity upon stimulation for several days *in vitro*.

Table 3.4. Comparison of individual CD8 T cell responses from MTB infected donors identified with either a 6-day proliferation assay or an *ex vivo* IFNγ Elispot assay.

Donor ID	Proliferation assay	Ex vivo IFNγ Elispot assay	MTB status
TB-1001	No response	No response	TB
TB-1002	E2	C15	TB
TB-1018	No response	E14	TB
TB-1043	E6, E9	No response to ESAT6 peptide pool	TB
TB-1045	No response	No response	TB
TB-1010	No response	E5, E10, E12	TB
TB-036	No response	C17	TB
17-1015	C6, C15	No response to CFP10 peptide pool	LTBI
17-1003	No response	E2, E12, E13	LTBI
17-1028	No response	C11	LTBI

3.2.5 Increased breadth and magnitude of MTB-specific proliferative CD4 T cell responses in LTBI, compared with TB donors

The above results indicate CD8 T cell proliferation is impaired in individuals with TB, compared with LTBI. To begin to address whether there was also evidence of differential proliferative capacity of CD4 T cells in LTBI and TB donors, CD4 T cell proliferation was analyzed in the OG-based proliferation assay described above. The information gathered from this subset was used to create a CD4 T cell peptide map for CFP10 and ESAT6. For TB donors, only one donor responded to any given peptide, whereas an immunodominant CFP10 peptide, C16, elicited a response from 6/20 LTBI donors (Figure 3.8A). Four ESAT6 peptides (E6, E12, E13, and E15) elicited a proliferative CD4 T cell response in 4/20 LTBI donors (Figure 3.8B). Apart from peptide E17, each peptide was recognized by CD4 T cells from at least one LTBI donor, thus indicating the broad level of immunogenicity for both of these proteins. Similar to CD8 T cells, CD4 T cells from LTBI donors proliferated to a greater breadth of ESAT6 and CFP10 peptides overall than CD4 T cells from TB donors (Figure 3.9A). The magnitude of MTB-specific proliferating CD4 T cells was also greater in LTBI donors (Figure 3.9B). Overall, the results indicate that CD4 T cells in LTBI have an increased magnitude of proliferation compared to TB disease, and recognize a greater breadth of peptides in both CFP10 and ESAT6.

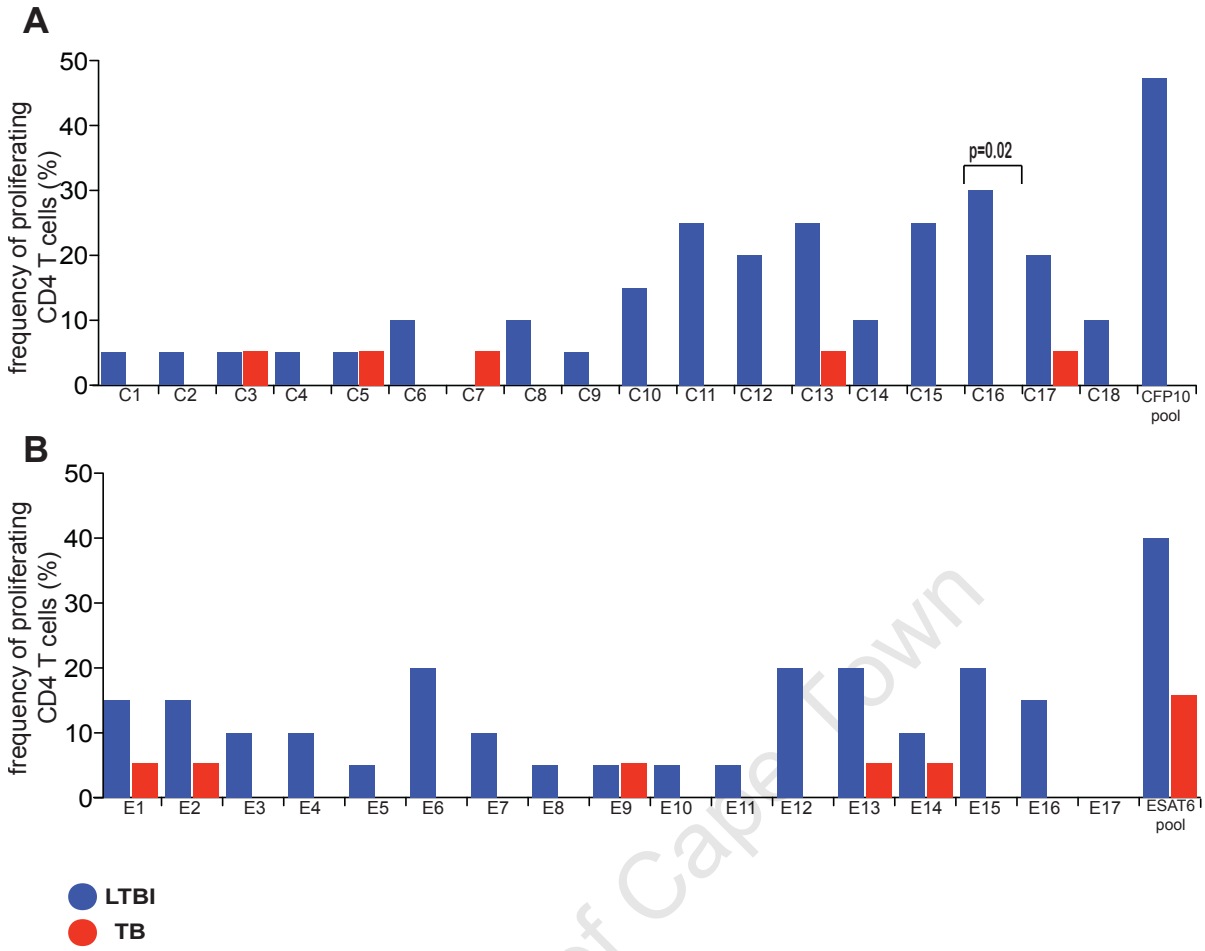


Figure 3.8. Frequency of recognition of individual 15-mer peptides in CFP10 and ESAT6 by CD4 T cells. PBMCs were incubated with individual overlapping 15-mer peptides from CFP10 (A) or ESAT6 (B) in a 6-day proliferation assay. The percentage of TB (n=19) and LTBI (n=20) donors who had a positive proliferative CD4 T cell response to each peptide is shown. Differences in the frequency of recognition of individual peptides between LTBI and TB donors were assessed using the Fisher's exact test.

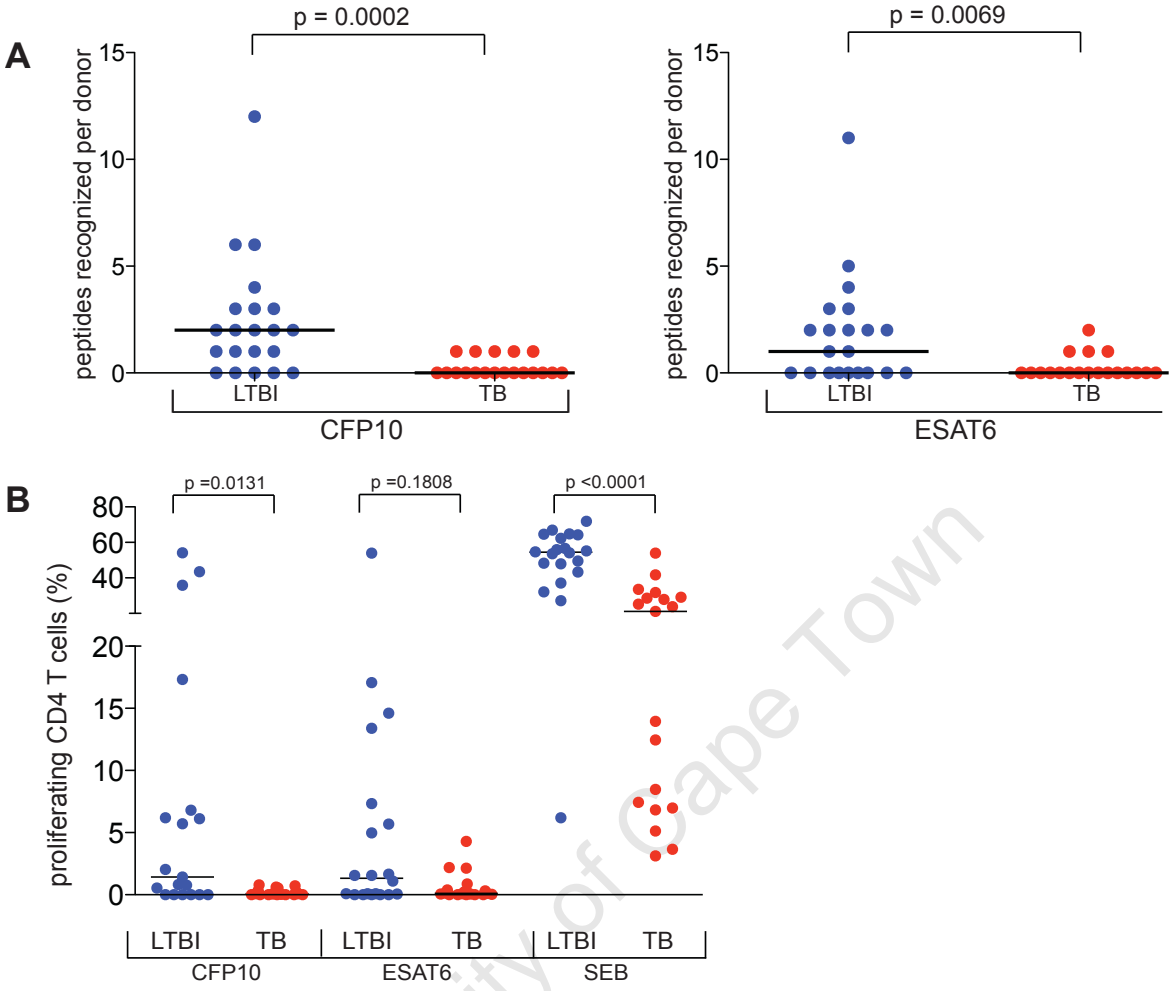


Figure 3.9. Greater breadth and magnitude of proliferative CD4 T cell responses in individuals with LTBI, compared to patients with TB. Individual overlapping peptides spanning the sequence of CFP10 and ESAT6, or CFP10 or ESAT6 peptide pools, were used to stimulate PBMCs from donors with LTBI (n=20) and TB (n=19) in a 6-day proliferation assay. (A) The number of peptides with a positive proliferative response for each donor is shown. (B) The frequency of proliferating (OG^{low}) CD4 T cells after background subtraction is shown. Cells were stimulated with CFP10 and ESAT6 peptide pools or SEB. Differences were assessed using a Mann Whitney test. Horizontal lines represent medians.

3.3 Discussion

Correlates of protection need to be identified in order for rational vaccine design. In MTB infection, identification of immune correlates of protection is based in part on the assumption that latently infected individuals have managed to control infection more successfully than individuals who progress to develop active TB disease. Therefore, factors identified in individuals with LTBI but not TB may correlate with protection against development of TB disease. Such factors include the phenotype and functional capacity of antigen-specific T cells, for example memory cell phenotype, cytotoxicity, proliferative capacity, and cytokine production. In this study, we found that CD8 T cells from TB donors exhibited impaired proliferative capacity compared with LTBI upon stimulation with MTB-specific proteins and SEB. It is hypothesized that reduced proliferation in individuals with TB upon SEB stimulation may be due to the previous exposure of these cells to an altered microenvironment that was caused by TB disease. Differential epitope specificity was also observed in the proliferation assay, with CD8 T cells from LTBI donors targeting different peptides than TB donors, for example E12 was recognized by CD8 T cells from LTBI donors and not TB disease donors. Moreover, LTBI donors had proliferative CD8 T cell responses to a greater breadth of peptides than TB disease donors. A decreased breadth and magnitude of CD4 T cell proliferative responses was also seen in individuals with TB, compared with LTBI.

