Development of novel T cell assays and assessment of immune recognition to latency associated *M.tuberculosis*-specific antigens Rv2660 and Rv2659

By

Lerisa Govender

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School of Child and Adolescent Health

Faculty of Health Sciences

University of Cape Town

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Dedication

To Sunny & Parvathi Govender,

Pa and Mummy, I know what you have done for me. I am eternally grateful…

Pa, although I will never be blessed to hear your guiding, encouraging voice again, I know that you are watching over me and will forever be my guiding light. This I dedicate to you especially Pa, for always listening so intently to me, when I babbled excitingly about my studies and adventures, I will never feel as significant again.

To Pa

Pa you were like the sun to me,
a sure thing, always there,
beaming light and warmth on our lives
And I knew for sure I was loved
If I could get another chance, another walk, another dance with you
I would play a song that would never, ever end
How I would love, love, love
To be with you again
I never dreamed that you would be gone from me
If I could steal one final glance, one final step, one final dance with you
I would play a song that would never, ever end
Because I would love, love, love
To dance with you again
Every night I fall asleep and this is all I ever dream
Love you and miss you

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Abbreviations

APC	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
BAL	Bronchoalveolar lavage
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CMI	Cell-mediated immunity
CFP-10	Culture filtrate protein 10
CFSE	Carboxyfluorescein succinimidyl ester
CFUs	Colony forming units
CO_2	Carbon dioxide
CV	Coefficient of variation
dH2O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	6 kDa early secretory antigenic target
FCS	Fetal calf serum
g	grams
HIV	Human immunodeficiency virus
IL	Interleukin
IFN-γ	Interferon gamma
LTBI	Latent tuberculosis infection
mM	Milimolar
M	Molar
M. bovis	*Mycobacterium bovis*
M.tb	*Mycobacterium tuberculosis*
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
MDR	Multidrug resistant

mL	Mililitre
min	Minute
MOI	Multiplicity of infection
NK	Natural killer
NO	Nitric oxide
OG	Oregon green
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative of *M.tb*
PHA	Phytohaemagglutinin
QFT™	QuantiFERON®-TB Gold In-Tube
RD	Region of difference
SEB	*staphylococcal enterotoxin B*
TB	Tuberculosis
TCR	T cell receptor
(T_C)	Cytotoxic T cells
TGF-β	Transforming growth factor β
T_H	T helper
TNF	Tumor necrosis factor
TT	Tetanus toxid
Uns	Unstimulated
ViViD	LIVE/DEAD Fixable Violet Dead Cell Stain
WHO	World Health Organization

Abstract

Nearly 130 years have elapsed since the discovery of *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis, yet today it is estimated that 1 in every 3 of the world's population is infected with this pathogen. In South Africa alone there were approximately 1000 new TB cases per 100 000 population in 2007, ranking the country second in incidence rate, globally. Hence research into new vaccine strategies to control the epidemic is vital. Current vaccines under development are prophylactic and designed to boost pre-existing immunity induced by the only licensed TB vaccine, BCG. A new approach is the development of a post-infection vaccine aimed at inducing an immune response that prevents progression to TB disease when administered to individuals latently infected with *M.tb*. This vaccine would have a dramatic impact on the worldwide TB burden.

Our objective was to address 2 areas in TB vaccinology, firstly a novel post-infection TB vaccine strategy, and secondly, optimal measurement of vaccine-induced responses using a new immunological assay.

The aim of the first study was to investigate human T cell responses to antigens that have been associated with *M.tb* latency. Rv2660 and Rv2659 were investigated, as these antigens are candidate antigens for a post-infection vaccine based on findings from *in vitro* models of *M.tb* suggesting preferential expression during latency *in vivo*. No information exists on the immune response to these antigens in *M.tb* infected or TB diseased individuals. Hence, we investigated the immune recognition of Rv2660 and Rv2659 in these 2 groups, and further characterised the nature of these antigen-specific T cell responses. We observed that (i) these antigens are significantly more likely to be recognised during *M.tb* infection compared with TB disease as shown by measurement of soluble IFN-γ in response to the specific antigens, (ii) *M.tb* infected persons had greater Rv2660- and Rv2659-specific $CD4^+$ T cell proliferation and associated cytokine expression

compared, with TB diseased persons. We propose that Rv2660 and Rv2659 may be candidates for incorporation into a post-infection vaccine.

The aim of the second study was to determine whether antigen-specific T cell proliferation could reliably be identified through analysis of the intracellular expression of the nuclear protein, Ki67. Antigen-specific proliferation is a critical function of memory T cells that is often used as a measure of vaccine immunogenicity and T cell functional capacity. A Ki67 lymphoproliferation assay was optimised, and results compared to those from conventional assays that assess BrdU incorporation or Oregon Green (a CFSE derivative) dye dilution. The Ki67 assay proved to be more sensitive than BrdU incorporation for detection of proliferating cells, whereas sensitivity was similar to the dye dilution of Oregon Green. Overall, our data suggest that intracellular Ki67 expression provides a specific, quantitative and reproducible measure of antigen-specific T cell proliferation *in vitro*. Importantly, the Ki67 assay requires small volumes of blood, and is a very practical and efficient assay for measuring vaccine induced T cell responses.

In conclusion, findings presented in this thesis will contribute significantly to the field of tuberculosis vaccinology.

Chapter 1: Literature Review

1.1 The immune system

The human immune system is constantly challenged by a variety of pathogenic microbes and foreign substances. A complex array of mechanisms evolved to enable the recognition and protection of the host from pathogenic microorganisms and viruses. This response is typically characterised by differentiation of foreign (or 'non-self') from 'self' elements, resulting in mechanisms for elimination of foreign material, while not compromising host tissue. This complex process is executed by two arms of the immune system: the innate immune response and the adaptive immune response. For the successful clearance of an invading pathogen, the immune system must act promptly and initiate these responses through involvement of a diverse array of leukocytes and their soluble secretory products (Figure 1.1) (Janeway CA, 2008; Chaplin, 2010).

1.1.2 The Innate Immune System

The innate immune response is non-specific and provides the first line of defence against foreign invaders. Professional phagocytic cells, which include monocytes, neutrophils, and macrophages, possess a primitive recognition system. Since the innate immune response makes use of invariant recognition molecules expressed on a large number of cells, it reacts swiftly to an invading pathogen. These innate cells are rapidly recruited to the site of infection by chemotactic signals (Janeway CA, 2008; Chaplin, 2010).

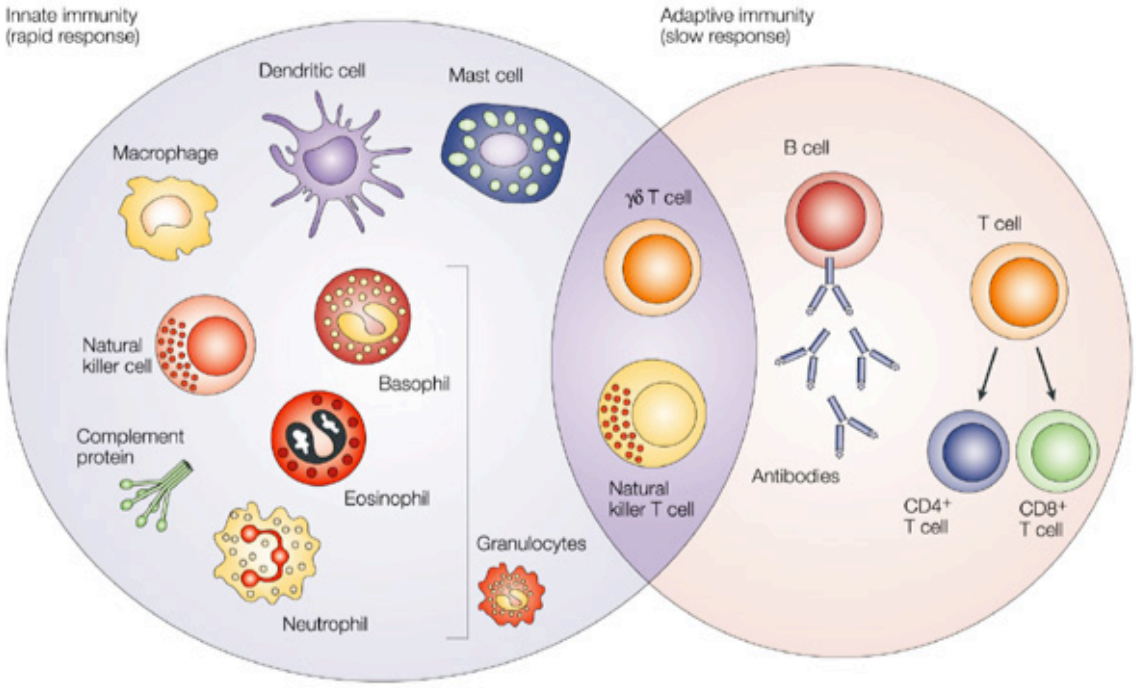

Figure 1.1 The main components of the innate and adaptive immune systems. The innate immune response functions as the first line of defence against infection. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory (From Tennant DA *et al*. Nature Reviews Cancer 2004).

Neutrophils accumulate at sites of infection to phagocytose and degrade microbes by producing reactive oxygen species (ROS) that are cytotoxic to pathogens. Monocytes and macrophage are highly phagocytic and are mobilized shortly after neutrophil recruitment to the inflammatory site to produce nitric oxide (NO), which is a critical effector molecule for killing microbial pathogens (Janeway CA, 2008).

Antigen presenting cells (APCs) such as dendritic cells (DC) and macrophages recognize pathogens via the expression of pathogen associated molecular patterns (PAMPs), which bind to pattern recognition receptors on or in the cells (PRRs). Upon engagement, the APCs ingest the microorganism, become activated, and release factors to enhance and instruct the subsequent adaptive immune response. Activated APCs migrate from the site of infection to regional lymph nodes where processed antigen (Ag) is presented to cognate T cells as peptides primarily via major histocompatibility

complex (MHC) class I or II molecules (Janeway CA, 2008; Iwasaki and Medzhitov, 2010).

1.1.3 The Adaptive Immune System

If the innate response is unsuccessful in eliminating the infection rapidly, the adaptive immune response is primed for pathogen control (Janeway CA, 2008). The hallmark of the adaptive immune response is antigen specificity and the capacity to develop long-lived memory, which would allow for a swift and efficient adaptive response upon secondary encounter with the antigen/ pathogen. The adaptive immune response is mediated by T and B lymphocytes, which develop in the thymus and bone marrow, respectively, and exit these primary lymphoid tissues as naïve lymphocytes.

T lymphocytes represent the primary effector cells of the cell-mediated immune (CMI) response and function primarily by secreting effector molecules and mediators. $CD4^+$ T cells, including T helper (T_H) 1, T_H2 T_H17 and regulatory T cell (Treg) subsets, recognise peptides presented by MHC II on APCs, and in addition to having effector function, have a critical role in coordinating or regulating the ensuing immune response. $CD8^+$ T cells recognise peptides bound to MHC I, and primarily have a cytotoxic function (McCullough and Summerfield, 2005; Iwasaki and Medzhitov, 2010). Other T cell subsets, such as gamma-delta T cells and NK T cells, may also play an important role in the adaptive response to microorganisms.

Naïve T cells circulate between the blood and secondary lymphoid tissue until encountering cognate antigen presented by APCs. For an antigen-specific T cell to become activated, 2 critical signals must be received. Firstly, the T cell receptor (TCR) engages the cognate peptide-MHC complex, and secondly, co-stimulation is provided via interaction between CD28 on the T cell and CD80 (B7.1) and CD86 (B7.2) on the APC. Other signals from the APC and the environment play a critical role in determining which of multiple distinct phenotypic and functional cells the naïve cell differentiates into. The

differentiation fate of these naïve T cells is largely decided by the cytokine milieu present, and may ultimately lead to a differentiation into T_H1 or T_H2 or T_H17 or Treg cells (Figure 1.2) (Dong 2006, Deenick et al 2007). The innate cell cytokine IL-12 activates the transcription factor STAT-4, which drives the differentiation of $CD4^+$ T cells into T_H1 cells, which typically produce the cytokines IFN-γ, TNF-α and IL-2, and provide protection against intracellular pathogens such as viruses and bacteria. The induction of T_H2 cells is initiated by IL-4-mediated activation of STAT-6, which typically produce the cytokines IL-4, IL-5 and IL-13, and provide protection against extracellular pathogens and parasites. $CD8^+$ T cells on the other hand, primarily kill pathogen-infected cells by releasing cytotoxic granules such as perforin and granzymes. There is overlap between $CD4^+$ and $CD8^+$ T cell function, as both subsets may produce cytokines or have cytotoxic function (Chaplin, 2010).