Antigen-specific CD8 T cells that produce cytokines but lack proliferative capacity are likely to be effector cells. These may be terminally differentiated fully functional cells, or terminally differentiated exhausted cells with reduced functionality, including reduced cytokine production and proliferation. Decreased PBMC proliferative capacity to CFP10 and ESAT6 peptide pools in individuals with TB disease, compared with LTBI, has been previously reported (Govender, Abel et al. 2010). The increased magnitude of proliferation in LTBI donors in the presence of low levels of *ex vivo* IFNγ production in CD8 T cells implies a central memory phenotype of these cells. However a limitation of these data is that specific memory markers were not evaluated, hence classification into memory subsets was not possible. Due to the lack of proliferative capacity upon stimulation, it is thought that terminally differentiated cells will not become long-lived memory T cells. It must also

be noted that the *ex vivo* frequency of MTB-specific CD8 T cells is low in LTBI donors, as measured by whole blood ICS assays with CFP10 and ESAT6 peptide pools (data not shown), but increases after a 6-day culture *in vitro*. The IFNγ production by CD8 T cells of TB donors in the absence of proliferation suggests that the phenotype of CD8 T cells in individuals with TB differs from that of LTBI.

A possible confounding factor that may contribute to differences in proliferation between the two groups is reduced viability in the TB donors after *in vitro* culture. However, T cell viability as measured by the frequency of $VIVID^{low}$ CD3+ cells after 6 days was similar in TB and LTBI donors (data not shown), suggesting that cell death in the assay system was not solely responsible for the decreased proliferation seen in TB donors.

We have shown that CD8 T cells in individuals with TB produce IFNγ upon short-term stimulation, but have a reduced proliferative capacity. IFNγ production is one of the last functions to cease in the event of T cell exhaustion, which results in dysfunctional T cell responses under conditions of high levels of persistent antigen stimulation (Wherry 2011). The reduced T cell proliferative capacity in the context of TB disease implies that MTB-specific CD8 T cells from these individuals may be functionally impaired. As the mycobacterial antigen load is greater in TB disease than LTBI, we initially hypothesized that high levels of antigen exposure would result in a greater breadth of recognition of CD8 T cell epitopes in the context of active TB disease compared with LTBI. Contrary to this hypothesis, there was a decreased breadth of recognition of CFP10 and ESAT6 epitopes among TB disease donors, as measured in the 6-day proliferation assay using cells isolated from peripheral blood. This is further evidence supporting the theory that CD8 T cells in TB disease are exhausted. However, it must be noted that MTB-specific CD8 T cells in TB disease may be trafficking to the site of disease in the lung, and therefore CD8 cells in the blood may not reflect the true breadth of responses present in these individuals.

The CFP10 peptides identified in this study correspond with previous data indicating the immunogenicity of the C-terminus region of this protein and its importance in cell surface binding (Renshaw, Lightbody et al. 2005). The $71\text{-}85^{th}$ aa position of CFP10 has been found to contain two epitopes by Shams *et al* (Shams, Klucar et al. 2004): IRQAGVQYSR (T1) and EISTNIRQA (T6). T1 corresponds to aa

76-85, so it is found in both C15 and C16 sequences used in this study. T6 corresponds to aa 71-79, so it is contained solely in C15. We found that peptides C15 and C16 were recognized by both CD4 and CD8 T cells in LTBI individuals. Lewinsohn *et al* used T cell clones to identify another CFP10 epitope corresponding to aa 2-11 (Lewinsohn, Zhu et al. 2001). We did not identify any MTB-infected donors in our study responding to this sequence in CFP10, possibly due to the small sample size or differential expression of particular MHC alleles between the two study populations.

We found that ESAT6 peptides were widely recognized across the protein by T cells in individuals with LTBI and TB disease, consistent with previous studies (Chapman, Munkanta et al. 2002). For example, it has been shown by Lalvani *et al* that aa 69-76 of ESAT6 is immunodominant in a donor with active TB disease (Lalvani, Brookes et al. 1998). These peptide-specific cells displayed cytolytic activity and produced IFNγ. This sequence is within peptide E14, which induced IFNγ production *ex vivo* in TB donors, but not proliferation in the assays performed in this study. Lalvani *et al* also identified aa 82–90 as immunogenic (Lalvani, Brookes et al. 1998); this sequence is found within E15, which was identified in the proliferation assay but not in the IFNγ assay as immunogenic. A minimal epitope corresponding to aa 21–29 of ESAT6 was found in an *ex vivo* Elispot (Pathan, Wilkinson et al. 2000). It corresponds to a sequence within E4, which was identified in this study as immunodominant in one TB donor, also within the *ex vivo* Elispot assay.

We were able to perform both the *ex vivo* Elispot and the proliferation assays in 7 TB donors and 3 LTBI donors. It was very interesting to note that donors with a response to individual peptides in the proliferation assay did not have a response to the same peptides or the corresponding peptide pool in the Elispot assay. This was reciprocal, as responses in the Elispot assay were not found in the proliferation assay within individual donors. This strongly suggests that cytokine production and proliferative capacity represent distinct populations of antigen-specific CD8 T cells in MTB infection, and that the relative dominance of these functionally distinct populations may be associated mycobacterial antigen load and MTB infection/disease status. It must be mentioned that the extent to which CD4 T depletion influenced CD8 T cell responses to peptide stimulation in this short term assay is unknown. CD4 T cells may be required for improved antigen presentation

and co-stimulation to CD8 T cells; hence their depletion may affect CD8 T cell recognition in the epitope mapping data. However we were still able to detect IFNγ production from CD8 T cells in response to stimulation with the 15mer peptides. A potential reason why CD4 T cell proliferation was greater in magnitude than CD8 proliferation may be that TB is an intracellular pathogen of macrophages, thus antigens are preferentially processed and presented by MHC II molecules. Hence CD4 T cells are the dominant response induced by MTB.

It is important to note the geographical differences in study populations between previous studies and this study, as HLA restrictions identified in North American and European cohorts may not be prominent in the South African population. Because we did not HLA type donors in this study, it was not possible to define the HLA restriction and minimal epitopes for these peptides. Another limitation of this study was that only IFNγ production and proliferation were used as markers of antigen specificity. As a result, antigen-specific CD8 T cells secreting other cytokines or with cytotoxic activity may be missed. Antigen-specific CD8 T cells commonly produce IFNγ in conjunction with other cytokines, and it is one of the last cytokines to stop being produced in the event of exhaustion. Additional limitations of this study include the small sample size and inter-individual variation in background cytokine production and proliferation. Furthermore, it is possible that NK or NKT cells may produce IFNγ in the Elispot assay, however results from whole blood *ex vivo* ICS assays indicate that CD4- and CD8-negative cells do not produce significant amounts of IFNγ upon stimulation with CFP10 and ESAT6 peptides (data not shown).

While low frequencies of IFNγ+ CD8 T cells are present in LTBI, they proliferate robustly upon stimulation *in vitro*. This suggests that the antigen specific cells in LTBI may have a central memory phenotype. This indicates that the *in vivo* environment in latently infected individuals may contain low levels of exposure to MTB antigens hence the antigen specific CD8 T cells are not chronically exposed to antigen. Because of this, the cells are not stimulated to become effector CD8 T cells, and instead maintain a long-term memory phenotype that is capable of robust proliferation upon antigen re-stimulation.

Overall these results highlight the need to further define factors contributing to the functional differences observed in MTB-specific CD8 T cell responses between individuals with LTBI and TB. In the next chapter the phenotype of antigen-specific CD8 T cells in individuals with TB were compared to LTBI donors in order to further characterize MTB-specific CD8 T cell responses that are associated with either protection from, or progression to active TB disease.

CHAPTER FOUR

Phenotypic characterization of *Mycobacterium tuberculosis*-specific CD8 T cells in latent and active TB disease

4.0 Overview

In the previous chapter, we observed that some MTB-specific CD8 T cells from individuals with TB disease produced detectable levels of IFNγ *ex vivo*, but lacked robust proliferative capacity, whereas the opposite was true of MTB-specific CD8 T cells from healthy individuals with latent MTB infection (LTBI). It is important to determine the phenotypes of MTB-specific CD8 T cells that are associated with functional capacity in the context of latent infection and active disease in order to better understand the role of CD8 T cells in control of MTB infection.

A multiparameter flow cytometry panel was designed and optimized to assess cell turnover, susceptibility to apoptosis and terminal differentiation/senescence in CD8 T cells from TB and LTBI donors. Bcl-2, Ki67, CD95, CD57, CD127 and IFNγ were measured in each group, and the antigen-specific CD8 T cells were compared to the total CD8 T cell population. We hypothesized that activated MTB-specific CD8 T cells in TB would display an effector phenotype with a high rate of turnover, and be more prone to apoptosis, compared with LTBI.

The results indicate that Bcl-2 and CD57 expression are increased in the total CD8 T cell population in individuals with TB disease, compared with LTBI, which may be due to inflammatory-mediated alterations of the tissue microenvironment. CD127 expression was increased on MTB-specific CD8 T cells, compared with the total CD8 T cell population, in individuals with LTBI, but not TB. Preliminary results shown in this chapter suggest that initiation of anti-TB treatment may reverse the phenotype seen in TB disease, implying that MTB-specific CD8 T cell phenotype is associated with mycobacterial antigen load.

Together these data suggest that MTB-specific CD8 T cells from individuals with TB have a short-lived effector phenotype relative to MTB-specific CD8 T cells from individuals with LTBI. This may explain in part the reduced proliferative capacity in TB disease, as shown in the previous chapter. Additionally, active TB disease state influences the total CD8 T cell population by increasing the expression of Bcl-2 and CD57. The mechanisms by which mycobacterial antigen load and disease status affect the phenotype and functional capacity of CD8 T cells should be further elucidated.

4.1 Introduction

Antigen stimulation in acute infection causes T cells to expand rapidly and then contract upon pathogen clearance, due to the withdrawal of cytokines required for proliferation and maintenance (Snow, Pandiyan et al. 2010). However in some instances the pathogen is not cleared, resulting in a chronic infection. During chronic infection, immune exhaustion characterized by a hierarchical loss of function has been described in antigen-specific T cells as a result of sustained T cell receptor (TCR) signaling by high antigen levels (Akbar and Henson 2011). The capacity to produce IL-2 is initially lost, followed by impaired proliferative ability, loss of TNFα production, and reduced cytotoxic activity. Only in the later stages of exhaustion is IFNγ production compromised (Wherry, Blattman et al. 2003), and eventually populations of exhausted antigen-specific T cells may be deleted. The functions lost may vary depending on the disease model, and are associated with antigen load and disease progression. For instance, human immunodeficiency virus (HIV)-infected long-term non-progressors (LTNP) display relatively limited T cell exhaustion compared with chronically infected adults with very high viral loads. The stage of infection (early vs. late) may also influence the level of exhaustion (Wherry, Blattman et al. 2003).

T cell exhaustion was first described in a mouse model of LCMV infection, in which virus-specific CD8 T cells did not have antiviral effector functions even though they expressed activation markers (Zajac, Blattman et al. 1998). Exhaustion has been studied in viral models of chronic infection such as LCMV, HIV, HCV and cytomegalovirus (CMV), in both animal and human models of infection (Fletcher, Vukmanovic-Stejic et al. 2005; Day, Kaufmann et al. 2006; Urbani, Amadei et al. 2006). T cell dysfunction due to exhaustion is associated with increased expression of inhibitory receptors such as PD-1 and CTLA-4 (Day, Kaufmann et al. 2006; Blackburn, Shin et al. 2009). Upon interaction with its ligands PD-L1 and PD-L2, PD-1 signaling inhibits activation, proliferation and effector functions of antigen-specific T cells. (Barber, Wherry et al. 2006) CTLA-4 is an inhibitory marker that is upregulated in CD4 but not CD8 T cells in HIV infection (Kaufmann, Kavanagh et al. 2007); it is co-expressed with PD-1 in most cells, and its blockade results in increased HIV-specific CD4 T cell function (Kaufmann, Kavanagh et al. 2007).

To date there has been little investigation of immune exhaustion markers in the context of MTB infection in humans. It has however been reported that PD-1 may play a role in regulating CD8 T cells in MTB infection. Blockade of the PD-1 signaling pathway resulted in an increased frequency of MTB-specific IFNγ-producing T cells, and enhanced degranulation of CD8 T cells from individuals with TB (Jurado, Alvarez et al. 2008). The expression of inhibitory markers does not always indicate functional exhaustion, as certain markers are also correlates of late stage differentiated, functional T cells (Akbar and Henson 2011). Although prolonged expression of inhibitory receptors is a key aspect of exhausted T cells, activated cells can transiently express these receptors as well (Agata, Kawasaki et al. 1996).

Terminally differentiated T cells have minimal proliferative capacity and are susceptible to apoptosis, however they may persist *in vivo* in the presence of appropriate survival signals (Akbar and Henson 2011). It has been suggested that this persistence allows sustained low level immune responses that will keep pathogen levels at a minimum (Snow, Pandiyan et al. 2010). CD57 is one of several markers of terminally differentiated CD8 T cells. CD57, also known as Leu-7, is found on natural killer (NK) cells as well as some CD3+ cells (Wood, Twigg et al. 2009). The function of CD57 on T cells is unknown, however a sequence within it, known as HNK-1, has been found in various other cells such as neural tissue in the eye, and in cell adhesion molecules (Wood, Twigg et al. 2009).

Previous studies in HIV infection have indicated that CD57 expression is a more accurate predictor of terminal differentiation than CD27 downregulation (Wood, Twigg et al. 2009). It has been suggested that CD57+ T cell frequencies could be used as a correlate of immune competence and risk for opportunistic infection (Focosi, Bestagno et al. 2010). Clonally expanded senescent T cells express CD57, have defects in proliferation (Wood, Twigg et al. 2009), and express high levels of Fas ligand, which renders cells susceptible to Fas-mediated apoptosis (Wood, Knox et al. 2005). Although CD57+ T cells and NK cells are less able to proliferate (Focosi, Bestagno et al. 2010), they are still able to produce IFNγ upon encounter with their cognate antigen (Brenchley, Karandikar et al. 2003). For example, CD57+ T cells secreted high levels of IFNγ after stimulation with HIV antigens (Appay, Almeida et al. 2007). The proportion of CD57+ T cells increases with age, and increased expression has been reported in chronic immune activation and in

immunocompromised individuals (Wood, Twigg et al. 2009). Increased expression of CD57 on the total CD8 T cell population has also been reported in individuals with newly diagnosed pulmonary TB, compared with healthy controls (Fateminasab, Shahgasempour et al. 2006).

Replicative senescence is a characteristic of terminally differentiated cells, and has been described as the loss of replicative capacity of antigen-specific T cells due to repeated antigen stimulation (Akbar and Henson 2011). Senescence occurs as a natural function of age and repeated cell division, hence it has been frequently studied in elderly populations (Fletcher, Vukmanovic-Stejic et al. 2005; Appay, Fastenackels et al. 2011). Apart from terminal differentiation, senescence may also be due to a cell remaining permanently in G_0 (resting phase) of the cell cycle. Cells may remain in the resting phase of the cell cycle temporarily or permanently and characteristically do not express the nuclear protein Ki67, whereas cells in the active phases of the cell cycle will express Ki67. Therefore Ki67 expression indicates the proliferative ability of a cell, and the proportion of Ki67+ cells in a given population identifies the fraction of this population that is currently cycling (Scholzen and Gerdes 2000). Increased Ki67 expression has been reported on total CD8 T cell populations from TB patients compared with healthy controls (Villacian, Tan et al. 2005). Surprisingly, an increase in Ki67 expression in these patients was seen in the absence of an increase in other cellular activation markers such as CD38 (Villacian, Tan et al. 2005). Ki67 expression is indicative of, but not solely responsible for proliferation, as other signals may also play a role in preventing or encouraging proliferation (Scholzen and Gerdes 2000). Senescence-related signalling has been suggested to lead to apoptotic susceptibility (Akbar and Henson 2011).

An indicator of susceptibility to apoptosis is Fas, also known as CD95 or Apo1. CD95 is a member of the TNF receptor family, all of which possess the death domain. Both CD95 and Fas ligand (CD95L) are induced in the course of an adaptive immune response, as they are necessary for contraction of the effector T cell population after a pathogen has been cleared (Hao and Mak 2010).

The importance of CD95 and CD95L in apoptosis was first identified in mice with mutations in genes encoding these molecules. These mutations resulted in a lymphoproliferative disorder with symptoms similar to systemic lupus erythromatosis

(Takahashi, Tanaka et al. 1994). In TST positive healthy individuals, MTB stimulation caused CD95 mRNA upregulation in both CD4 and CD8 T cells, and antibody blockade of the CD95-CD95L interaction decreased cytolytic function for both T cell populations by 25% (Canaday, Wilkinson et al. 2001). TCR re-stimulation can cause CD95-mediated apoptosis of antigen-specific T cells, thus sparing by-stander cells that bear CD95 (Snow, Pandiyan et al. 2010). Restimulation induced cell death (RICD) is particular to antigen-experienced cells that undergo repeated stimulation with their cognate antigen. Susceptibility to RICD in memory cells can be measured using an *ex vivo* stimulation assay in a chronic disease model and identifying changes in expression of pro- and anti-apoptotic markers relative to the non-specific population (Snow, Pandiyan et al. 2010). The prevention of CD95-mediated RICD of bystander cells involves the expression of molecules which counter-act the pro-apoptotic signals (Krueger, Fas et al. 2003). Bcl-2 is a well-known anti-apoptotic molecule capable of counteracting the effects of CD95-mediated signaling (Hao and Mak 2010; Snow, Pandiyan et al. 2010).

Bcl-2 has been studied in the context of chronic lymphocytic leukemia, which involves the pathological accumulation of T lymphocytes. Bcl-2 accumulation results in reduced apoptosis (Wiegers, Kaufmann et al. 2011). Certain pro-apoptotic proteins influence the mitochondrial outer membrane potential, causing permeabilization and release of toxic molecules into the cytosol, thus leading to apoptosis. Anti-apoptotic proteins prevent this permeabilization, thus preventing apoptosis. It has been suggested that the presence of Bcl-2 may increase the half-life of memory CD8 T cells (Kurtulus, Tripathi et al. 2011). MTB infection of mice results in increased CD95 expression, and decreased Bcl-2 expression in total CD4 T cells, relative to a control group of uninfected mice, suggesting greater susceptibility to apoptosis upon MTB infection (Das, Vohra et al. 1999).

IL-7 signaling results in Bcl-2 upregulation within T cells that bear the IL-7 receptor (Nanjappa, Kim et al. 2011). Expression of the alpha chain of the IL-7 receptor (IL-7Rα, CD127) plays a non-redundant role in the maintenance of memory T cells via homeostatic proliferation (Nanjappa, Kim et al. 2011; Pellegrini, Calzascia et al. 2011). CD127 expression above a certain threshold has been associated with proliferation, whereas expression below this threshold results in cell survival, but not cell proliferation (Palmer, Mahajan et al. 2011). Therefore if memory T cells do not

posses adequate levels of CD127, they will not proliferate, and the immunological memory represented by that memory pool will cease to exist (Wherry 2011). HIV-infected LTNPs maintain highly functional proliferating CD8 T cells, in contrast with impaired proliferative capacity and low CD127 expression by HIV-specific CD8 T cells in individuals with high viral loads (Wherry, Day et al. 2006; Day, Kiepiela et al. 2007).

Effector CD8 T cells transiently downregulate homeostatic cytokine receptors such as CD127 (Shin and Wherry 2007). However, mouse models of acute viral infection indicate high CD127 expression of a small subset of effector T cells during acute infection identifies populations of effector cells that will differentiate into long-lived memory cells (Kaech, Tan et al. 2003). $CD127^{high}$ cells were found to express higher levels of anti-apoptotic molecules including Bcl-2 (Kaech, Tan et al. 2003), and CD127 expression and Bcl-2 expression were found to correlate in LCMV-specific CD8 T cells during memory cell development (Kaech, Tan et al. 2003). Additionally, administration of IL-7 to MTB-infected mice increased their survival (Maeurer, Trinder et al. 2000).

In this chapter, we used an *ex vivo* flow cytometry-based intracellular cytokine staining and phenotyping assay to characterize the expression of a novel panel of markers in CD8 T cells from individuals with LTBI and TB. This panel incorporates several relevant markers, and hence allows us to develop an idea of the interplay between senescence, terminal differentiation, proliferation, apoptotic susceptibility and antigen specificity. The results from these studies provide important insight regarding the reduced functional capacity of MTB-specific CD8 T cells in individuals with active TB disease compared with asymptomatic latent infection.

4.2 Results

4.2.1 Participant characteristics

A total of 30 participants were recruited for these studies (Table 4.1). Ten adults with active pulmonary TB disease (TB) who had received less than one week of anti-TB treatment were recruited. Additional blood samples were obtained at two months and six months after initiation of treatment in five of these donors for use in a longitudinal analysis. Twenty healthy asymptomatic adults with latent MTB infection (LTBI) were recruited; LTBI participants were identified by a positive response to ESAT6 and/or CFP10 in an IFNγ release assay (IGRA), and no previous history of TB diagnosis or treatment.

Table 4.1. Participant characteristics.

Characteristics	TB	LTBI
Subjects (n)	10	20
Female, n (%)	1 (10%)[a]	15 (75%)
Smear positive (%)	8 (80%)	N/A
Smear negative (%)	2 (20%)	N/A
Age[b], (range)	41.8 (26-60)[c]	25.9 (18-43)

[a] p = 0.0014, compared with LTBI

[b] Values denote mean (range)

[c] p <0.0001, compared with LTBI

N/A: not applicable.

4.2.2 *Intracellular cytokine staining and phenotyping assay*

In order to compare the phenotype of CD8 T cells between LTBI and TB donors, PBMCs were stimulated *ex vivo* for 6 hours with CFP10 or ESAT6 peptide pools to identify MTB-specific T cells; cells were stimulated with CMV pp65 peptide pool and SEB as non-MTB antigen-specific and positive control antigens, respectively. Stimulated cells were analyzed by flow cytometry and antigen-specific CD8 T cells were identified as CD8+IFNγ+ T cells. The expression of CD127, Bcl-2, CD95, CD57 and Ki67 was assessed on the total CD8 T cell and antigen-specific CD8 T cell populations. Representative flow plots illustrating the gating strategy in one LTBI donor are shown in Figure 4.1.

It is possible that differences in T cell viability can affect the expression of markers, as well as the number of cells acquired. In order for valid comparisons to be performed between the two groups, it is important that cell viabilities are similar. Figure 4.2 shows that there are similar frequencies and number of viable lymphocytes (CD3+VIVID^{low} cells) acquired between the two groups.

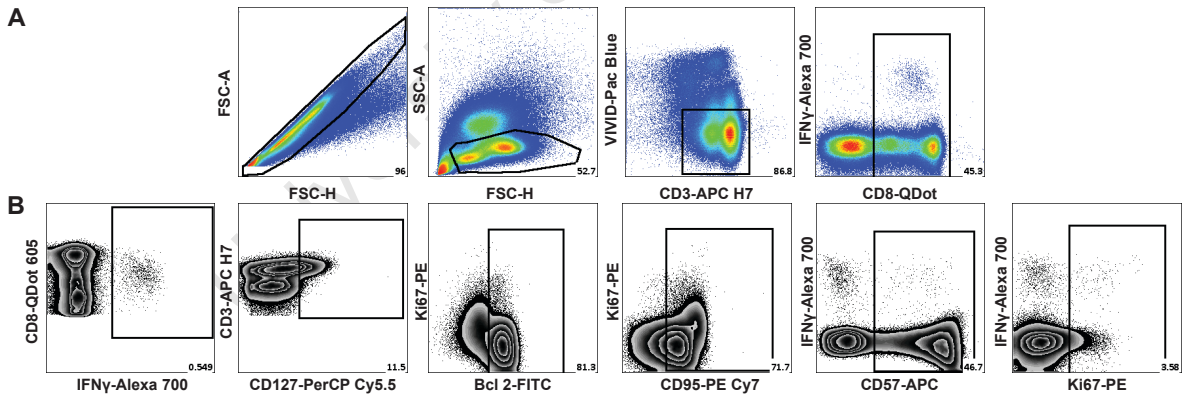


Figure 4.1. Flow cytometry gating strategy. Representative flow plots from individual with TB following stimulation with CFP10 peptide pool. (A) Singlets and lymphocytes were gated on based on forward scatter and side scatter characteristics, live (VIVID^{low}) CD3+ cells were gated on, followed by CD8+ cells. (B) After this preliminary gating, IFNγ, CD57, CD127, Bcl-2, Ki67 and CD95 positive subsets were identified.