Two additional subsets of T cells have recently been described, T_H17 and regulatory T cells (Tregs). In the presence of transforming growth factor β (TGF-β) and interleukin 6 (IL-6), naïve $CD4^+$ T cells differentiate into T_H17 cells, which produce interleukin 17 (IL-17). (Many other cytokine signals that results in T_H17 differentiation have also been described.) T_H17 cells mainly produce the pro-inflammatory cytokine IL-17, which has been implicated in the induction of autoimmune diseases, and mediates a protective role against extracellular bacteria (Weaver et al., 2007). It has been shown that IL-23 is important in maintaining T_H17 cells and hence regulating the expression of IL-17 (Harrington et al., 2006). Tregs suppress effector immune responses and immune-mediated pathology, thereby maintaining immune system homeostasis. These cells express FOXP3 and high levels of CD25 on the surface, and *in vitro* experiments indicate that TGF-β and IL-10 are partly involved in the immunosuppressive activity of this population (Wan, 2010).

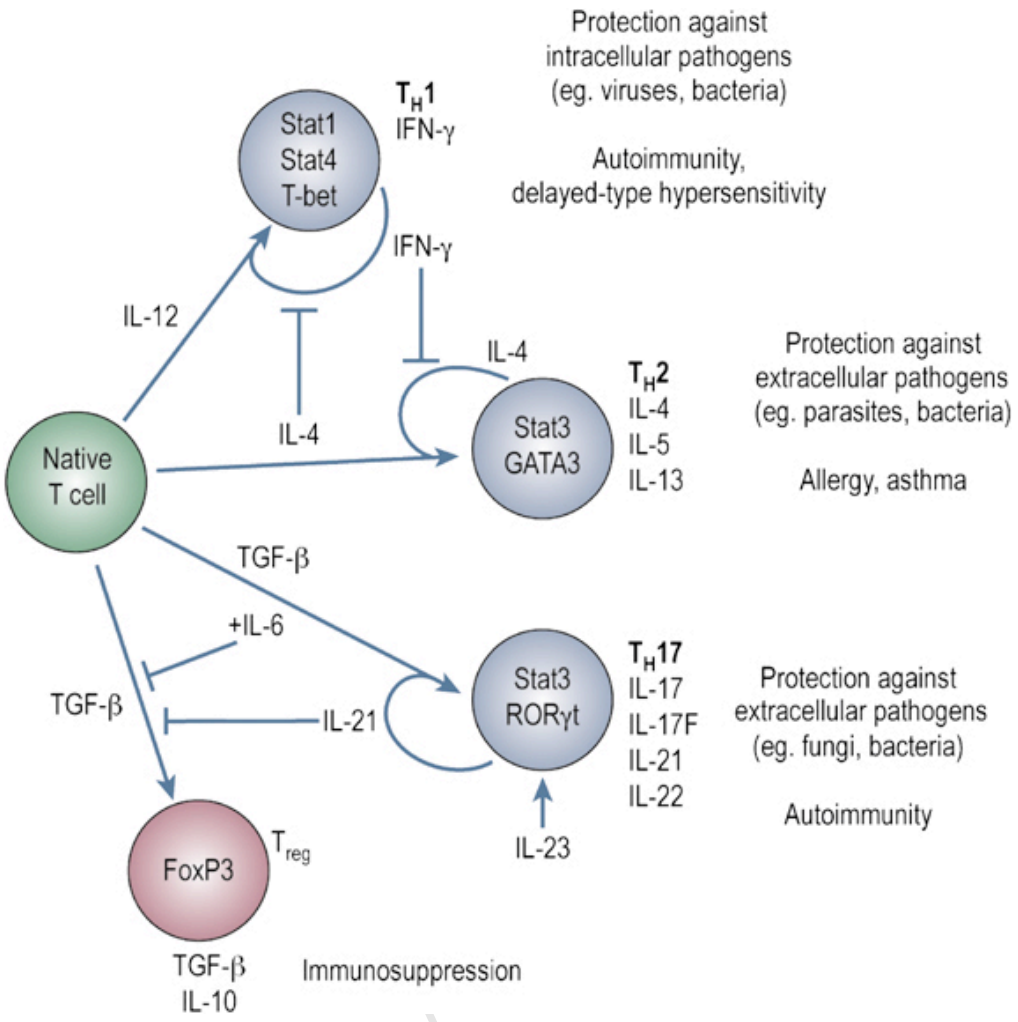

Figure 1.2 Overview of the molecular requirements for T cell differentiation and the functional roles of the subsets. (From Deenick EK *et al* ,Immunology Cell Biology 2007).

B lymphocytes represent the primary effector cells of the humoral immune response, and can directly recognise foreign material or antigen via surface immunoglobin receptors. Once a naïve B cell encounters antigen, the cell becomes activated, clonally expands, and generates antigen-specific antibodies. Activated B cells may differentiate into plasma cells or memory B cells. Plasma cells refer to a population of terminally differentiated B cells with the capacity to produce and release large amounts of antibodies. These antibodies may play a role in the opsonisation of pathogens, which facilitates enhanced phagocytosis, or may neutralise pathogens by preventing cellular infection. Memory B cells, on the other hand, are long-lived B cells that expand swiftly when restimulated with the same antigen, and mediate rapid effector function (Janeway CA, 2008).

B and T_H cells are in continuous interaction with each other. B cells can be activated in either a T_H dependent or independent manner. Upon encounter with antigen B cells under the influence of cytokines produced by T_H cells develop into plasma cells that secret a distinct class of antibodies, this maturation of B cells is largely governed by T_H2 cells. Antibody class switching however; is influenced by different T_H cells, with T_H1 cells favouring the production of opsonising antibodies and T_H2 cells favouring the production of neutralizing antibodies. This interaction is one of many which indicate that multiple cellular populations are involved in eliciting a specific immune response.

1.2 Tuberculosis

The pathogen identified to be the causative agent of tuberculosis (TB) disease, *Mycobacterium tuberculosis* (*M.tb*), was initially described by Robert Koch in 1882. It has subsequently been characterized as a gram-positive, slow growing, facultative intracellular pathogen (Manganelli et al., 1999). Along with other members of the *Mycobacterium* genus, *M.tb* has a unique waxy cell wall of which the majority of the constituents are mycolic acids, which make up more than 50% of the bacteria's dry weight (Brennan and Nikaido, 1995). This allows the bacteria to retain basic dyes in the presence of acid alcohols, hence their classical acid fastness (Allen, 1992).

1.2.1 Epidemiology

TB is a major global health concern and is responsible for more deaths than any other curable infectious disease. Epidemiologists estimate that 2 billion of the world's population is latently infected with *M.tb,* and that 9.27 million new TB cases occurred in 2007 alone (WHO report, 2009). The majority of these patients were from South East Asia and Sub-Saharan Africa. In 2007, the incidence rate in Sub-Saharan Africa was 325 cases per 100,000 people, which was twice the rate of South East Asia. The statistics are far more alarming in South Africa, which has an incidence rate of approximately 1,000 cases per 100,000 people per annum (Figure 1.3).

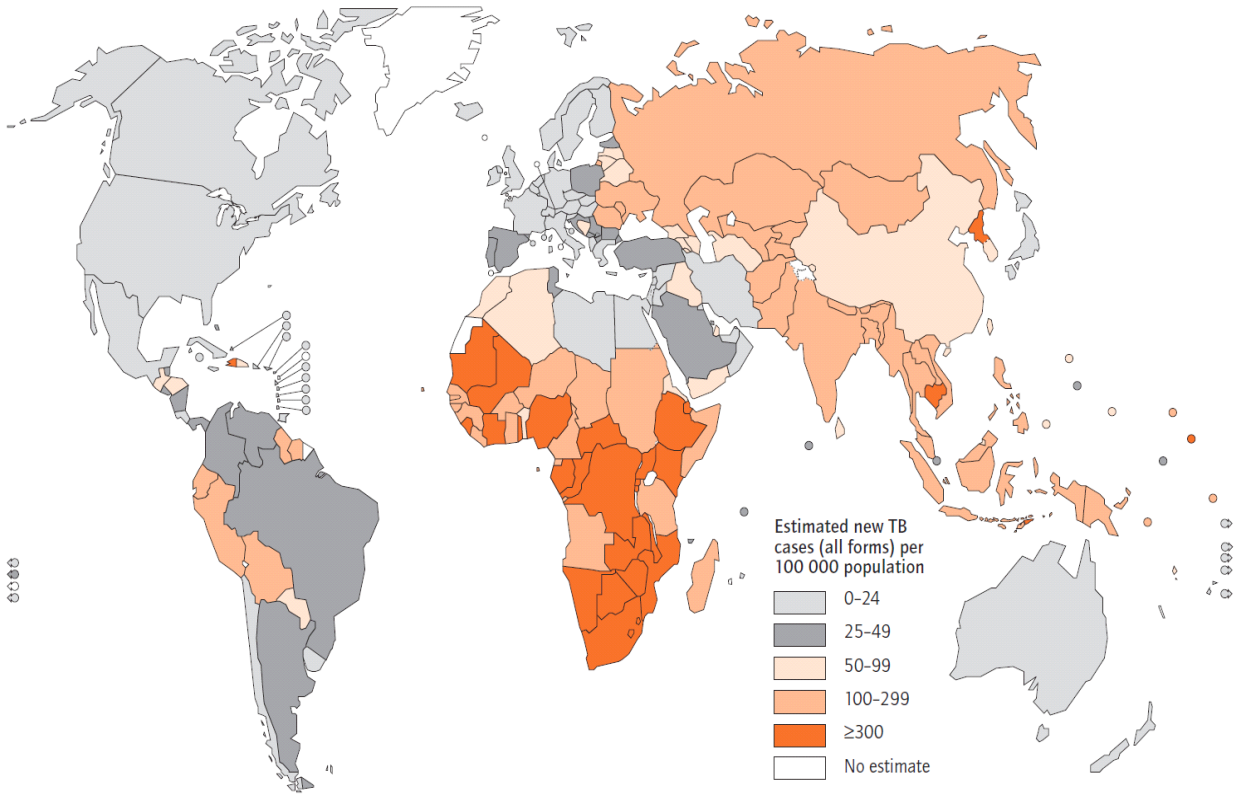

Figure 1.3 The global distribution pattern of new TB cases in 2007 indicates that developing countries are most affected (Map derived from the WHO Report, Global tuberculosis control-surveillance, planning, financing 2007).

According to the global distribution pattern, the vast majority of new TB cases occur in developing countries, a phenomenon strongly associated with human immunodeficiency virus (HIV) co-infection (Figure. 1.4). Whereas the lifetime risk of developing TB disease following infection is 10% in HIV-uninfected persons, the *annual* risk of developing TB is 10% in individuals co-infected with HIV. Among other factors, depletion of CD4 T cells are likely to be responsible, as these cells are critical for protection against TB. HIV-infected persons demonstrate both increased rates of reactivation of latent TB and more rapid progression to active disease. TB is currently the major cause of mortality amongst HIV infected individuals (Skeiky and Sadoff, 2006).

The eradication of TB seems even more challenging considering the fact that some strains are resistant to anti-TB drugs. These include the multidrug resistant (MDR) bacilli, resistant to at least isoniazid and rifampicin, and

extensively drug resistant (XDR) bacilli, resistant to isoniazid, rifampicin, and at least 3 classes of second-line drugs. MDR and XDR in particular is of major concern since treatment options are limited and expensive, while outcomes are poor, particularly in HIV-infected persons (Gandhi et al., 2006). The World Health Organisation (WHO) has classified 27 countries, including South Africa as global priority settings for the improved management of MDR-TB.