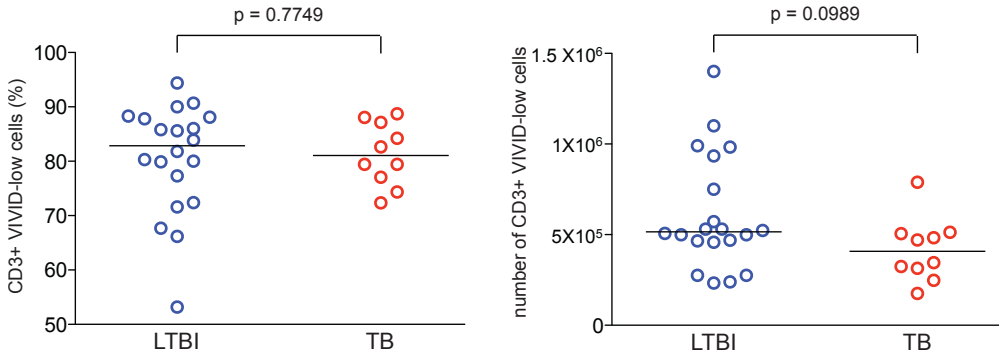


Figure 4.2. T cell viability is similar in LTBI and TB PBMCs. Viability of cryopreserved PBMCs was assessed in LTBI (n=20) and TB (n=10) CD3+ lymphocytes using flow cytometry. Left: frequency of viable CD3+ T cells as a percentage of total lymphocytes. Right: number of viable CD3+ lymphocytes acquired for each donor. Statistical differences were assessed using the Mann Whitney test. Horizontal lines represent the median.

4.2.3 Activated Ki67+ CD8 T cells in peripheral blood ex vivo do not differ by disease state

In order to identify whether differences in the activation status of cells could explain the reduced proliferation seen in TB donors relative to LTBI, total CD8 T cell populations were analyzed for Ki67 expression. The proportion of *ex vivo* Ki67+ CD8 T cells (actively dividing) was similar between groups (Figure 4.3), indicating that reduced proliferation in TB was not due to an increased proportion of actively dividing CD8 T cells, as measured by intracellular Ki67 expression, in peripheral blood *in vivo*.

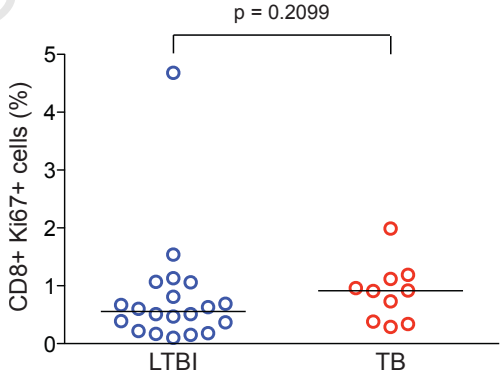


Figure 4.3. Ki67 expression in total CD8 T cells does not differ between disease states. The total CD8 T cell population in LTBI and TB donors was analyzed *ex vivo* using flow cytometry. The proportion of Ki67+ CD8 T cells is shown. Statistical differences were assessed by the Mann Whitney test. Horizontal lines represent the medians.

4.2.4 Increased CD57 and Bcl-2 expression by CD8 T cells in TB donors, compared with LTBI

Differences in the microenvironment due to inflammation during active disease may cause phenotypic changes in the total CD8 T cell population. In order to identify whether MTB infection and disease state influenced the phenotype of the total CD8 T cell population, expression of CD95, CD57, Bcl-2 and CD127 was compared between TB and LTBI donors using flow cytometry. In the total CD8 T cell population, susceptibility to Fas-mediated cell death, as measured by CD95 expression, was not different between TB and LTBI donors. However, CD57 expression was significantly higher in TB donors, compared with LTBI (Figure 4.4).

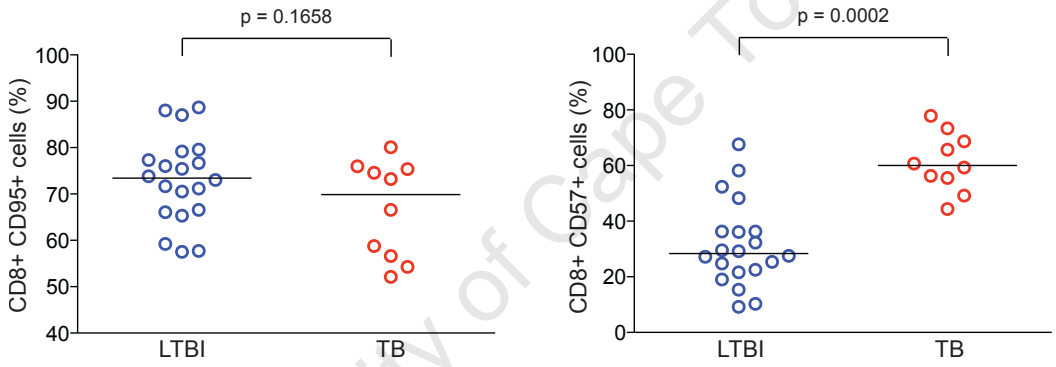


Figure 4.4. CD8 T cells in individuals with TB express higher levels of CD57, but not of CD95, compared with LTBI. The expression of CD95 and CD57 on total CD8 T cell populations was analyzed by flow cytometry. The percentage of CD95+ CD8 T cells is shown on the left graph and the percentage of CD57+ CD8 T cells is shown on the right graph. Horizontal lines represent the medians. Statistical differences were assessed by the Mann Whitney test.

CD57 expression has been shown to be a function of age, with low expression on lymphocytes in infants, whereas adults constitutively express CD57 in 15%-20% of lymphocytes (Focosi, Bestagno et al. 2010). The median age of the TB donors in this study was higher than LTBI donors (Table 4.1). To determine whether CD57 expression by CD8 T cells is associated with age in this study, we correlated the frequency of CD8+CD57+ T cells in LTBI and TB donors with age and found a significant but weak positive correlation (Figure 4.5), suggesting the higher levels of CD57 expression seen in individuals with TB may at least be partially a function of

their increased age relative to the LTBI donors. Unfortunately we did not have access to samples from younger TB patients, therefore we could not compare age-matched donors. In the future we could specifically recruit older LTBIs that are age-matched to the TB group to try to eliminate this possible confounder in CD57 between the two groups.

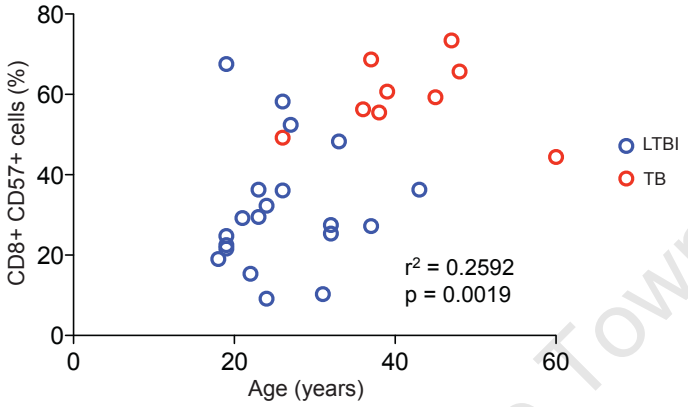


Figure 4.5 Association between CD57 expression on CD8 T cells and age. The frequency of CD57+ cells in the total CD8 T cell population was correlated with age in years for both LTBI (blue circles; n=20) and TB donors (red circles; n=10). A linear regression analysis was performed.

CD127 expression on CD8 T cells was similar between the groups, despite increased Bcl-2 expression in individuals with TB (Figure 4.6). This indicates the involvement of a CD127-independent pathway of Bcl-2 upregulation. However, a linear regression analysis indicated CD127 expression was moderately related to Bcl-2 expression, independent of disease status (Figure 4.7). These data suggest that although a greater proportion of the total CD8 T cell population in TB donors is terminally differentiated, these cells may be protected from Fas-mediated programmed cell death, as evidenced by lack of CD95 upregulation and increased Bcl-2 expression, compared with LTBI donors.

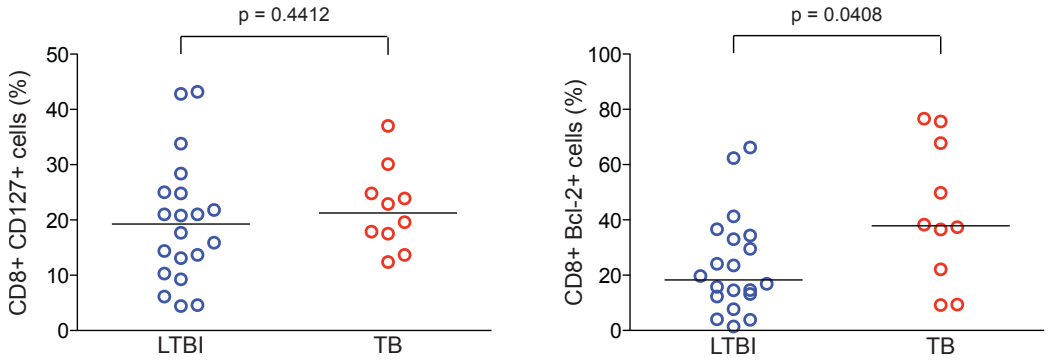


Figure 4.6. CD8 T cells in individuals with TB express higher levels of Bcl-2, but not of CD127, compared with LTBI. The expression of CD127 and intracellular Bcl-2 in total CD8 T cell populations was analyzed by flow cytometry. The percentage of CD127+ CD8 T cells is shown in the left graph and Bcl-2+ CD8 T cells in the right graph. Horizontal lines represent the medians. Differences were assessed with the Mann Whitney test.

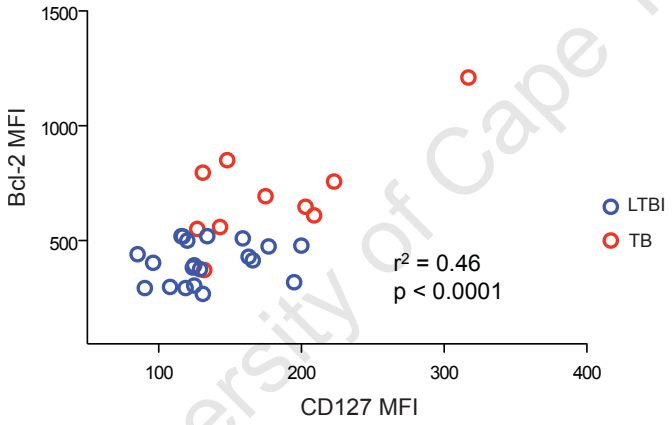


Figure 4.7. CD127 expression influences Bcl-2 expression. A linear regression was performed on CD127 and Bcl-2 expression on total CD8 T cell populations from both LTBI (blue circles; n=20) and TB donors (red circles; n=10).

4.2.5 Increased expression of CD127 on MTB-specific CD8 T cells in latent infection

We next investigated phenotypic differences in MTB-specific CD8 T cells between individuals with TB and LTBI. PBMCs were stimulated for 7 hours with ESAT6 or CFP10 or CMV phosphoprotein-65 (pp65) peptide pools, after which IFNγ production was detected using flow cytometry, and used to identify antigen-specific CD8 T cells. 5/10 TB donors and 7/20 LTBI donors had a positive response in this assay to either ESAT6 or CFP10. 10/10 TB donors and 18/20 LTBI donors had a CMV-specific CD8 T cell response, and all donors had a response to the positive control *Staphylococcal* enterotoxin B (SEB).