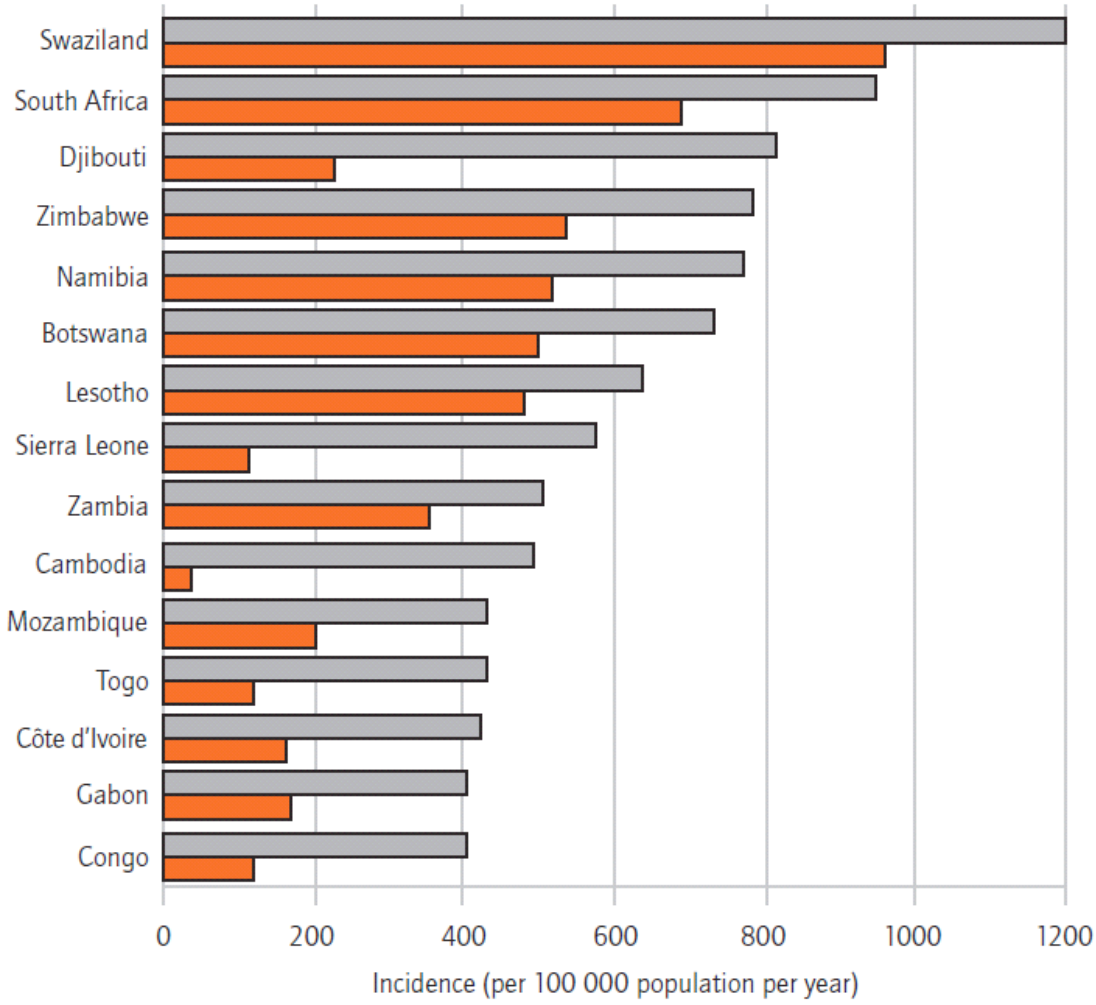

Figure 1.4. **Fifteen countries with the highest estimated tuberculosis incidence rates per capita in 2007.** Grey bars indicate tuberculosis incidence for all ages and the orange bars indicate the corresponding co-infection with HIV among adults aged 15-49 years. (Graph derived from the WHO Report, Global tuberculosis control-surveillance, planning, financing 2007).

1.2.2 Host pathogen interaction

In order to understand the success of *M.tb* infection and to develop effective intervention strategies, it is critical to gain insight into both the pathogenesis of infection and disease, and the host immune response to control the infection.

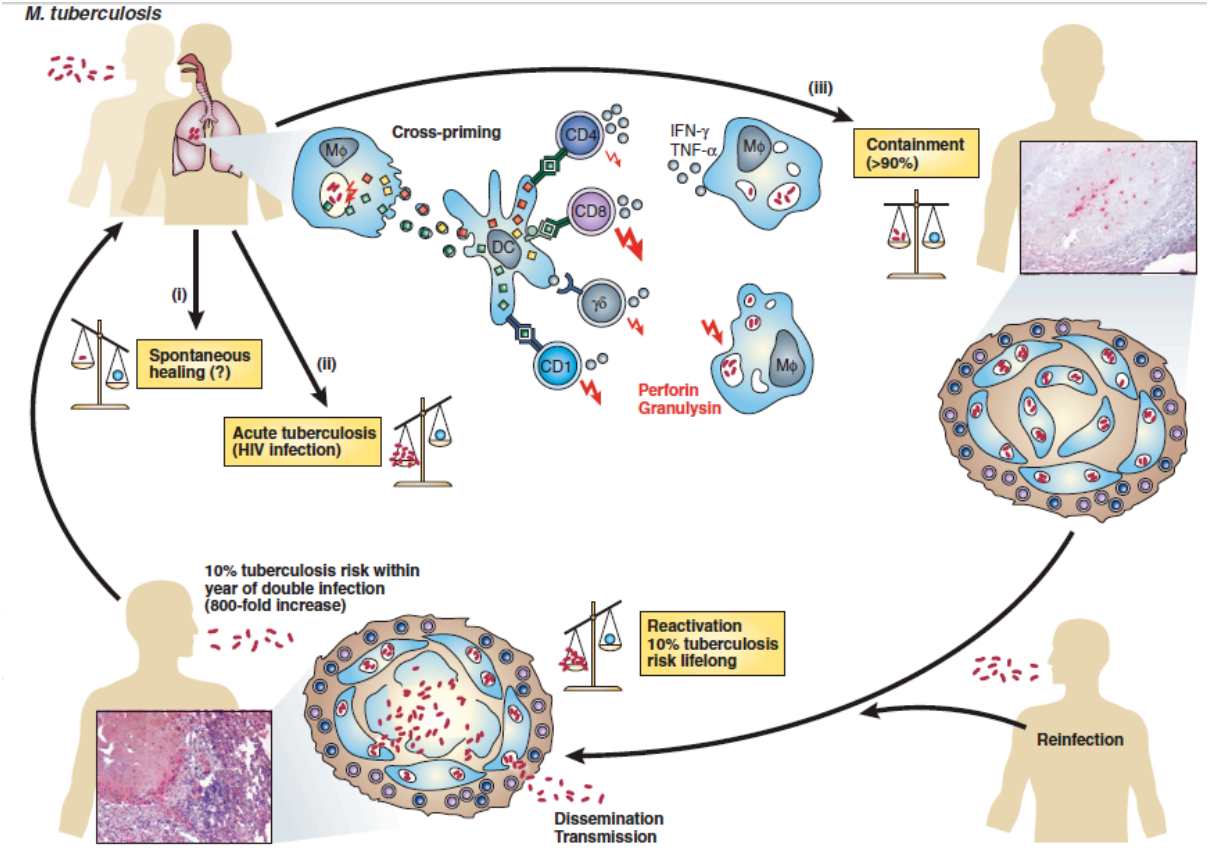

Figure 1.5 Different outcomes of *M. tuberculosis* infection and underlying immune mechanisms. (From Kaufmann, Nature Medicine. Reviews, 2005)

The main route of infection with *M.tb* is via the aerosol route, i.e., inhalation of infected airborne micro-droplets. Once the pathogen reaches the lungs, it is immediately recognised and phagocytosed by alveolar macrophages, resulting in sequestration within a phagosome (Russell, 2001). The bacterium is recognised by macrophages through mainly toll-like and C-type lectin receptors (Akira et al., 2006; Dulphy et al., 2007). These receptors recognise PAMPs, which are invariant molecular structures expressed by pathogens. Following Infection the immune response mounted by the host can result in differential outcomes, which are depicted in Figure 1.5. Firstly, the infection

can be eliminated by the host immediately after inhalation; it is difficult to know how often this occurs, while the mechanisms underlying this clearance have not been fully elucidated (Bhatt and Salgame, 2007). Secondly, *M.tb* bacilli may be contained by the immune response within a specialised pathophysiological structure in the lung, called a granuloma. The pathogen may remain quiescent within granulomas for many years, while the host remains clinically asymptomatic; this is referred to as latent TB infection (LTBI), and has occurred in up to one third of the world's population (WHO report, 2009). Thirdly, infection can lead to TB disease early after infection, or much later in life. Disease is likely to manifest in genetically susceptible individuals, or in persons with immune deficiency, which may be relative (e.g., very young or old age) or overt (e.g., HIV infection). HIV infection increases the risk of developing TB disease 800-fold (Russell, 2001; Kaufmann et al., 2005). In the diseased individual, *M.tb* had escaped containment in the granuloma, resulting in local dissemination to the rest of the lungs, where the inflammatory response subsequently caused local symptoms such as a cough with sputum production, or systemic symptoms such as weight loss, severe wasting disease, night sweats and fever (Kaufmann et al., 2010).

1.2.3 Latent *M.tb* infection

As with any manifestation of TB, latency is the result of a dynamic interaction between host and pathogen. A distinctive feature of *M.tb* bacilli is the ability to persist in a latent state within a granuloma. During this latent *M.tb* infection there are no signs or symptoms of the disease and the host is unable to transmit the infection. The significance of latent TB infection is that it does not remain latent in all individuals and they represent a reservoir of *M.tb* with the potential for reactivation and causing disease (Ehlers, 2009; Lin and Flynn, 2010).

Two models of latency have been proposed: (i) A programmed response of *M.tb* to the host environment induces a metabolic adaptation in the bacterium for long-term, symbiotic survival, and (ii) The host immune response drives

the bacterium into this alternate metabolic state, allowing dormancy or slow replication only (Hanekom and Ernst, 2008).

It was initially postulated that *M.tb* bacilli are actively replicating during TB disease and in a non-replicating state during clinical latency. However, there is now growing evidence from state-of-the-art medical imaging such as computed tomography that during latency, some *M.tb* bacilli within granuloma lesions remain metabolically active. This suggests that similar to active TB, there may be a range of infection states under the umbrella of latency, making this a more dynamic process than previously imagined. This implies that LTBI should be studied further to understand the dynamic range, and which infection state would put a person at risk of future disease (Barry et al., 2009; Ehlers, 2009).

(i) Diagnosis of latent *M.tb* infection

Current diagnosis of LTBI relies on detection of immunological memory to *M.tb* antigens. A classical diagnostic test such as the tuberculin skin test (TST) can be used to diagnose *M.tb* infection: delayed type hypersensitivity response is measured 48–72 hours after intradermal injection of purified-protein derivative (PPD). A limitation of the TST is that interpretation of a result is often complicated by the detection of cross-reactive immune responses in individuals vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) or exposed to environmental mycobacteria (Arend et al., 2001; Gallant et al., 2009). A recent diagnostic test, the QuantiFERON®-TB Gold In-Tube (QFT™) detects IFN-γ release by a memory T cell response to the *M.tb*–specific antigens, early secretory antigen-6 (ESAT-6), culture filtrate protein-10 (CFP-10) and TB7.7 (Mori et al., 2004). Although this test has shown to be very useful in low TB incidence areas, current evidence does not support superior performance over the TST in settings of high TB incidence (Kariminia et al., 2009).

1.3 Immunity to tuberculosis

M.tb has evolved ways to adapt to the hostile host immunity in order to survive. As mentioned above, after inhalation, *M.tb* is engulfed by alveolar macrophages and DCs, which migrate to the draining lymph nodes where they present mycobacterial antigens to cognate T cells (Chan and Flynn, 2004).

1.3.1 Cellular immunity to *M.tb*

There is extensive evidence to support a central role of T_H1 immunity in host protection against *M.tb* infection. Mouse studies and evidence obtained from individuals susceptible to mycobacterial infection reveal that the IL-12/IFN-γ pathway is indispensable for protection against infection (Altare et al., 1998; Cooper et al., 1993; Cooper et al., 1997; Flynn et al., 1993; Jouanguy et al., 1996; Newport et al., 1996). Multiple components of the immune system act in concert to induce an optimum response including T cells, activated macrophages, and cytokines such as interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor (TNF-α). The macrophage-activating and "protective" T_H1 phenotype is driven by the local release of interleukin-12 (IL-12) from infected cells (Ladel et al., 1997).

(i) Macrophages as the effectors of T_H1 immunity

The macrophage is central to the control of *M.tb* infection. *M.tb* multiply in resting macrophages, but activation of infected macrophages with IFN-γ results in induction of mycobacteriostatic or mycobacteriocidal states *in vitro* (Flesch and Kaufmann, 1987). TNF-α and IFN-γ act synergistically to activate macrophages, and subsequently induce production of reactive nitrogen intermediates (RNIs), one of the antimicrobial defence mechanisms (Ding et al., 1988). Nitric oxide (NO) is generated from L-arginine via the enzymatic action of the inducible isoform of nitric oxide synthase (NOS2) (Ding et al., 1988) and its importance in protective immunity was confirmed in studies where mice deficient of NOS2 activity were shown to be susceptible to *M.tb*

infection (Chan et al., 1995; Flynn et al., 1998; MacMicking et al., 1997; Scanga et al., 2001). The relevance of this mechanism in human infection remains controversial: however, there are reports of NOS2 induction and/or RNI production by human macrophages (Bonecini-Almeida et al., 1998; Nicholson et al., 1996; Rockett et al., 1998). Moreover, production of reactive oxygen species (ROS) is required for optimum host protection against *M.tb*. Mice incapable of expressing NADPH-oxidase, and therefore no ROS, were shown to be more susceptible to *M.tb* compared with WT mice (Cooper et al., 2000). Another mechanism of *M.tb* elimination is a vitamin-D dependent pathway which leads to the induction of the antimicrobial peptide, cathelicidin, which results in the killing of *M.tb* (Liu et al., 2007).