Compared to the total CD8 T cell population, CD127 expression was increased on MTB-specific CD8 T cells in LTBI donors, indicating an increased capacity for homeostatic proliferation (Figure 4.8A, B). In contrast, there was no difference in CD127 expression between MTB-specific and total CD8 T cells in individuals with TB (Figure 4.8A, B). However when compared to each other, CD127 expression was similar in MTB-specific CD8 T cells between TB and LTBI donors (Figure 4.8C). In order to identify whether increased or maintained CD127 expression was particular to MTB-specific CD8 T cells in LTBI or TB respectively, CD127 expression was compared in this subset to CMV-specific CD8 T cells, and SEB-stimulated cells within the same cohort (Figure 4.8D). In LTBI donors, MTB-specific CD8 T cells displayed greater CD127 expression levels relative to the CMV and SEB controls. This difference was not seen in TB disease (Figure 4.8D). Together these data indicate CD127 expression on antigen-specific CD8 T cells may be reflective of the amount of antigen exposure *in vivo*.

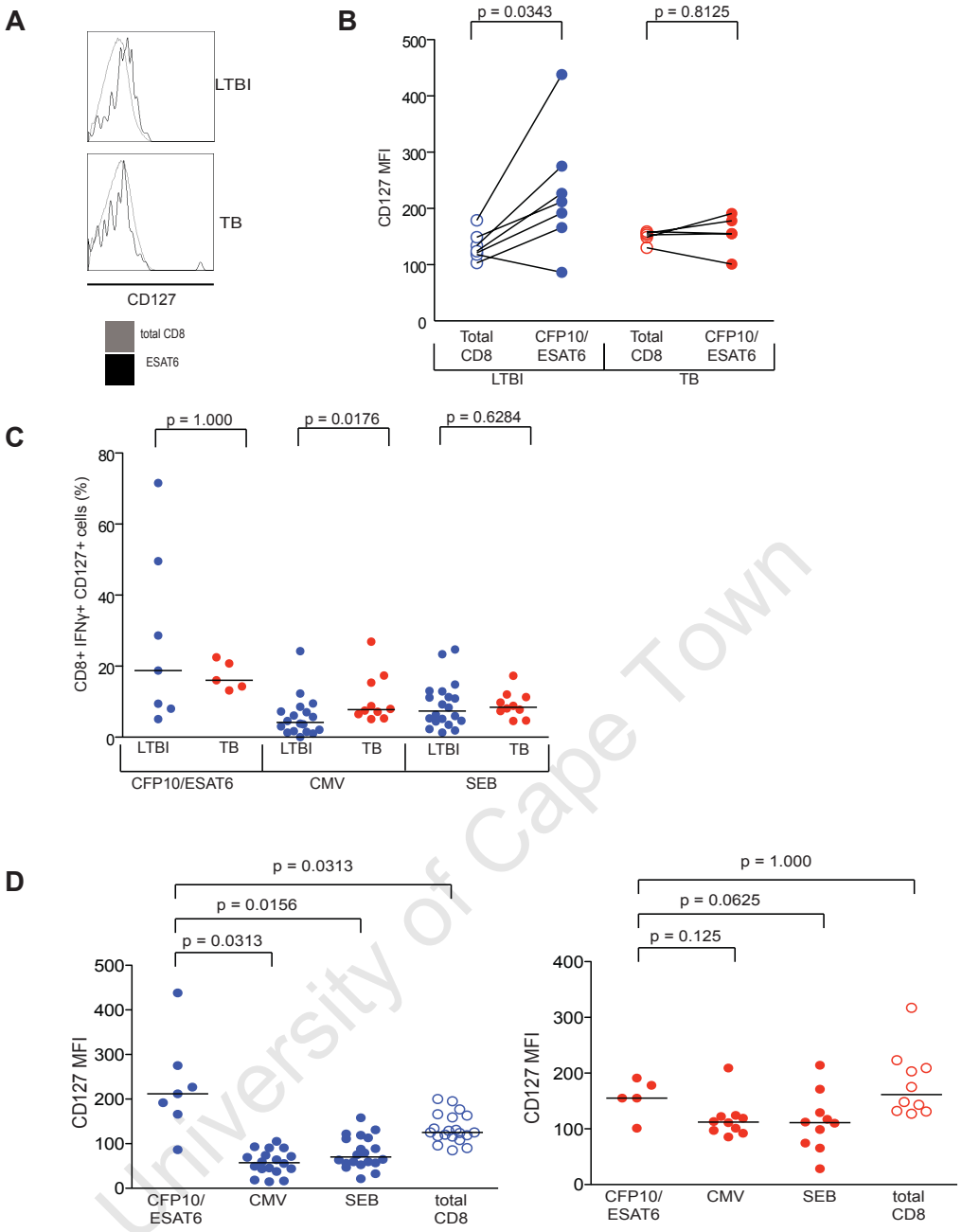


Figure 4.8. Increased CD127 expression on MTB-specific CD8 T cells is associated with latent infection. After 7-hour stimulation with ESAT6 or CFP10 peptide pools, CMV pp65 peptide pool, or SEB, CD127 expression was determined on CD8+IFNγ+ cells and total CD8 T cells. (A) Representative histograms from one LTBI and one TB donor indicating CD127 expression of MTB-specific CD8+IFNγ+ cells (black line) and the total CD8 T cell population (grey line). (B) After stimulation with ESAT6 or CFP10, CD127 expression on CD8+IFNγ+ cells was compared to the total CD8 T cell population in LTBI and TB. (C) The frequency of CD8+IFNγ+ CD127+ cells was compared between groups using a Mann Whitney test. Horizontal lines represent the medians. (D) Comparison of CD127 expression by CD8+IFNγ+ T cells between the various antigens for LTBI donors (left panel) and TB donors (right panel). Statistical differences in B and D were determined by the Wilcoxon matched pairs test. Horizontal lines represent the medians.

4.2.6 Ki67 expression is similar between antigen-specific CD8 T cells in individuals with LTBI and TB

We next compared turnover of MTB-specific CD8 T cells in TB and LTBI donors by analysis of Ki67 expression (Figure 4.9A, B). If a greater proportion of MTB-specific CD8 T cells were activated in the context of active TB disease, then Ki67 expression should be increased in the MTB-specific cells from TB donors relative to LTBI donors. Contrary to this hypothesis, Ki67 expression levels did not differ between groups (Figure 4.9C). Additionally, there were no differences in Ki67 expression when comparing the total CD8 T cell population to MTB-specific CD8 T cells or between antigen stimulation conditions in either group (Figure 4.9B, D), implying that the percentage of actively dividing MTB-specific CD8 T cells circulating in peripheral blood may not be directly correlated with the other phenotypic and functional differences observed between the two groups.

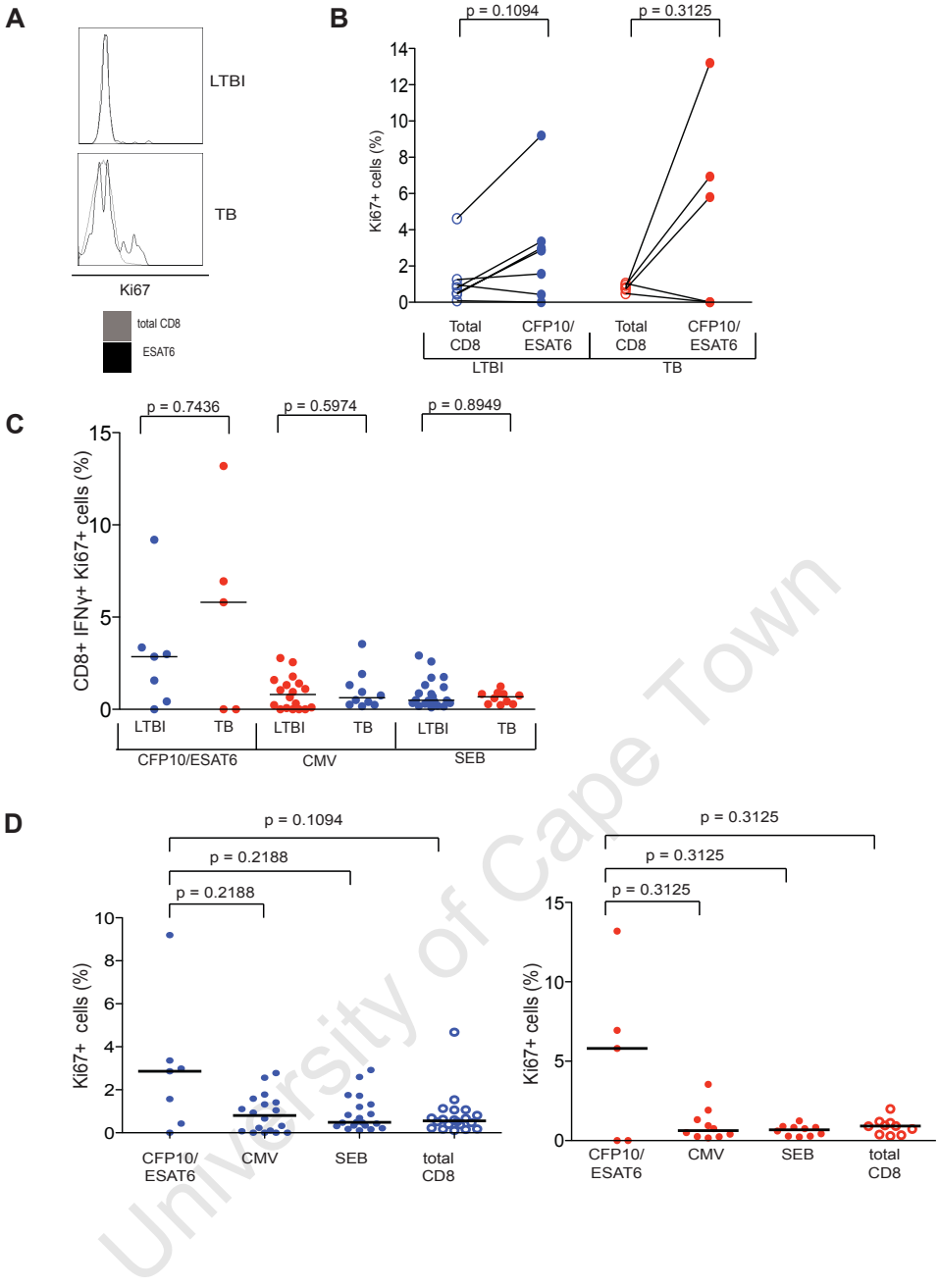


Figure 4.9. Ki67 expression in antigen-specific CD8 T cells is similar to total CD8 T cells and does not differ between latent and active TB. Intracellular Ki67 expression was measured in antigen-specific CD8 T cells following a 7-hour stimulation with ESAT6 or CFP10 peptide pools, CMV pp65 peptide pool or SEB, and in the total CD8 T cell population. (A) Representative histograms of Ki67 expression in MTB-specific CD8+IFNγ+ T cells (black line) and total CD8 T cells (grey line) from one LTBI and one TB donor. (B) After stimulation with ESAT6 or CFP10 peptide pools, the percentage of Ki67+ cells in MTB-specific CD8+IFNγ+ cells was compared to the percentage of Ki67+ cells in the total CD8 T cell population in LTBI and TB donors. (C) The frequency of CD8+IFNγ+ Ki67+ cells for each antigen specificity was compared between LTBI and TB donors; statistical differences were assessed using a Mann Whitney test. (D) Comparison of Ki67 expression by CD8+IFNγ+ T cells between the various antigens for LTBI donors (left panel) and TB donors (right panel). Statistical differences in B and D were determined by the Wilcoxon matched pairs test. Horizontal lines represent the medians.

4.2.7 Expression of CD95 and Bcl-2 in MTB-specific CD8 T cells indicates increased susceptibility to RICD in TB compared with LTBI

To test whether the antigen-specific CD8 T cells in individuals with active TB disease are more susceptible to apoptosis, CD95 and Bcl-2 expression levels were analyzed by flow cytometry. Compared to the total CD8 T cell population, Bcl-2 expression was similar in MTB-specific cells in both LTBI and TB (Figure 4.10A, B), whereas CD95 expression was increased on MTB-specific CD8 T cells compared with total CD8 T cells (Figure 4.11A, B). When MTB-specific CD8 T cells were compared between LTBI and TB donors, no significant differences were found in the expression of these two markers (Figure 4.10C, 4.11C). However, when using paired analyses to compare MTB-specific CD8 T cells to CD8 T cells of other antigen specificities within each cohort, interesting patterns of expression were found. MTB-specific T cells in LTBI donors trended to display increased Bcl-2 expression relative to CMV and SEB stimulated cells and total CD8 T cells in the same individuals, whereas-MTB specific CD8 T cells in TB donors trended to display decreased Bcl-2 expression relative to other antigen stimulated CD8 T cells and total CD8 T cells (Figure 4.10D). Together these data suggest MTB-specific CD8 T cells may be more susceptible to apoptosis compared with CD8 T cells of other antigen specificities within the same individual.