(ii) $CD4^+$ T cells

T_H1 cells

It is well established that $CD4^+$ T cells are essential for protective immunity against *M.tb*. AIDS patients with low $CD4^+$ T cell counts are much more likely to develop active TB disease than HIV-uninfected individuals, providing evidence of the importance of $CD4^+$ T cells in anti-TB immunity (Aaron et al., 2004). Mice deficient in $CD4^+$ T cells have diminished ability to control infection further indicating the importance of this subset in protective immunity against *M.tb* (Caruso et al., 1999; Tascon et al., 1998). Importantly, Feng *et al.* showed that the adoptive transfer of $CD4^+$ T cells conferred protection to *M.tb* in RAG mice, which are deficient in B and T cells (Feng and Britton, 2000).

IFN-γ produced by $CD4^+$ T cells is central for the activation of anti-mycobacterial macrophages as described above (Cooper et al., 1993). TNF-α on the other hand, acts synergistically with IFN-γ to fully activate macrophages, and for the initiation and maintenance of granulomas (Cooper et al., 1993). TNF gene deletion (Bean et al., 1999) and TNF neutralization studies (Eriks and Emerson, 1997; Kindler et al., 1989) have demonstrated

that TNF plays a critical role in protective immunity against *M.tb* infection. In the absence of TNF, mice challenged with either avirulent *M. bovis* or virulent *M.tb* were shown to succumb to infection more rapidly, compared with wild type mice (Bean et al., 1999; Jacobs et al., 2000). Although IL-2 has little direct effector function, the cytokine promotes expansion of $CD4^+$ and $CD8^+$ T cells; hence, IL-2 serves to amplify effector T-cell responses. In addition, IL-2 produced by T cells can enhance natural killer cell activity that could contribute to the early control of infection (Seder et al., 2008).

So-called "polyfunctional" $CD4^+$ T cells, which co-express the cytokines IFN-γ, TNF-α and IL-2, have recently achieved prominence in the immunological literature. Darrah *et al* showed in mice that following primary infection with another intracellular pathogen, *Leishmania major*, or after vaccination against this organism, the generation of polyfunctional T cells that secrete IFN-γ, IL-2 and TNF strongly correlated with protection against subsequent challenge. This study emphasized that measuring the magnitude of IFN-γ producing $CD4^+$ T cells alone was not sufficient to predict protection (Makedonas and Betts, 2006; Darrah et al., 2007; Seder et al., 2008). In addition, it has been shown in a murine TB model that more polyfunctional cells at the site of infection associated with a more protective immune response against TB (Forbes et al., 2008).

T_H17 cells

T_H17 cells have been reported by several groups to mediate a role during *M.tb* infection. Immunization of mice with a novel vaccine comprising an immunodominant epitope derived from ESAT-6 resulted in significant protection of vaccinated mice upon *M.tb* infection. This protection was shown to be mediated, in part, by specific T_H17 cells in the lung, which subsequently induced recruitment of IFN-γ producing T_H1 cells, through up-regulation of the chemokines CXCL9, CXCL10, and CXCL11(Wozniak et al., 2006; Khader et al., 2007). A study by Scriba *et al.* reported that mycobacteria-specific T_H17

cells were present in healthy *M.tb*-exposed and TB diseased patients; however, T_H17 frequencies were lower in the latter group, indicating a potential protective role against TB disease development (Scriba et al., 2008). Further studies are required to understand the role of this pro-inflammatory cytokine in protection against TB for interventions such as novel TB vaccines.

Regulatory T cells

Jaron *et al.* have shown that depletion of Tregs in BCG vaccinated mice results in, a reduction of the bacterial burden after challenge with *M.tb*., indicating that Tregs inhibit the *M.tb*-specific effector response and have a negative impact on immune control (Jaron et al., 2008). A clinical study investigating the role of Tregs showed that TB diseased patients had higher frequencies of Tregs in peripheral blood compared to healthy uninfected individuals. Furthermore, *ex vivo* depletion of Tregs from PBMC resulted in increased numbers of ESAT-6- and CFP-10–specific IFN-γ producing T cells. This may indicate that the expanded Treg population in TB patients may contribute to suppression of T_H1 immunity (Guyot-Revol et al., 2006). The role of Tregs may be explained by a recent study by Shafiani *et al*, who used a murine model to demonstrate that even small numbers of *M.tb*-specific Tregs might actually impair immune protection by delaying the priming of *M.tb*-specific effector T cells, and hence their accumulation at the primary site of infection, the lung. The delay in the recruitment of *M.tb*-specific effector T cells in the lung results in a prolonged bacterial expansion and may explain the higher bacterial burden observed in mice injected with *M.tb*-specific Tregs (Shafiani et al., 2010).

(iii) $CD8^+$ T cells

M.tb antigens can be presented to $CD8^+$ T cells primarily via the MHC–I pathway. Evidence from several studies has confirmed the importance of $CD8^+$ T cells in the immune responses against *M.tb*. A study in non-human primates showed that depletion of $CD8^+$ T cells compromised BCG vaccine-

induced immune control of *M.tb* replication in the animals, and this led to a significant decrease in the vaccine-induced immunity against TB (Chen et al., 2009). Experiments carried out by Flynn *et al.* showed that mice deficient in β2-microglobulin, which therefore lack MHC class I molecules and consequently fail to develop functional $CD8^+$ T cells, were more susceptible to *M.tb* (Flynn et al., 1992). Although *M.tb* resides within phagosomes, several mechanisms have been proposed to explain how the organism could gain access to the cytoplasm, leading to presentation of mycobacterial peptides via the MHC-I pathway. Myrvik and colleagues suggested that *M.tb* was able to perforate the phagosomal membrane in order to use the nutrients in the cytosol. It was postulated that *M.tb* in the cytoplasm would result in enhanced MHC class I presentation to $CD8^+$ T cells (Myrvik et al., 1984). $CD8^+$ T cells may also be activated through cross-presentation of antigens (Schaible et al., 2003). Macrophages infected with *M.tb* undergo apoptosis and vesicles containing *M.tb* antigens can then be taken up by bystander DC, which are able to present the antigens via the MHC-I pathway, leading to the priming of $CD8^+$ T cells.

Effector functions of $CD8^+$ T cells include amongst others, production of IFN-γ and TNF-α (Caruso et al., 1999; Scanga et al., 2000), which induce macrophage activation, as well as lysis of infected cells. $CD8^+$ T cells lyse infected cells by releasing perforin which forms pores on the cell membrane surface (Stenger et al., 1997). This reduces the pool of infected cells and causes the release of bacteria, which can be killed more efficiently by activated macrophages. Alternatively, $CD8^+$ T cells may directly kill the intracellular bacteria directly by releasing granulysin (Stenger et al., 1998), however this happens only in the presence of perforin, which allows the granulysin to gain access to the cell. Experiments by Stenger *et al.* demonstrated a decrease in viability of intracellular mycobacteria when infected macrophages were incubated with perforin and granulysin (Stenger et al., 1997).

1.3.2 Humoral immunity to *M.tb*

Many questions regarding the role of B cells during *M.tb* infection remains unanswered. Since *M.tb* resides within phagosomes, the dogma has been that only cell-mediated responses are important in protective immunity against *M.tb*. However, BCG vaccination induces anti-lipoarabinomannan (LAM) IgG1 (de Valliere et al., 2005), and administration of anti-LAM antibodies results in increased survival of mice after challenge with *M.tb* (Teitelbaum et al., 1998). Furthermore, anti-mycobacterial antibodies were shown to enhance innate immunity, by allowing improved uptake and subsequent killing of mycobacteria by macrophages in the presence of specific antibodies. Moreover, coating of mycobacteria with specific antibodies resulted in more effective processing and presentation by DCs via Fc receptors resulting in an enhanced ability to stimulate $CD4^+$ and $CD8^+$ T cells (de Valliere et al., 2005). Presumably, anti-TB antibodies mediate a more relevant role when *M.tb* is extracellular, e.g., upon lysis of infected macrophages, and during dissemination. Antibodies may also play an indirect role in controlling inflammation caused by infection with *M.tb*. Maglione *et al* found that adoptive transfer of B cells resolved the inflammatory exacerbation observed in B-cell-deficient mice upon airborne challenge with *M.tb* (Maglione et al., 2007). Antibodies may therefore have a variety of protective effects during infection with intracellular pathogens (Maglione and Chan, 2009).

It is important to note that a complex orchestrated interaction of multiple cell types, including innate cells and T cell subsets, in balance, is likely to be important for optimal immunity against *M.tb* (Kaufmann et al., 2005; Hanekom et al., 2007).

1.4 Vaccination Strategies against *Mycobacterium tuberculosis*

1.4.1 Current strategies

There is an urgent need for an effective vaccine to prevent TB disease; this is likely the most sustainable solution to control the TB epidemic. BCG is the

only licensed vaccine currently available. BCG is an attenuated form of *Mycobacterium bovis,* which was generated after multiple *in vitro* passages of the organism (Reece and Kaufmann, 2008). Despite being safe and currently the most widely used vaccine in the world, its efficacy remains variable (Fine, 1995; Behr, 2002). BCG provides approximately 80% protection against severe forms of TB in infants, such as meningitis and miliary disease, but confers variable protection against pulmonary TB disease at all ages. It is estimated that the efficacy of BCG to protect adults against lung TB disease varies between 0 and 80% (Colditz et al., 1994; Brandt et al., 2002).

One of the main reasons accounting for the variability of BCG may be that pre-exposure to environmental mycobacteria may induce immunity that would clear BCG before it can induce an immune response. It has been shown in mice that exposure to environmental mycobacteria can result in cross-reactive immunity, which leads to more rapid clearance of BCG upon vaccination and reduced immunogenicity as a consequence (Brandt et al., 2002). The discrepancies in BCG efficacy may also be explained by strain variability (Behr, 2002), as significant differences in the genome of different BCG strains have been described (Brosch et al., 2007), which may influence immunogenicity (Aguirre-Blanco et al., 2007) and virulence (Kroger et al., 1995). The variable efficacy of BCG may also be attributable to genetic differences in host populations (Fine, 1998).

1.4.2 New vaccination approaches

Given the variable efficacy of BCG in providing protection against TB disease, intensive research is being carried out to develop an improved vaccine or improved vaccination strategy. New vaccines would have to be at least as effective as BCG (Reece and Kaufmann, 2008). Current vaccines under development are designed to either improve BCG or boost immunity induced by BCG. These could be combined in heterologous prime-boost vaccination strategies (McShane et al., 2004). The prime vaccine is likely to remain BCG or will perhaps be replaced with a modified BCG to become more effective, or

even an attenuated *M.tb*. It is likely that this vaccine will continue to be administered at birth. It is proposed that a different (i.e., heterologous) boost vaccine will be administered at a later time point. This vaccine will comprise antigens present in BCG and *M.tb*. These vaccines are designed as prophylactic (pre-exposure) vaccines and, hence, are aimed at stimulating an immune response that controls subsequent infection more effectively than the immune response stimulated during natural infection. It is probable that these vaccines might prolong the duration of asymptomatic infection, thus delaying disease outbreak due to reactivation or re-infection. (Brennan et al., 2007; Barker et al., 2009). Post-infection vaccines are also in the pipeline and these will be aimed at LTBI individuals to prevent progression to TB disease. A post-infection vaccine will contain proteins that are primarily expressed during the latent stage of infection, and is likely to boost the immune response induced after natural infection with *M.tb* (Lambert et al., 2009). The addition of latency associated antigens to well-established prophylactic vaccines (i.e., the vaccine candidates under clinical testing today) have been suggested as the basis for a future generation of multi-stage TB vaccines with activity against all stages of infection (Andersen, 2007a).

(i) Prophylactic vaccines

(A) Recombinant BCG (rBCG) and attenuated *M.tb* strains are two approaches being explored to replace the standard BCG with an improved priming vaccine. These vaccines are based on strains of BCG currently in use, or are derived from attenuation of *M.tb* through deletion of genes associated with virulence (Lambert et al., 2009). rBCG30 is an example of a new TB vaccine which aims to induce a more potent immune response by overexpressing a key *M.tb* antigen (Rao et al., 2003). This rBCG vaccine, overexpressing the immunodominant Ag85B controlled a virulent *M.tb* challenge more effectively than mice immunized with normal BCG, had lesser pathology in lungs and spleens, and survived longer (Horwitz et al., 2000; Horwitz and Harth, 2003). Importantly, rBCG30 has limited replication *in vivo* in SCID (severe combined immune deficiency) compared to parental BCG,

which may translate to better safety for administration to HIV infected individuals (Tullius et al., 2008; Lambert et al., 2009).

A different approach involves the use of a mutant strain of *Mycobacterium bovis*. The Δ *ureC hly*$^{+}$ rBCG (VPM1002) is a urease C deficient mutant expressing the listeriolysin gene (Grode et al., 2005). The bacterium is unable to inhibit phagolysosome formation, given that urease C is important in neutralising phagosome acidity, which is critical for lysosome fusion (Schaible et al., 1998). Lysteriolysin, which originates from the organism *Listeria monocytogenes,* induces pore formation and therefore allows the organism to escape from the phagosome into the cytoplasm (Conradt et al., 1999; Grode et al., 2005). It was postulated that BCG in the cytoplasm would result in enhanced MHC class I presentation to CD8 T cells. Subsequent preclinical studies in mice, guinea pigs and non-human primates have demonstrated a superior CD8 T cell mediated response when Δ*ureC hly*$^{+}$ rBCG was compared with normal BCG. In addition the rBCG showed greater safety and efficacy against an aerosol infection with *M.tb* than the parent BCG strain in immunodeficient mice (Grode et al., 2005),

Several other live attenuated candidates are also being assessed in preclinical studies. MTBVAC01 is a live attenuated *M.tb* that is a double mutant deficient in the virulence genes phoP and fadD26 (Perez et al., 2001; Soto et al., 2004). This vaccine has proven to be safe in severe combined immunodeficiency (SCID) mice and has conferred better protective efficacy, compared with BCG (Martin et al., 2006). Verreck, et al, in a non-human primate model have shown that MTBVAC01 was well tolerated and afforded protection against challenge with a virulent *M.tb* (Verreck et al., 2009). A major concern for attenuated *M.tb* vaccines is the potential to revert to a virulent form hence these vaccines are required to undergo rigorous safety testing.

(B) Viral vectored vaccines containing antigens present in both BCG and *M.tb,*

and are aimed at enhancing immunity induced by BCG. Live vectors such as Adenovirus (Ad) and modified Vaccinia Ankara (MVA) have been engineered to be non-replicating and to deliver relevant antigens into the host. Pre-exposure to environmental mycobacteria may affect the efficacy of whole mycobacterial vaccines like BCG or attenuated *M. tuberculosis* (Fine, 1998); live viral vectors remain an ideal alternative in such settings. Two examples of viral vectored vaccines include the MVA85A and Areas402 vaccines, the backbone of which are replication-deficient viruses. These vaccines contain antigens expressed by both BCG and *M.tb* and are aimed at enhancing immunity induced by BCG, which is given at birth.

MVA85A comprises modified vaccinia Ankara and antigen 85A (McShane et al., 2004). Ag85A and B are part of the immunodominant Ag85 complex and are involved in mycobacterial cell wall synthesis (Belisle et al., 1997). MVA85A was the first viral vectored TB vaccine to be tested in humans. The clinical trials in adolescents and adults have shown that MVA85A is a safe vaccine that induces durable T cell responses, including an IFN-γ^+TNF-α^+IL-2^+, polyfunctional T cell response (McShane et al., 2004; Sander et al., 2009). MVA85A is undergoing multiple phase I/II trials in Africa, including trials in HIV infected patients and a phase IIb trial in infants (Lambert et al., 2009). Aeras-402 comprises an Adenovirus, serotype 35, that expresses a fusion protein of Ag85A, Ag85B and TB10.4 (Hussey et al., 2007). TB10.4 is a member of the ESAT-6 family of proteins and has been shown to be immunogenic (Skjot et al., 2002). The vaccine is in several Phase I/IIa trials in Africa, USA and Europe. Thus far, Aeras-402 has been shown to be safe, and able to induce a polyfunctional CD4 T cell response as well as a durable and robust CD8 T cell response in healthy adults (Abel et al., 2010). One concern is that previous natural exposure to a cross-reactive strain of adenovirus may hinder responses to adenovirus-based vaccines. Since the Ad35 serotype has a low prevalence, ranging from 3%-5% in developed countries and 20% in Africa, this vaccine may escape this limitation (Lambert et al., 2009).

(C) Subunit vaccines are designed to contain recombinant proteins that are administered with a T_H1-inducing adjuvant. Ideally, a subunit vaccine would comprise broadly recognised T cell epitopes to ensure the broad coverage of different populations (Weinrich Olsen et al., 2001; Skeiky et al., 2004). Examples of subunit vaccines include the Hybrid 1(H1) and the Mtb72F (M72) vaccines. H1 contains a fusion protein of Ag85B and ESAT-6 and the adjuvant IC31. Both Ag85B and ESAT-6 are strongly recognised by the host immune system during primary infection and have been shown to be protective when used as vaccines in animal models with IC31, a combination of a leucine-rich peptide named KLK and a synthetic oligonucleotide, named ODN1a (Weinrich Olsen et al., 2001; Aagaard et al., 2009). M72 contains the antigens Mtb32 and 39 expressed as a recombinant fusion polyprotein, together with the adjuvant ASO1E. This vaccine has been shown to induce robust T cell and antibody responses in healthy adults, (Von Eschen et al., 2009) and to protect mice, guinea pigs and nonhuman primates against challenge with virulent *M.tb* (Skeiky et al., 2004). Several Phase I and II clinical trials have been completed and results indicate cell mediated responses induced by M72 persisted for at least 6 months post vaccination (Lambert et al., 2009)

(ii) Post-infection vaccine

Recent TB vaccine design strategies include the development of post-infection vaccines that incorporate antigens presumed to be expressed during latency (Andersen, 2007b). The aim of this vaccine strategy is to target latently infected individuals that represent a reservoir of *M.tb* with the potential for reactivation and a source of disease transmission. Currently, the vast majority of vaccine candidates tested are aimed at preventing primary disease and/or reactivation of latent infection through prophylactic vaccines. Although these vaccines allow for maintenance of a stable infection, they do not achieve sterile eradication of *M.tb*. Post-infection vaccines may not only maintain latent infection and thus prevent reactivation of TB disease but also eradicate dormant bacteria and hence induce sterile clearance of *M.tb*. To

date there has been no consistent success in the attempts to use prophylactic vaccine candidates as post-infection vaccines (Aagaard et al., 2009). It is hypothesized that inducing robust T cell responses to latency associated antigens (see below) may enhance immunological control of latent TB. Therefore, a rational strategy might be to target latent infection with post-exposure vaccines expressing latency-, starvation-, or resuscitation-associated antigens.

The search for novel latency associated antigen(s) has been facilitated by expression profiling of *M.tb* laboratory strains cultured under conditions that mycobacteria encounter *in situ* during latent infection. Latency antigens can be defined as those expressed by dormant or slowly replicating *M.tb* bacilli, grown under *in vitro* conditions that mimic the granuloma. The dormancy survival regulon (DosR) is a distinct set of 48 genes that have been shown to be upregulated under conditions of hypoxia and nitric oxide stress. Starvation antigens expressed by *M.tb* under *in vitro* conditions of nutrient starvation are known as antigens of the starvation stimulon compromising antigens Rv2656-2661 (Betts et al., 2002). Resuscitation antigens are expressed during reactivation of dormant bacilli, which subsequently resume active metabolism. This process is facilitated by so-called resuscitation promoting factors (rpfs) that are secreted from slowly replicating bacteria (Downing et al., 2005).

The screening for recognition of antigens expressed by dormant or slowly replicating *M.tb* in latently infected individuals is the first step. Thus far clinical studies have focused on antigens of the DosR. T cell recognition of selected DosR antigens has been demonstrated in latently infected individuals from a non-TB endemic area (Leyten et al., 2006). In addition, immune recognition of five DosR antigens, namely, Rv1733, Rv0081, Rv1735c, Rv1737c and Rv3131, in latently infected individuals from TB endemic regions of South Africa, Uganda and Gambia have also been shown (Black et al., 2009). The current study is the first to investigate human recognition of antigens from the starvation stimulon. It is important that candidate *M.tb* antigens from these

antigenic groups initially be screened in human populations to assess the breadth of immune recognition. In addition, since cell mediated immune responses are known to be essential for the control of *M.tb*, antigens inducing a strong T_H1 response during natural infection should be chosen as candidate antigens for potential inclusion in post-infection vaccines.

1.5 Optimal measurement of vaccine responses

Efficient and sensitive measurement of T cell responses is absolutely critical for the assessment of new vaccines, and for assessment of the human immune response to *M.tb* (Hanekom et al., 2004). At SATVI, we are actively involved in assessing novel TB vaccines in clinical trials, and multiple immunological studies are conducted to gain insight into the immune response to TB infection and disease. It is critical that the appropriate assay is employed to address a specific question. The assay system chosen to measure vaccine-induced responses, may be guided by the aspect of T cell immunity to be measured; for example, longer-term assays may be better for measuring central memory T cell responses thought to be critical for long-term protection induced by vaccines. The assay system used can also be dictated by what is practical in the setting; for example, if incubation of whole blood or PBMC isolation early after blood collection is not possible, it may be wiser to perform longer-term diluted whole blood or PBMC assays, to minimise a potentially significant effect of processing delay on assay outcomes. Blood volume restriction in infants, compared to adults, may further dictate assay choice (Hanekom et al., 2008). T cell assays that are widely used in vaccine trials include 5 to 7 day whole blood or PBMC-based assays of proliferation, overnight or 48 hour enzyme-linked immunosorbent spot assays (ELISPOT) and 6 to 18 hour-incubation of whole blood or PBMC with specific antigens to measure T-cell-specific cytokine expression (Hanekom et al., 2008).

In short-term assays, whole blood or PBMC are typically incubated with specific antigens for 6 to 18 hours. Brefeldin-A or monensin is added for the last few hours of incubation to capture cytokines intracellularly. Hanekom *et*

al. developed and optimised a whole blood intracellular cytokine detection assay (WBA) for assessing T cell responses. This assay is utilized in all our vaccine trials and facilitates the harmonization of vaccine assessment in the TB field. In the WBA, blood is collected and processed within 2 hours of collection, and incubated with antigen for 12 hours. The vaccine-specific response measured directly *ex vivo,* provides a "snapshot" of the host immune response and results reflect the *in vivo* state of circulating T cells (Hanekom et al., 2004; Hanekom, 2005). Also, the effector function of specific cells is assessed without a prolonged period of cell activation, not allowing time for cellular division, resulting in more quantitative outcomes. The WBA is a sensitive and reliable way of measuring mycobacteria specific and vaccine-specific T cell immunity. For short-term assays it is important that whole blood is incubated and PBMC isolated and incubated as soon as possible after collection, to avoid loss of sensitivity. This appears to be of particular concern when protein or whole bacterial or viral antigens need to be processed, and may be less important for peptide antigens (Hanekom et al., 2008).

In long-term assays, diluted whole blood or PBMC is incubated with specific antigens for 5 to 7 days. Proliferation of T cells in the diluted whole blood culture may be measured by thymidine incorporation, or alternatively, by detecting BrdU incorporation into nucleic acids. PBMC may be stained with the fluorescent dyes CFSE or Oregon Green (OG), prior to incubation, allowing detection of proliferation by dilution of the dyes. CFSE and OG bind non-specifically to intracellular proteins and each cellular division decreases the fluorescence intensity of the cell by half. These assays may also be adapted to measure the cytokine-producing potential of the proliferating cells, by adding PMA/ionomycin, which causes calcium influx with cytokine production, and Brefeldin-A, which blocks export of proteins, including cytokines (Hanekom et al., 2008). The capacity for antigen-specific T cells to proliferate and clonally expand, which is important in long-lived protective immunity, may constitute a different aspect of an *ex vivo* measurable immune response (Rosenberg et al., 1997). Also, the expansion of cells may increase

sensitivity, i.e., ability to measure a response not measurable with other systems. The monitoring of vaccine-induced T cell proliferative potential may be important for determining vaccine take, memory function and long-term persistence of vaccine-specific responses. Hence, novel TB vaccine candidates should be assessed for their potential to induce durable responses in recipients; an outcome that can be optimally measured by a proliferation assay adapted for large studies in the field.

1.6. Current studies

In this thesis, we attempt to address two relevant areas in TB vaccinology. Firstly, the screening of human populations for an immune response to novel *M.tb* latency-associated antigens, and the characterisation of this immune response. The long-term goal would be inclusion of promising candidates into a post-infection vaccine. Secondly, we address the development and optimisation of a novel immunological assay for measurement of vaccine-specific responses.