Although CD95 expression was increased in MTB-specific CD8 T cells from both LTBI and TB cohorts, compared with the total CD8 T cell populations (Figure 4.11B), the increase in CD95 expression could be attributed to short-term activation *in vitro*, and we thus compared CD95 expression on MTB-specific CD8 T cells to CD95 expression on CD8+IFNγ+ following SEB stimulation within the same individuals (Figure 4.11D). The increase in CD95 expression on MTB-specific CD8 T cells relative to total CD8 T cells in TB donors could not necessarily be attributed to activation, as MTB-specific CD8 T cells from these donors trended towards higher CD95 expression than SEB-stimulated cells, suggesting that this increase is related to re-activation following chronic/long term antigen stimulation, and not solely due to *in vitro* stimulation. (Figure 4.11D).

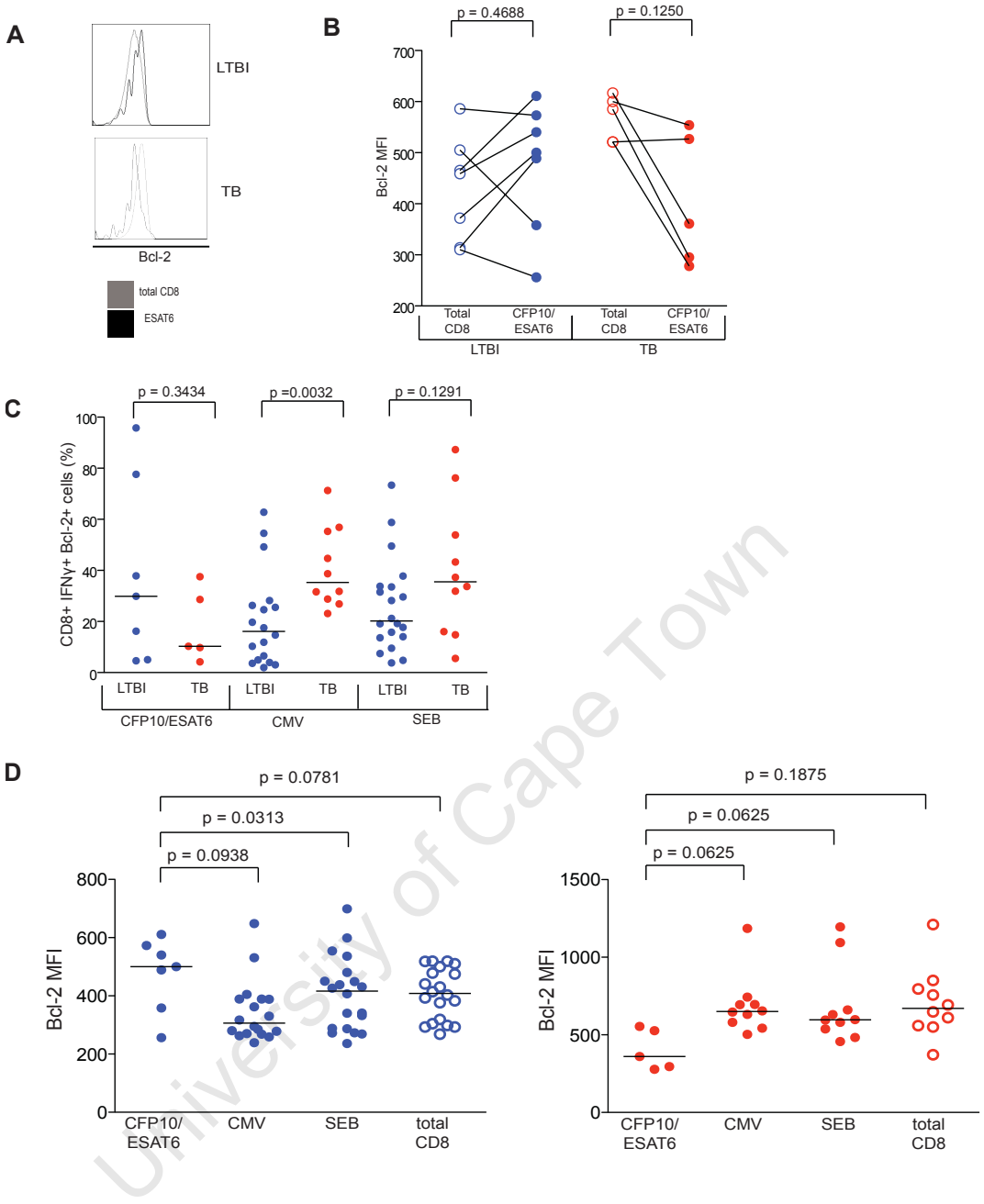


Figure 4.10. Differential patterns of Bcl-2 expression in MTB-specific CD8 T cells between individuals with LTBI and TB. Intracellular Bcl-2 expression was measured in total CD8 T cells and antigen-specific CD8+IFNγ+ T cells by flow cytometry following a 7-hour stimulation with ESAT6 or CFP10 peptide pools, CMV pp65 peptide pool or SEB. (A) Representative histograms of Bcl-2 expression in total CD8 T cells (grey line) and MTB-specific CD8+IFNγ+ T cells (black line) from one LTBI and one TB donor. (B) After stimulation with ESAT6 or CFP10, Bcl-2 MFI on CD8+IFNγ+ cells was compared to the total CD8 population in LTBI and TB donors. (C) The frequency of CD8+IFNγ+ Bcl-2+ cells was compared between LTBI and TB donors using a Mann Whitney test. (D) Comparison of intracellular Bcl-2 expression in CD8+IFNγ+ T cells between the various antigens for LTBI donors (left panel) and TB donors (right panel). Statistical differences in B and D were determined by the Wilcoxon matched pairs test. Horizontal lines represent the medians.

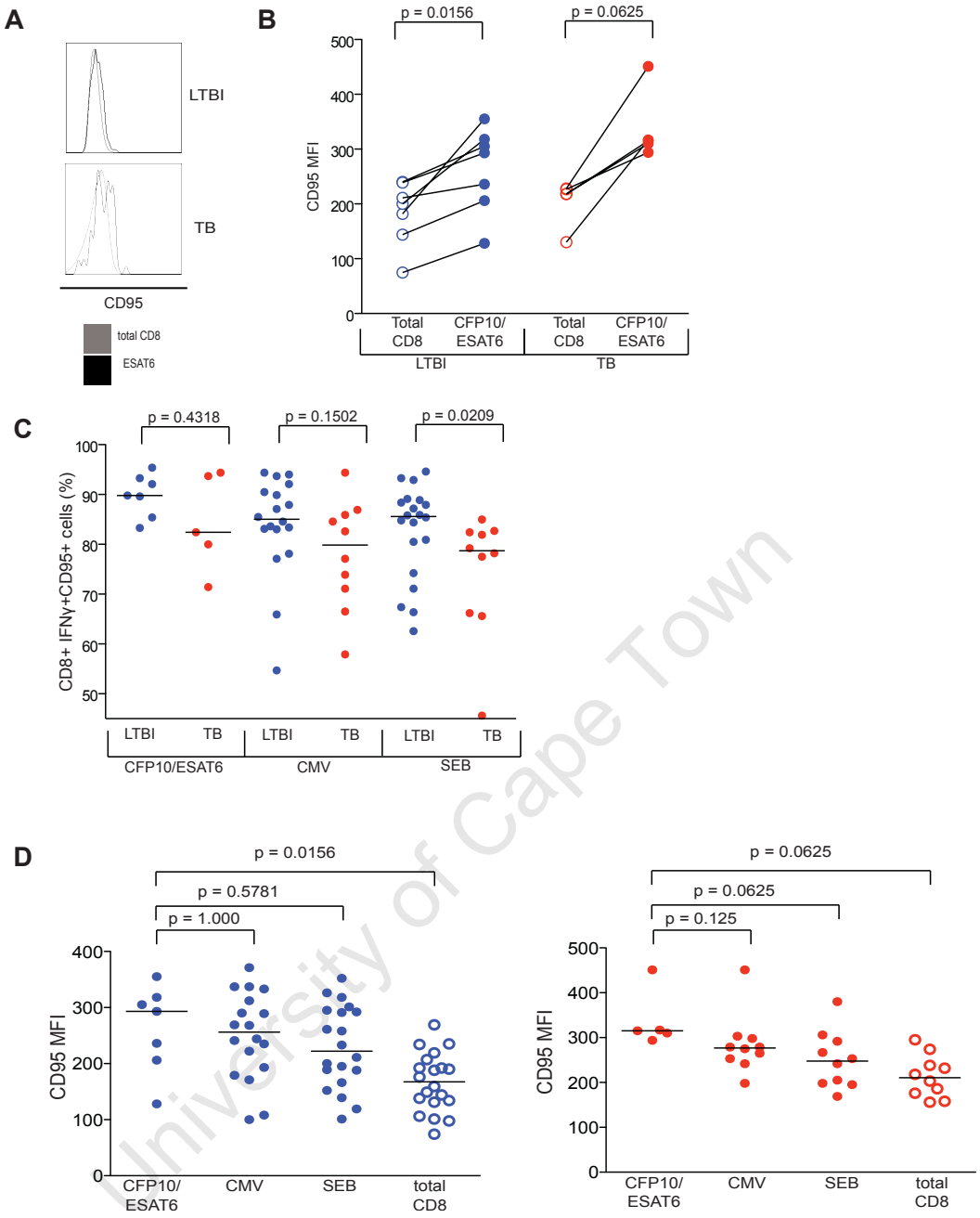


Figure 4.11. CD95 expression is increased on MTB-specific CD8 T cells relative to total CD8 T cells in LTBI and TB donors. CD95 expression was measured on total CD8 T cells and antigen-specific CD8+IFNγ+ T cells following a 7-hour stimulation with ESAT6 or CFP10 peptide pools, CMV pp65 peptide pool or SEB. (A) Representative histograms of CD95 expression on total CD8 T cells (grey line) and MTB-specific CD8+IFNγ+ T cells (black line) from one LTBI and one TB donor. (B) After stimulation with ESAT6 or CFP10, CD95 expression on CD8+IFNγ+ T cells was compared to the total CD8 T cell population in LTBI and TB donors. (C) The frequency of CD8+IFNγ+ CD95+ cells was compared between TB and LTBI donors using a Mann Whitney test. (D) Comparison of CD95 expression by CD8+IFNγ+ T cells between the various antigens for LTBI donors (left panel) and TB donors (right panel). Statistical differences in B and D were determined by the Wilcoxon matched pairs test. Horizontal lines represent the medians.

4.2.8 CD57 expression does not differ between antigen-specific CD8 T cells and total CD8 T cells in LTBI and TB donors

When comparing total CD8 T cell populations between LTBI and TB donors, it was found that CD57 was expressed by a significantly higher frequency of CD8 T cells from TB donors than LTBI donors (Figure 4.4). We next compared CD57 expression on MTB-specific CD8 T cells from LTBI and TB donors. For both LTBI and TB donors, there were no differences in CD57 expression when comparing MTB-specific CD8 T cells to the total CD8 T cell population (Figure 4.12A, B). There was no differential expression of CD57 between MTB-specific CD8 T cells from LTBI and TB donors, however CMV and SEB stimulated CD8 T cells expressed higher levels of CD57 in TB than in LTBI donors (Figure 4.12C). This may be a reflection of the increased global CD57 expression as seen in the total CD8 T cell population of TB donors. There were no significant differences when comparing MTB-specific CD8 T cells to CMV and SEB stimulated cells within the same individuals (Figure 4.12D). Together these results suggest that TB disease is associated with a global increase in CD57 expression, which is not restricted solely to MTB-specific CD8 T cells.