1.6.1 Study 1 (Chapter 3)

Objective: To investigate human T cell responses to *M.tb* latency associated antigens in latently infected and TB diseased patients.

Specific aims: (i) To determine human antigen recognition of *M.tb* latency associated antigens Rv2659 and Rv2660, by measurement of soluble IFN-γ response to the specific antigens, and hence determine the proportion of responders to these antigens, and (ii) To determine the cytokine expression and proliferative capacity of T cells recognising latency associated antigens in LTBI and TB diseased persons.

1.6.2 Study 2 (Chapter 4)

Objective: To determine whether antigen-specific lymphoproliferation, after long-term culture, can be identified through the analysis of Ki67 expression in T cells.

Specific aims: (i) To determine the kinetics of Ki67 expression in T cells following long-term culture with antigen, (ii) To determine how Ki67 as an indicator of antigen-specific T cell proliferation compares with standard proliferation assays, (iii) To determine how the functional profiles of Ki67 expressing, proliferating T cells compare with those of proliferating cells detected with standard assays, and (iv) To evaluate performance of the Ki67-based assay for longitudinal assessment of immune responses to vaccination in infants.

Chapter 2: Assay Optimisation

The aim of this chapter was to determine which T cell assay system resulted in optimal measurement of Rv2660 and Rv2659-specific T cell responses. To optimally measure the immune response to the novel latency associated antigens Rv2659 and Rv2660 (Chapter 3), both short-term whole blood (intracellular cytokine expression) and long-term PBMC and whole blood (lymphoproliferation) assays were compared. The outcomes of short-term assays, often called "directly *ex vivo*" assays, reflect the *in vivo* state of circulating T cells. Additionally the effector function of specific cells is assessed without a prolonged period of cell activation, not allowing time for cellular division. The result may be considered a quantitative reflection of immune status in peripheral blood (Hanekom et al., 2008). On the other hand, lymphoproliferation assays allow for the expansion of antigen-specific cells, enabling the measurement of rare populations that might otherwise be undetectable. The capacity of antigen-specific T cells to proliferate and produce cytokines may be assessed. Furthermore, it has also been proposed that central memory cells may be more optimally measured with lymphoproliferation assays (Hanekom et al., 2008). After stringent optimisation of these 2 assay systems, the results were compared to determine the most sensitive and practical assay to characterise the immune response to the latency associated antigens Rv2660 and Rv2659.

2.1. Materials and Methods

2.1.1 Blood collection

Blood was collected from healthy adults with a positive QuantiFERON®-TB Gold In-Tube (QFT™) test, all of whom had no history of TB disease. Blood was collected in sterile tubes containing heparin to avoid blood clotting. The blood was collected using a UCT Research Ethics Committee-approved protocol, which included written informed consent obtained from all participants.

2.1.2 Short-term whole blood assay

We first conducted a short-term whole blood assay to determine direct *ex vivo* responses to Rv2660 and Rv2659. Whole blood was collected in heparinized syringes and processed within 2 hours of collection. Briefly, 500μL undiluted whole blood was incubated for 12 hours at 37°C with Rv2660 peptide pool (20mers, overlapping by 10 amino acids, final concentrations of 1, 3 and 10μg/mL/peptide), or Rv2659 peptide pool (20mers, overlapping by 10, final concentration 1, 3 and 10μg/mL/peptide) (provided by Staten Serum Institute) or viable BCG (reconstituted from the vaccine vial, Danish strain, Staten Serum Institute, 1.2 x 10^6 CFU/mL) and the co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences, 0.5μg/mL each). These antibodies are known to enhance the frequency of antigen-specific cytokine expression.

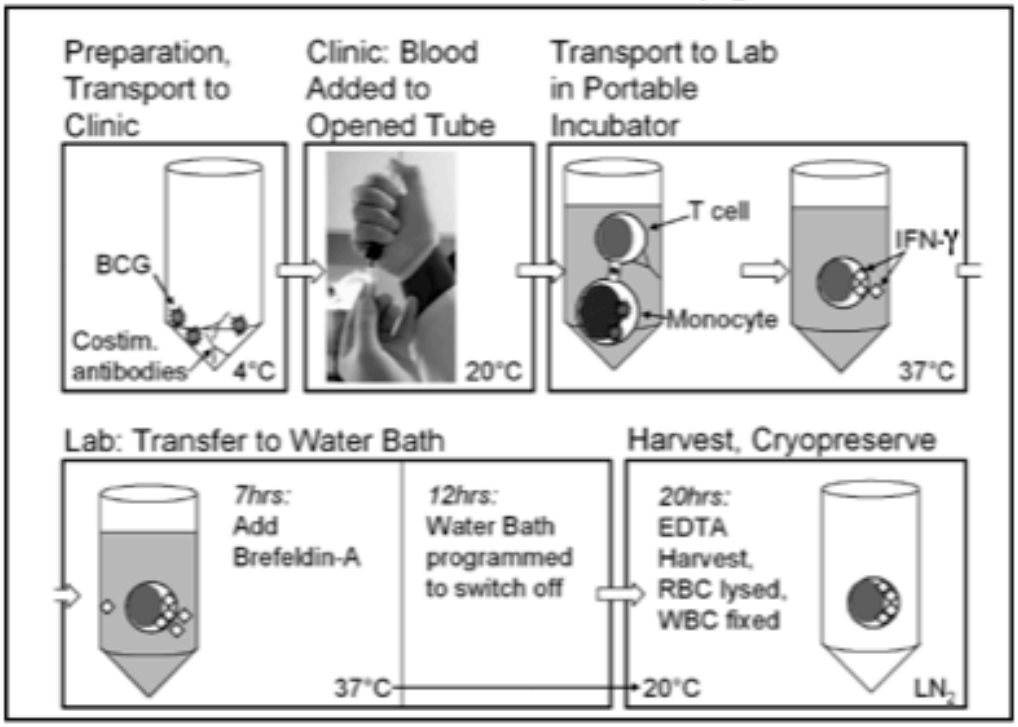

Figure 2.1. Flow diagram depicting the whole blood intracellular cytokine assay (Hanekom *et al.*).

Five-hundred microlitres of blood incubated with Staphylococcal enterotoxin B (SEB, Sigma-Aldrich, 10μg/mL) and 500μL incubated with the co-stimulatory antibodies alone (unstimulated) served as positive and negative controls, respectively. After 7 hours, 100μL supernatant was collected and stored at -80°C. Brefeldin A (Sigma, 10μg/mL) was added for the last 5 hours of

incubation. Following EDTA harvest, red blood cells were lysed and white cells fixed with BD FACS Lysing Solution (BD Biosciences), and the cells cryopreserved. Please refer to figure 2.1 for a flow diagram of the method.

2.1.3 Six-day whole blood BrdU incorporation assay

Next we determined whether the expansion of Rv2660 and Rv2659-specific T cells would allow greater sensitivity in the measurement of the immune response to these antigens. Whole blood (125µL diluted 1:10 in warm RPMI 1640) was incubated with Rv2660 peptide pool (as in section 2.1.2), Rv2659 peptide pool (as in 2.1.2) or 1 x 10^5cfu/mL viable Danish BCG, in duplicate wells in 96 well culture plates for 6 days at 37°C with 5% CO_2. On day 6 (day 3 for PHA), 10µmol/L BrdU (Sigma-Aldrich) was added for the last 5 hours of culture. To induce cytokine expression and capture the cytokines intracellularly, 10ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 1.5µg/mL ionomycin (Sigma-Aldrich) and 1.5µg/mL Brefeldin A (Sigma-Aldrich) were also added during the last 5 hours of culture. Control antigens included 1µg/mL phytohaemagglutinin (PHA; positive control, Sigma-Aldrich), medium only (Uns, negative control) or, for intracellular cytokine assays, medium with PMA and ionomycin (Uns-PI). On day 6, 100µL supernatant was collected and stored at -80°C. Cells were harvested with 2mM EDTA (Sigma-Aldrich) and red blood cells lysed and fixed using BD FACS Lysing Solution (BD Biosciences), according to the manufacturer's instructions and cryopreserved until analysis.

The assay was further optimised by:

1) Staining of white blood cells with a viability dye, LIVE/DEAD Fixable Violet Dead Cell Stain Kit (ViViD, Invitrogen). Prior to staining with the viablity dye, red blood cells were lysed using an ammonium chloride-lysing solution which contained no fixative. This was followed by fixing and cryopreservation of cells.

2.1.4 Flow cytometric analysis of processed whole blood

Cryopreserved cells were later thawed and washed in phosphate buffered saline (PBS, BioWhittaker), and permeabilised using Perm/Wash Solution (BD Biosciences). The following monoclonal antibodies were used for phenotypic and/or intracellular cytokine staining: CD3-QDot 605 (UCHT1), CD8-PerCPCy5.5 (SK1), IFN-γ-Alexa Fluor 700 (B27), TNF-α-PECy7 (MAb11), IL-2-APC (MQ1-17H12), anti-BrdU-FITC (B44). ViViD, which fluoresces in the Pac-Blue channel was compensated using PacBlue (UCHT1) antibody. All antibodies were from BD Biosciences except for CD3-QDot 605, which was from Invitrogen. Samples were acquired on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA), configured with 3 lasers and 10 detectors, using the FACS Diva 6.1 software. Compensation settings were defined using anti-mouse kappa beads (BD Biosciences) that were stained with the respective fluorochrome–conjugated antibodies.

2.1.5 Six-Day PBMC Oregon Green assay

Next, a 6-day PBMC assay was performed to determine whether whole blood and PBMC-based lymphoproliferation assays both result in expansion of Rv2660 and Rv2659-specific T cells. PBMC were isolated by density gradient centrifugation and cryopreserved in 10% DMSO (Sigma-Aldrich) in heat-inactivated fetal calf serum (Adcock Ingram). PBMC were thawed in 12.5% human AB serum/ RPMI containing 2.5μg/mL DNase (Sigma-Aldrich) and stained with 10μg/mL of Oregon Green (OG) (Molecular Probes, Invitrogen). The PBMC were resuspended in 12.5% AB serum in RPMI, and 200,000 cells in 200μL medium were rested overnight at 37°C, 5% CO_2 in a 96 well plate. Duplicate wells containing cells were either stimulated with BCG at an MOI of 0.01, Rv2660 peptide pool (as in 2.1.2), Rv2659 peptide pool (as in 2.1.2), or SEB (0.05μg/mL, positive control) or no antigen (negative control), for 6 days at 37°C with 5% CO_2. All antigens were obtained from the Staten Serum Institut, Denmark. After incubation, plasma was collected and stored at -80°C until further use. PBMC were then incubated with PMA (50ng/mL) and ionomycin (250ng/mL) to induce cytokine production, and Brefeldin A

(10μg/mL) was added for a further 5 hours. PBMC were harvested, stained for viability with ViViD, fixed with FACS Lysing Solution, and cryopreserved until later analysis.

2.1.6 Flow cytometric analysis of processed PBMC

Fixed, cryopreserved PBMC were thawed, washed with PBS and permeablised with Perm/Wash Solution for 10 minutes. PBMC were stained with the following antibodies: anti-CD3 Qdot605 (clone UCHT1) anti-CD8 Cy5.5PerCP (SK-1) and anti-IFN-γ AlexaFluor700 (B27), (all from BD Biosciences) for 1 hour at 4°C. After washing, cells were acquired on a LSRII flow cytometer (BD Biosciences), as in 2.1.4 Oregon Green and ViViD were compensated with FITC and PacBlue respectively. Data was analyzed using Flowjo 8.8.4 (Treestar). See figure 2.2 for illustration of method.

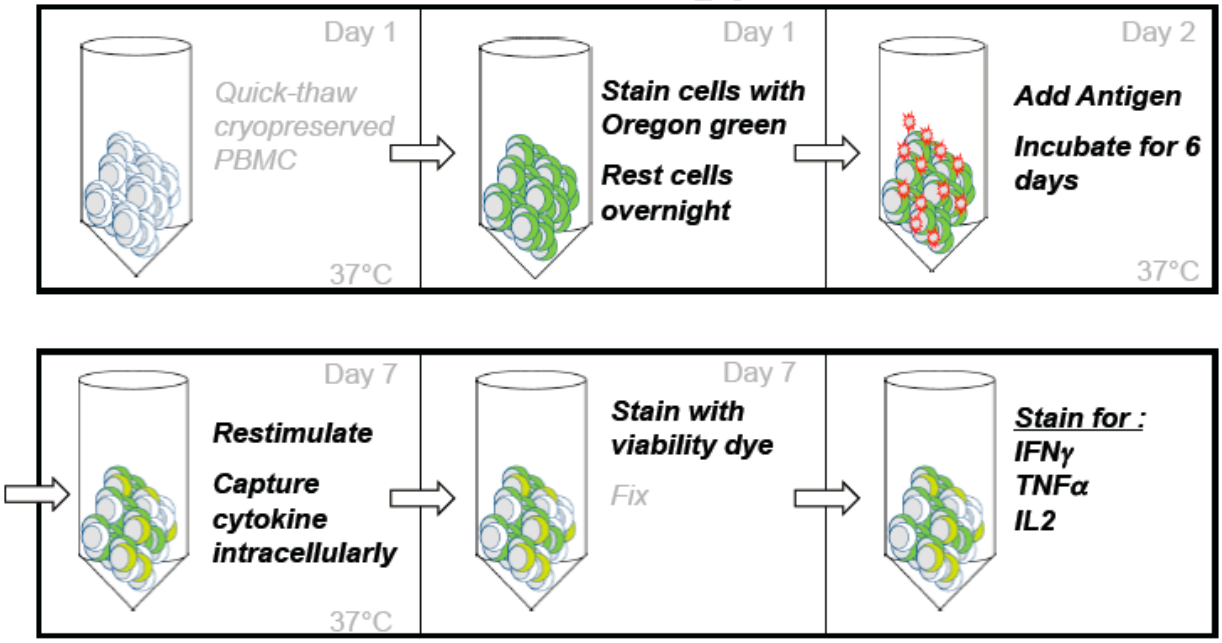

Figure 2.2. Flow diagram depicting the PBMC–based 6-day Oregon Green intracellular cytokine assay. (Diagram obtained from Alana Keyser, SATVI 2010)

2.1.7 IFN-γ measurement in supernatants

To determine whether Rv2660 and Rv2659 were recognised by LTBI individuals, soluble IFN-γ responses to the antigens were measured as indication of an antigen recognition. Briefly, 96-well plates were coated with 2μg/mL of anti-IFN-γ mAb (Pharmingen) for 2 hours at 37°C, and blocked with

1% BSA (Roche diagnostics) in PBS for 1 hour at 37°C. IFN-γ standard (15.6-2000pg/mL) (Pharmingen) and supernatants derived from the whole blood assay were added to the pre-coated plates, and incubated at 4°C overnight. After washing, appropriate wells were incubated with 1μg/mL biotin-labeled mouse anti-human IFN-γ mAb (Pharmingen) for 45 mins at room temperature (RT), followed by streptavidin peroxidase for 30 mins. OPD Fast (Sigma) was utilized as substrate, and the reaction stopped with 2M H_2SO_4 after 40 mins development. Plates were read at 405 nm on a Versemax ELISA plate reader using Softmax Pro software Version 4.7.1. The limit of detection of the IFN-γ ELISA was 15.6pg/mL.

2.1.8 IL-2 measurement in supernatants

As for IFN-γ, soluble IL-2 measurement was also performed as an antigen recognition screen. The IL-2 ELISA was carried out similarly to the IFN-γ ELISA with the following exception: Plates were coated with 4μg/mL of anti-IL-2 mAb (Pharmingen). The IL-2 standard (15.6-1000pg/mL) (Pharmingen) and supernatants were incubated at 4°C overnight. After washing, appropriate wells were incubated with 1μg/mL biotin-labelled mouse anti-human IL-2 mAb (Pharmingen). The limit of detection of the IL-2 ELISA was 15.6pg/mL.

2.2. Results

2.2.1 Detection of soluble IL-2 and IFN-γ depends on the duration of the assay

To assess whether Rv2660 and Rv2659-induced IL-2 and IFN-γ can be detected by both long and short-term PBMC and whole blood assays, supernatants from both the short-term and long-term assays were collected and cytokine levels determined by ELISA.

Supernatant from the direct *ex vivo* assay whole blood assay revealed that IFN-γ was only detected after incubation with the highest concentration (10μg/mL/peptide) of Rv2660 and Rv2659 (Figure 2.3A); however, IL-2 was detected at all 3 concentrations of the antigens, albeit at low levels (Figure 2.3B). Conversely, analysis of a long-term whole blood assay revealed that IFN-γ but not IL-2 (Figure 2.3C and D) was detected in the supernatants at all concentrations of antigens. IFN-γ was also detectable in supernatants from PBMCs incubated with Rv2660 and Rv2659 at all 3 concentrations for 6 days (Figure 2.3E), which verified that results from whole blood and PBMCs could assess similar IFN-γ levels after stimulation with viable BCG and SEB after 6 days of culture.

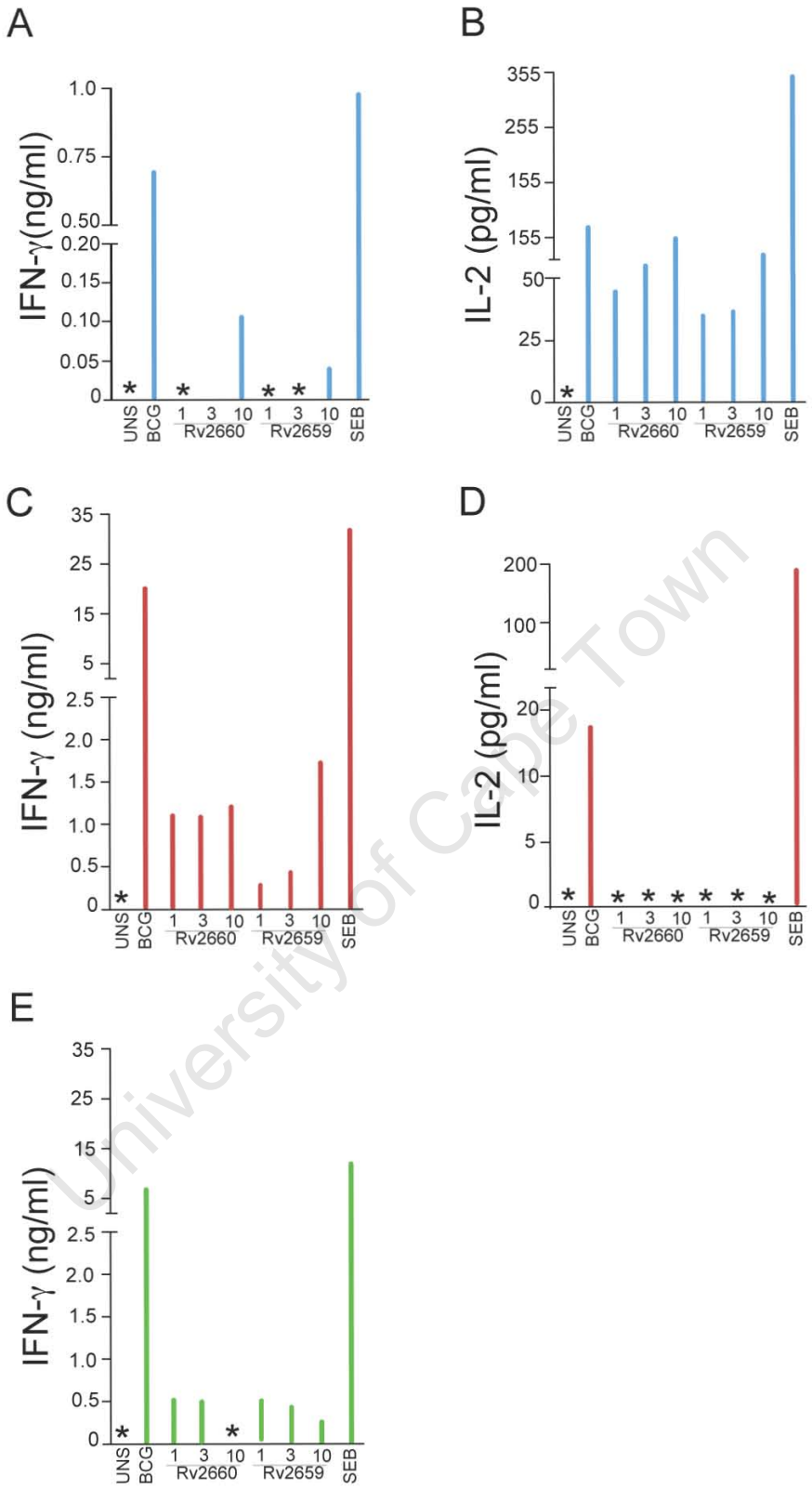

Figure 2.3. Detection of soluble IL-2 and IFN-γ in short- and long-term assays, by ELISA. Detection of IFN-γ (A) and IL-2 (B) from supernatants of direct *ex vivo* whole blood assay. Detection of IFN-γ (C) and IL-2 (D) from supernatants of a 6-day whole blood assay. Detection of IFN-γ (E) from supernatants of Oregon Green PBMC assay. (n=3) for all assays (*undetectable).

2.2.2 Rv2660 and Rv2659-specific T cell responses are barely detectable using the direct ex vivo intracellular cytokine assay

To be able to more comprehensively characterise the antigen-specific T cell response to Rv2660 and Rv2659, multiparameter flow cytometry was utilized. Whole blood incubated for 12 hours with Rv2660 and Rv2659 resulted in minimal T cell responses, which were barely above background levels (Uns) (Figure 2.4). We concluded that the latency antigen specific response detected by the short-term intracellular cytokine assay was too small to allow reliable measurement and characterisation of the T cell response.

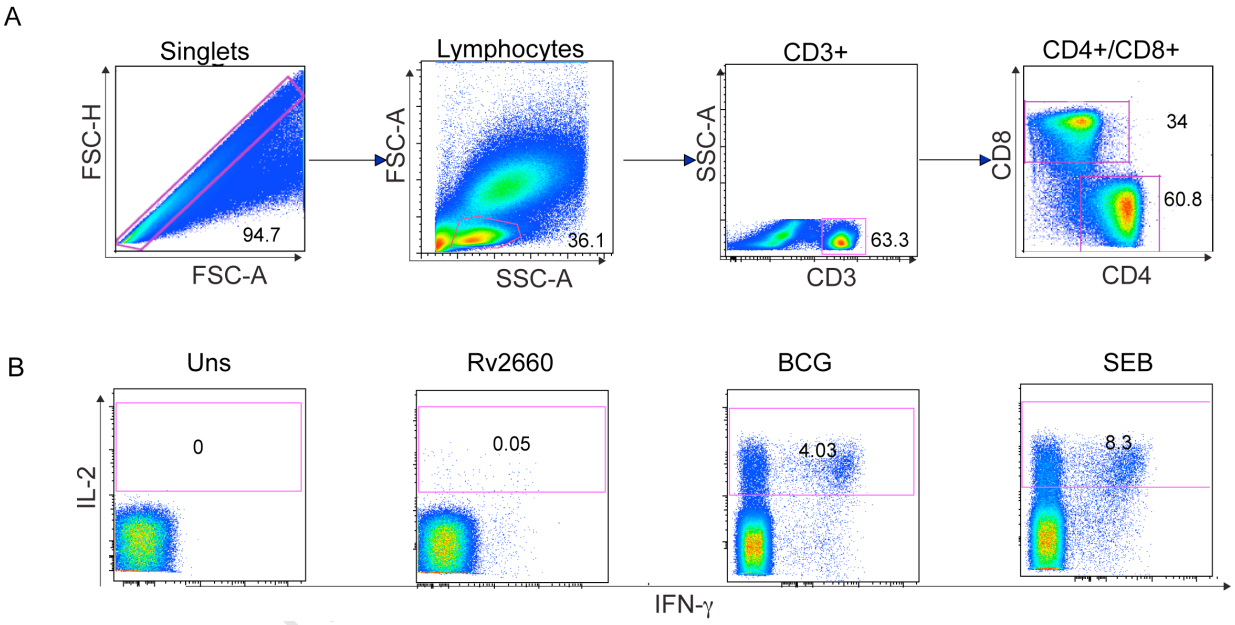

Figure 2.4. Gating strategy and detection of latency antigen specific CD4$^+$ T cells producing IFN-γ and/or IL-2 from the direct *ex vivo* whole blood assay. (A) Doublet cells were excluded by gating on forward scatter-height (FSC-H) against forward scatter-area (FSC-A). CD3$^+$ lymphocytes were selected by gating on CD3$^+$ against side scatter area (SSC-A). CD3$^+$ T cells were further differentiated into CD4$^+$ and CD8$^+$ T cell subsets by staining for CD8$^+$ and CD4$^+$ T cells, and then cytokine expression patterns were analyzed. (B) Representative dot plots of cytokine co-expression patterns in unstimulated or antigen-specific CD4$^+$ T cells. Results are representative of two healthy LTBI adults.