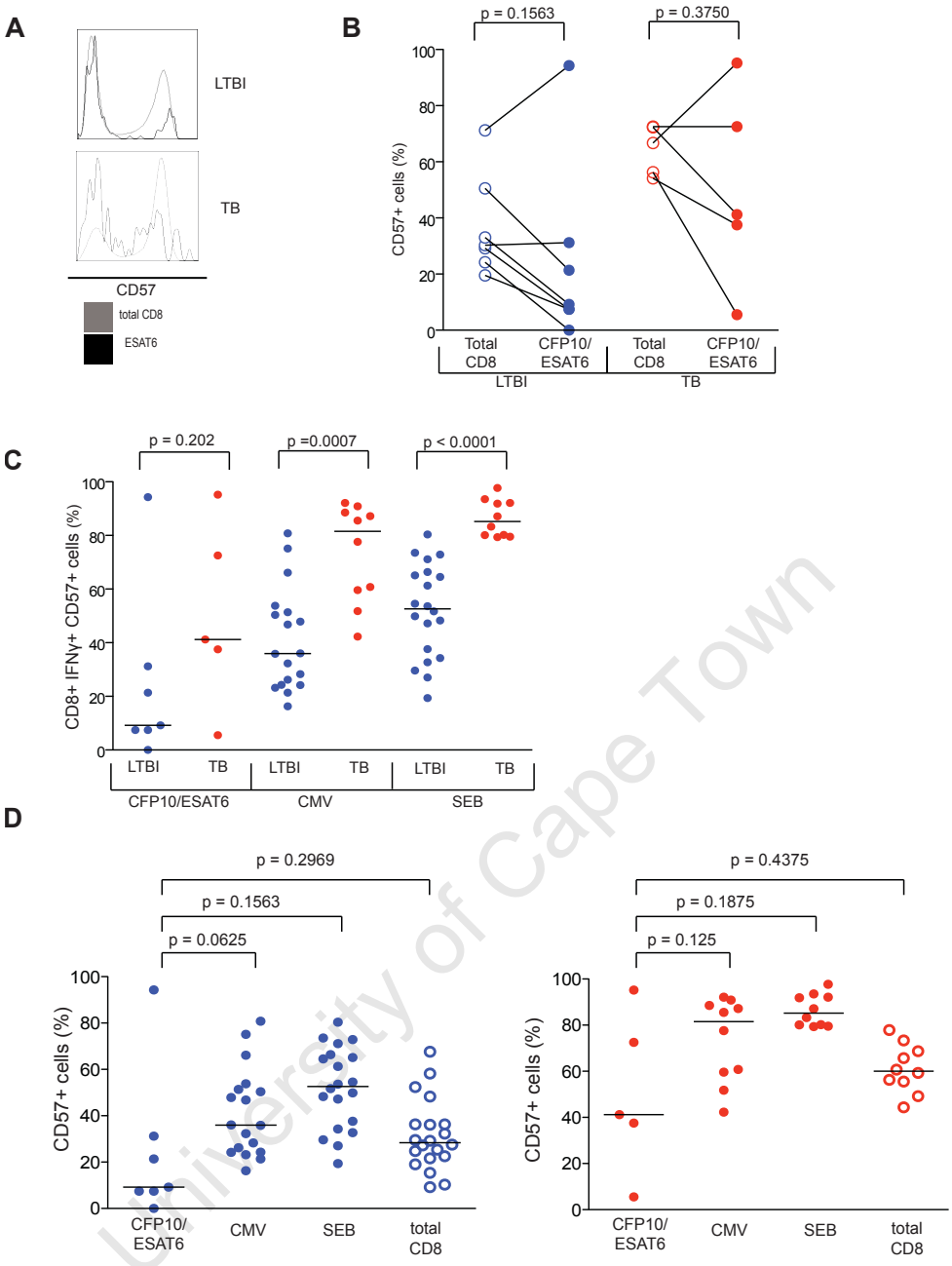


Figure 4.12. CD57 expression in CD8 T cells does not differ by antigen specificity in LTBI and TB donors. CD57 expression was measured on total CD8 T cells and antigen-specific CD8+IFNγ+ T cells following a 7-hour stimulation with ESAT6 or CFP10 peptide pools, CMV pp65 peptide pool or SEB. (A) Representative histograms of CD57 expression on total CD8 T cells (grey line) and MTB-specific CD8+IFNγ+ T cells (black line) from one LTBI and one TB donor. (B) The frequency of CD57+ cells in antigen-specific CD8+IFNγ+ cells was compared to the total CD8 T cell population in LTBI and TB donors. (C) The frequency of CD8+IFNγ+ CD57+ cells was compared between LTBI and TB donors using a Mann Whitney test. (D) Comparison of the percentage of CD8+IFNγ+ CD57+ cells between the various antigens for LTBI donors (left panel) and TB donors (right panel). Statistical differences in B and D were assessed by the Wilcoxon matched-pairs test. Horizontal lines represent the medians.

4.2.9 Initiation of anti-TB treatment influences the expression of markers of apoptosis and senescence

To begin to address whether reduction in bacterial load following initiation of anti-TB treatment could alter the MTB-specific CD8 T cell phenotype seen in individuals with TB disease, expression of CD127, Ki67, Bcl-2, CD95 and CD57 was measured longitudinally at <1 week, 2 months, and 6 months after initiation of treatment in 5 individuals. One of the donors did not have a detectable MTB-specific CD8 T cell response at any of the time points; another donor only had a detectable MTB-specific CD8 T cell response at the 2 month and 6 month time points. In the remaining donors, it was found that susceptibility to apoptosis of MTB-specific CD8 T cells seems to decrease with treatment, as evidenced by increased CD127 and Bcl-2 expression and decreased Ki67 and CD95 expression over time; however, due to the sample size, these trends did not reach statistical significance in MTB-specific or total CD8 T cell populations (Figure 4.13A, B). Overall, these preliminary findings suggest the effector phenotype of MTB-specific CD8 T cells is associated with mycobacterial antigen load, and successful response to anti-TB treatment may promote differentiation of MTB-specific CD8 T cells with greater long-term memory capacity.

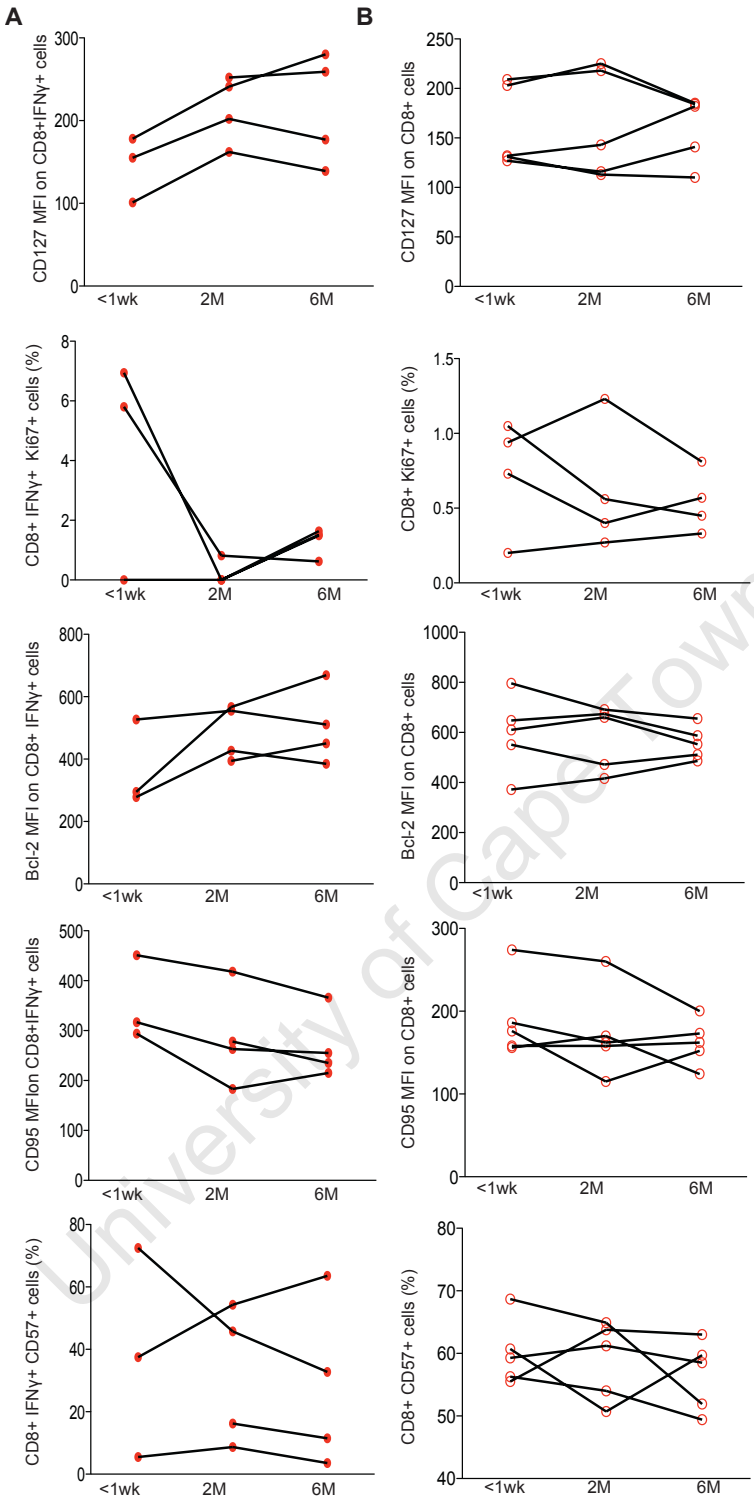


Figure 4.13. Apoptotic susceptibility of MTB-specific CD8 T cells may decrease with time on TB treatment. A longitudinal analysis was performed on CD8 T cells from blood taken at <1 week, 2 months, and 6 months following initiation of anti-TB treatment. Expression of CD127, Ki67, Bcl-2, CD95, and CD57 was analyzed by flow cytometry on MTB-specific CD8 T cells (A; n=4) and total CD8 T cells (B; n=5). No significant differences were found between total CD8 T cell populations over time using a Friedman repeated measures test.

4.3 Discussion

Chronic viral infections have been shown to result in reduced CD8 T cell function (Shin and Wherry 2007). The trends shown in this exploratory research suggest that TB disease state is associated with increased apoptotic susceptibility and senescence of MTB-specific CD8 T cells; moreover, a longitudinal analysis of individuals with TB disease suggests that the long-term memory capacity of MTB-specific CD8 T cells may at least be partially restored following reduction in mycobacterial load by successful anti-TB treatment.

Apoptotic susceptibility was defined by an increased sensitivity to Fas-mediated apoptosis through increased expression of CD95, and reduced levels of the anti-apoptotic molecule Bcl-2. Bcl-2 expression was increased in the total CD8 T cell population of TB donors compared to LTBI donors. It is hypothesized that this may be a protective reaction against the pyroptotic environment associated with the inflammatory milieu of active TB disease, which may prevent death in bystander lymphocytes.

In TB donors, we found that MTB-specific CD8 T cells displayed increased CD95 expression compared with total CD8 T cells, which could not solely be explained by *in vitro* activation, and reduced Bcl-2 expression compared to CMV and SEB stimulated CD8 T cells. This corresponds with findings by Klinger *et al*, who measured apoptosis in peripheral blood monocytes of TB diseased patients following infection with BCG or stimulation with heat-killed MTB. They showed that apoptosis was associated with reduced Bcl-2 mRNA and protein expression, in the absence of a corresponding decrease in the expression of pro-apoptotic molecules that would counter-act Bcl-2 signaling (Klingler, Tchou-Wong et al. 1997). In healthy LTBI donors, we showed that MTB-specific CD8 T cells displayed increased Bcl-2 expression relative to CD8 T cells of other antigen specificities within the same individuals, thus suggesting reduced apoptotic susceptibility of MTB-specific CD8 T cells in the context of latent infection. Together these data suggest that MTB-specific CD8 T cells in LTBI donors may have Bcl-2-mediated protection against apoptosis.

Based on the down-regulation of Bcl-2 and the high CD95 expression levels in MTB-specific CD8 T cells from TB donors, it was expected that CD127 expression would be reduced in these CD8 T cells within this cohort. Contrary to this hypothesis,

CD127 expression on MTB-specific CD8 T cells was similar to that of the total CD8 T cell population in TB donors, and increased relative to the total CD8 T cell population in the LTBI donors. This maintenance/increase of CD127 expression was unique to MTB-specific cells, as CMV-specific CD8 T cells and SEB-stimulated CD8 T cells from both groups tended to display reduced CD127 expression compared with the total CD8 T cell population. It was hypothesized that increased CD127 expression in MTB-specific CD8 T cells from LTBI donors represents a positive regulatory mechanism that prevents RICD, thereby promoting maintenance and survival of this pool of memory CD8 T cells in the context of low mycobacterial antigen loads in LTBI donors. If this is true, it would follow that the lack of increased expression of CD127 in MTB-specific CD8 T cells from TB donors may render these cells more susceptible to apoptotic cell death.

Because Bcl-2, IL-7 and Fas pathways are interrelated in terms of cell survival and homeostasis, the differential expression of one molecule may lead to differential expression of the other two molecules. The experiments in this study are observational in nature, and thus cause and effect relationships cannot be currently established; however this preliminary research will allow for comparisons to be made between human subjects and experimental animal models.

Similar frequencies of Ki67+ CD8 T cells in TB and LTBI donors implies that these groups have a similar rate of cell turnover of CD8 T cells circulating in peripheral blood *in vivo*. Therefore the limited proliferation seen in TB disease donors in 6-day *in vitro* proliferation assays must be caused either by the absence of further pro-proliferation signals, or increased apoptosis. Although the results were not statistically significant due to our small sample size, Ki67 expression in MTB-specific CD8 T cells decreased with time on treatment in TB diseased individuals, and the median Ki67 frequency was higher in MTB-specific CD8 T cells in TB disease compared to LTBI, which is consistent with previous findings showing that Ki67 is expressed in higher amounts in active TB compared to latent infection (Villacian, Tan et al. 2005). It is important to note that this study was performed on peripheral blood, which precludes analysis of cells that have migrated to the lungs during active disease. Therefore increased frequencies of Ki67+ MTB-specific CD8 T cells may have been present but undetectable, due to sequestration in the lungs at the site of active disease.

With regard to senescence, CD57 expression has been shown to be related to the natural waning of the immune system with age (Kudlacek, Willvonseder et al. 2000), and it has been suggested that increased CD57 expression levels can be used as a correlate of susceptibility to infection (Focosi, Bestagno et al. 2010). Our findings corroborate with this, as TB donors had an increased frequency of CD57+ CD8 T cells compared with LTBI donors. It is well known that elderly people are more susceptible to infectious diseases, purportedly due to a waning of immune system function with time (Focosi, Bestagno et al. 2010). The fact that CD57 expression is not different in MTB-specific CD8 T cells compared to the total CD8 T cell population within each group is surprising, as we hypothesized that high antigen levels in TB disease may drive MTB-specific CD8 T cells to become more terminally differentiated, as reflected by an increase in CD57 expression.

There are several caveats to consider when interpreting these data, including the small sample size, particularly in the TB donors, which will need to be increased in order to validate these preliminary findings. Additionally, the use of IFNγ as a marker for antigen specificity will exclude antigen-specific CD8 T cells that do not produce IFNγ. Moreover, detection of intracellular IFNγ production requires short-term antigen stimulation, which may affect the expression of phenotypic markers on stimulated CD8 T cells. However, in the absence of tetramers, IFNγ production is an ideal marker for antigen specificity in chronically stimulated, potentially exhausted CD8 T cells because IFNγ production is one of the last functions to be lost in exhausted T cells. Extremely exhausted, pre-apoptotic CD8 T cells from individuals with high viral loads in chronic HIV infection have been shown to produce IFNγ (Akbar and Henson 2011). In this research, the characterization of polyfunctional cytokines would be useful in order to identify how functional cells of specific phenotypes are. IL-2 production and IL-2 receptor expression would be of particular interest, as this cytokine is associated with lymphocyte proliferation. Unfortunately we did not have the capacity to measure multiple cytokines in our multi-color CD8 T cell phenotyping flow cytometry panels. The limitation of looking at CD8 T cell responses in the blood of individuals with pulmonary TB is that MTB-specific effector cells are expected to migrate to the site of infection, resulting in the detection of only re-circulating effector and/or memory cells in the periphery.

The differences between CD8 responses in active versus latent TB reflect inherent differences between the nature of effector and memory responses. For example in active TB the cells produce IFNγ ex vivo and proliferate poorly, which is characteristic of a late-stage differentiated phenotype. In latent infection proliferation of CD8 T cells is robust, whereas *ex vivo* cytokine production is poor, which implies a memory phenotype. Post treatment responses suggest possible restoration of the memory pool in individuals with TB, and further indicate that the effector and memory phenotype of CD8 T cells is related to mycobacterial antigen load.

Overall these data indicate that during active pulmonary TB disease, the effector memory pool of MTB-specific cells in TB donors have a phenotype indicative of the early-intermediate stage of exhaustion, where proliferative capacity is low and pro-apoptotic susceptibility is high, relative to the total CD8 T cell population. By contrast, LTBI donors display a phenotype indicative of increased cell survival. In LTBI donors, apoptotic susceptibility, as measured by CD127, CD95 and Bcl-2 expression, does not seem to be increased in MTB-specific CD8 T cells compared to the total CD8 T cell population. These results suggest that maintenance of MTB-specific CD8 T cells with long-term memory capacity may be associated with successful immune control of MTB infection. Further studies are warranted to determine whether changes in expression of these markers on MTB-specific CD8 T cells is associated with loss of immune control and subsequent progression to active TB disease. Additionally, further longitudinal studies on anti-TB treatment would shed light on the relationship between MTB-specific CD8 T cell phenotype and functional capacity with mycobacterial antigen load.

CHAPTER FIVE

Overall discussion and conclusion

Immune correlates of protection from TB disease progression must be identified in order to give better direction to preventative and therapeutic research. By detailed analysis of CD8 T cells in individuals with LTBI and TB disease, this project provides information on the functional capacity, breadth, magnitude and phenotype of MTB-specific CD8 T cells, as well as how these attributes differ in the context of latent and active TB disease.

Overall we have shown that CD8 T cells in LTBI have a greater proliferative response to immunodominant MTB proteins, and they recognize a greater breadth of peptides, compared with individuals with TB. MTB-specific CD8 T cells from individuals with TB disease respond to individual CFP10 or ESAT6 peptides by IFNγ production *ex vivo*, but maintain poor proliferative capacity, and express markers indicative of effector cells that may be in the early stage exhaustion. Our preliminary longitudinal analysis of a small number of individuals with TB on anti-TB treatment suggests that the expression of these markers may be reversed with increasing time on treatment. We have established and optimized flow cytometric panels for the characterization of effector functions of antigen-specific CD8 T cell responses, and these panels may be of relevance to apply to other cohorts of adults with TB and/or HIV infection to further understand the role of CD8 T cells in chronic infections.

The information provided by this study must be assessed while keeping in mind several factors, including the sequestration of effector T cells to the lung within active disease, small sample sizes, and the use of IFNγ as a marker for antigen specificity.

It is established that during active pulmonary TB, MTB-specific T cells will be sequestered to the site of infection, which is the lung. Wilkinson *et al* have shown that MTB specific IFNγ-producing cells are present at up to 15-fold higher concentrations in the broncho-alveolar lavage (BAL) fluid of patients with pleural TB compared to peripheral blood using an *ex vivo* Elispot assay (Wilkinson, Wilkinson et al. 2005). In patients with sputum smear-negative, culture-positive pulmonary TB, no differences were found in the distribution of CD4 or CD8 T cells between peripheral blood and BAL fluid. However, ESAT6 and CFP10-specific T cells were more concentrated in BAL fluid, with a BAL fluid: peripheral blood ratio of 9.9 and 8.9, respectively (Jafari, Ernst et al. 2008). This implies that there is a relatively low

frequency of MTB-specific T cells circulating in peripheral blood. However re-circulation of antigen-specific T cells, and the use of highly sensitive assays, allowed for the detection of adequate numbers of antigen-specific CD8 T cells in both groups of LTBI and TB donors. Additional studies of cells isolated from the lungs by BAL should be performed in our donors to further characterize the phenotype and functional capacity of MTB-specific CD8 T cells that traffic to the site of infection and disease.

With regards to sample size, it is known that increased numbers reduce the possibility of chance results. Current studies are ongoing with additional donors to verify these preliminary findings in larger cohorts. When establishing the cohorts, participants were not selected according to age or gender, and the donors were not HLA-typed. This randomization is the ideal method of sample gathering, as it is likely to produce demographics that are representative of each group of individuals. As a result, there were more males in our TB group, and this group was older than the group of LTBI donors.

Because donors were sampled from the same community and represent similar ethnicities, we have assumed that the distribution of HLA types is similar between cohorts. Studies such as this one lay the groundwork for future studies that will characterize in detail the HLA restrictions of the most commonly targeted immunodominant CD8 T cell epitopes in MTB-infected individuals. Mapping and HLA restriction of CD8 T cells is necessary for the generation of tools such as MHC class I tetramers, which can be used to track individual CD8 T cell populations over time during TB disease treatment. Such tools can also be highly useful in evaluating TB vaccine immunogenicity or isolating epitope-specific populations for a detailed microarray analysis, thus allowing for a better characterization of the phenotype and function of these cells in latent and active disease.

The use of IFNγ as a marker of antigen specificity implies that CD8 T cells with other functions, such as cytotoxicity, will not be detected. This can be remedied in future research by measuring the production of cytotoxic molecules such as perforin, granzymes, granulysin, as well as CD107 expression; in addition, cytotoxicity assays measuring killing of MTB-infected cells will give an indication of

how effective MTB-specific CD8 T cells may be in controlling bacterial replication *in vivo*.

Not all extraneous variables can be accounted for in any given human immunology study. Therefore the results provided are observational in nature, and often cause and effect relationships are difficult to establish in this context. Because T cell phenotype and function is dynamic, there is an underlying uncertainty as to the validity of cross-sectional analysis of antigen-specific T cells at a single point in time. This is compounded by the fact that MTB infection is not static but rather represents a dynamic process, even in the absence of clinical symptoms (Barry, Boshoff et al. 2009). Longitudinal studies are therefore important, for example to identify biomarkers or immunological factors indicative of successful treatment response in TB patients on anti-TB treatment, or to identify factors associated with recurrence or relapse of disease in these patients. Longitudinal studies of latently infected individuals prior to development of TB disease are important to identify immunological factors or biomarkers that precede development of disease, and may be useful indicators of risk of disease progression.

Lastly, there are several follow up questions that this project has generated: (1) in Chapter three a large breadth of subdominant peptide responses were observed in a sensitive proliferation assay, whereas an *ex vivo* Elispot assay showed distinctly immunodominant peptides in each donor, and minimal responses to non-immunodominant peptides. It would be interesting to study the role of CD8 T cells targeting subdominant epitopes, and whether they are a correlate of protection, as has been suggested in other models of chronic infections such as HIV (Frahm, Kiepiela et al. 2006; Im, Hong et al. 2011). (2) CD127 expression was higher in MTB-specific CD8 T cells compared to the total CD8 population, the CMV specific population and the SEB-stimulated population in LTBI, but not in TB. Latently infected individuals have lower antigen levels, and in theory memory capacity may be preserved, compared with individuals with TB. Additionally, Bcl-2 expression in MTB-CD8 T cells from LTBI donors was increased relative to other CD8 T cell populations within this cohort; the opposite trend in Bcl-2 expression was seen in MTB-specific CD8 T cells from TB donors. Future studies may involve testing whether these differences are due to a skewing of the response to an effector phenotype in TB disease, compared with a long-term memory phenotype in latent

infection. (3) In the context of these results, further studies are warranted to specifically evaluate markers of exhaustion, such as PD-1, CTLA-4 and LAG-3, in these cohorts in order to address whether chronic MTB infection and high antigen loads in TB disease can drive CD8 T cells to a state of functional exhaustion.

In conclusion, these results represent a detailed characterization of MTB-specific CD8 T cells with regard to antigen specificity, functional capacity and phenotype in the context of latent and active TB disease in humans. This project has positively influenced the community in which it was performed, and generated results which allow for the development of a platform for future studies to delineate associations between MTB-specific CD8 T cell function and disease progression using prospective, longitudinal cohorts sampled prior to and following development of TB disease.

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