2.2.3 Cell viability is compromised after long-term incubation of whole blood with antigen

Cell death occurs during longer-term (up to 7 days) incubation of whole blood or PBMC, hence we assessed exclusion of dead cells with viability dye ViViD. This dye works by binding to amines that are on the cell membrane.

Apoptosis results exposure of intracellular amines on the cell membrane; therefore, dead cells (ViViD^{high}) will fluoresce at a higher intensity for ViViD than live cells (ViViD^{low}). Figure 2.5 shows that after long-term stimulation of whole blood with a potent antigen such as viable BCG, cell viability is compromised - approximately 20% cell death was detected. These dead cells could be excluded by gating on CD3^{+} and ViViD^{low} cells (Figure 2.5). In the absence of a viability dye, total CD3^{+} cells will include both viable and non-viable T cells (Figure 2.5A). It is evident from panel A that dead cells fluoresce in the FITC channel (BrdU), by binding non-specifically to antibodies This is observed when comparing the frequency of proliferating cells in the total CD3^{+} population (Figure 2.5B, no ViViD) with the live CD3^{+} population (Figure 2.5C, ViViD included). The incorporation of ViViD into the analysis strategy results in clear delineation of positive and negative populations, and enables more reliable results to be generated (Figure 2.5C).

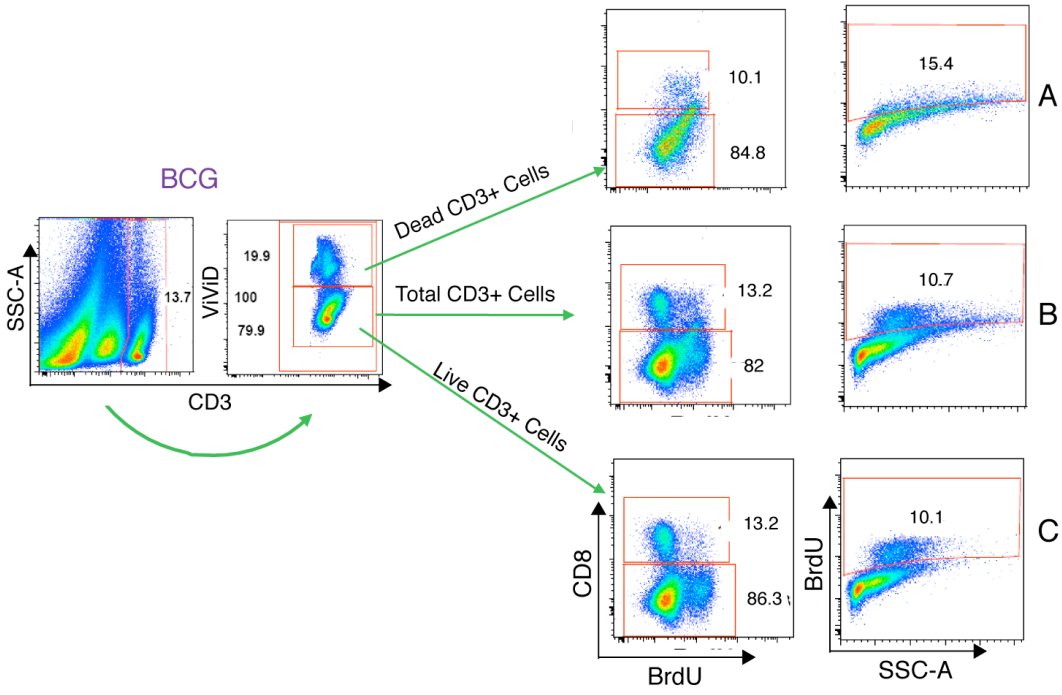

Figure 2.5. Incorporation of ViViD into a 6-day lymphoproliferation whole blood assay. Dead cells incorporate the viability dye, ViviD, and viable CD3^{+} T cells are selected by gating on ViViD^{low} cells. CD3^{+} T cells were further differentiated into proliferating (BrdU^{+}) CD4^{+} and CD8^{+} T cell subsets by staining for CD8^{+} (CD3^{+}CD8^{-} = CD4^{+} T cells). Optimisation experiments showed that using only a CD8 antibody resulted in clear delineation of CD8^{+} and C8^{-} (CD4^{+}) populations. Results are representative of two healthy LTBI adults

2.2.4 Expansion of Rv2660 and Rv2659-specific T cells by 6-day whole blood lymphoproliferation assay

Since the short-term WBA resulted in a barely detectable frequency of Rv2659- and Rv2660-specific T cells, a lymphoproliferation assay was assessed. This would allow expansion of rare antigen-specific T cell populations. Rv2660- and Rv2659-specific T cells were expanded for 6 days in culture with their respective peptide pools, and were detected by the incorporation of BrdU. The potential of specific (i.e., proliferating) $CD4^+$ to produce IFN-γ and TNF-α was subsequently determined from the $BrdU^+$ T cells. We showed that after polyclonal activation proliferating Rv2660-specific $CD4^+$ T cells and associated cytokine production were detectable using this assay (Figure 2.6). Background levels were very low, and stimulation with viable BCG or PHA (positive control) resulted in significant T cell proliferation and cytokine expression.

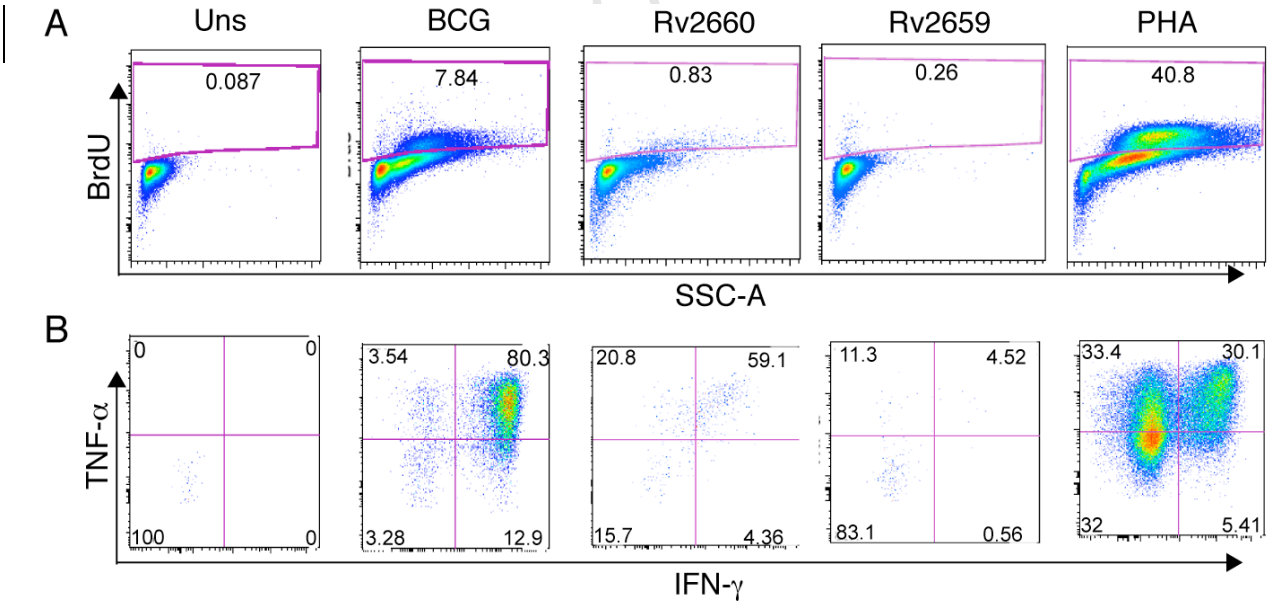

Figure 2.6. Expansion of Rv2660- and Rv2659-specific T cells in a 6-day BrdU whole blood lymphoproliferation assay. Frequency of $CD4^+$ $BrdU^+$ proliferating cells expressing IFN-γ and/or TNF-α upon stimulation with the indicated conditions. Proliferating $CD4^+$ T cells are selected by gating on BrdU against SSC-A, and then cytokine expression patterns were then determined after polyclonal activation with (PMA/Ionomycin) in proliferating T cells. The numbers within the gated areas indicate the frequency of BrdU+ (A) (proliferating) or the frequency of $CD4^+$ T cells producing cytokines (B). Results are representative of two healthy LTBI adults.

2.2.5 Expansion of Rv2660- and Rv2659-specific T cells by PBMC-based 6-day Oregon Green assay

Since only PBMC were available for analysis from one group to be studied, TB diseased patients, (Chapter 3), a PBMC-based assay was optimised to investigate immune responses to the novel antigens. Specifically, a PBMC-based Oregon Green (OG) 6-day assay was assessed to ascertain whether this assay would be practical and reliable for detecting Rv2660- and Rv2659-specific T cell responses.

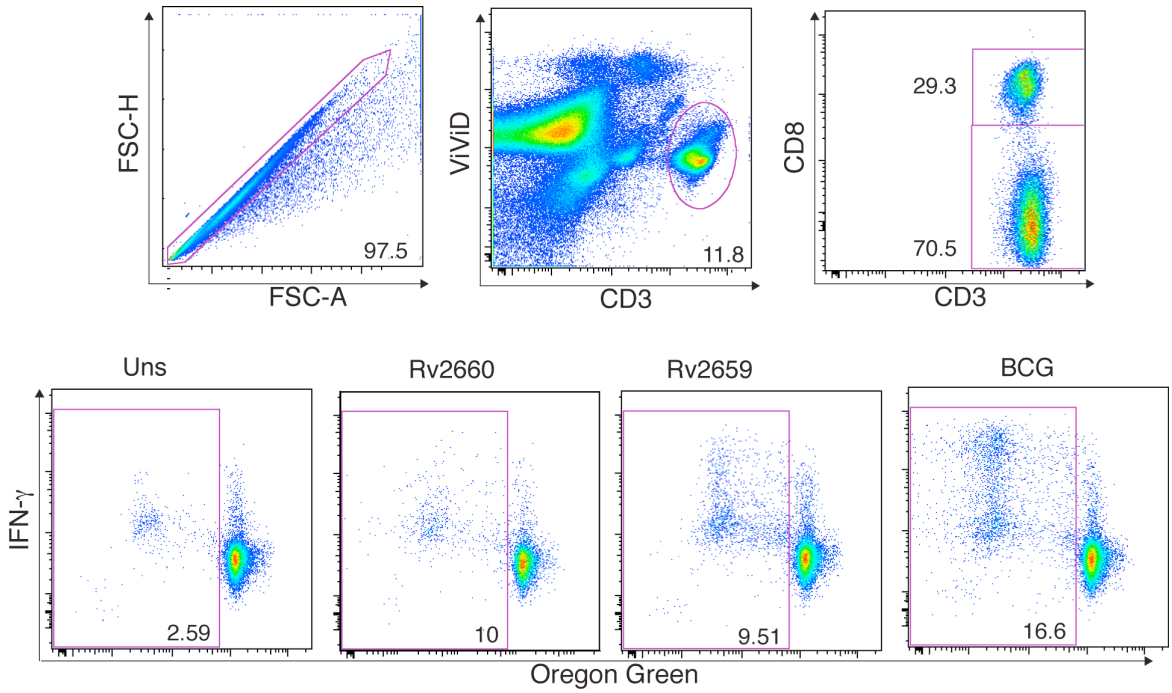

Figure 2.7. Expansion of Rv2660- and Rv2659-specific T cells in a PBMC-based 6-day Oregon Green assay. Frequency of $CD4^+$ proliferating cells expressing IFN-γ. PBMC were isolated, labelled with OG, and either left unstimulated or stimulated with the antigens indicated. Doublet cells were excluded by gating on forward scatter-height (FSC-H) against forward scatter-area (FSC-A), and viable T cells were then selected by gating on $CD3^+$ against ViViD. $CD3^+$ T cells were further differentiated into $CD4^+$ and $CD8^+$ T cell subsets by staining for $CD8^+$ ($CD3^+CD8^-$ = $CD4^+$ T cells, $CD3^+CD8^+$ = $CD8^+$ T cells). Proliferating (OG^{low}) CD4 T cells were then selected by gating OG against IFN-γ after polyclonal activation with (PMA/Ionomycin). Results are representative of three healthy LTBI adults.

Similarly to the BrdU whole blood assay, the PBMC OG assay resulted in expansion of Rv2660- and Rv2659-specific T cells, and enabled reliable detection of these populations (Figure 2.7). Therefore, the OG

lymphoproliferation assay was selected to characterise Rv2660- and Rv2659-specific T cell responses for the study (Chapter 3).

2.3. Discussion

It is critical that the appropriate assay is employed to address a specific question, and that the assay is carefully optimised to ensure the most sensitive and reliable outcomes. To this end, several assays were compared to determine which system allowed optimal measurement of Rv2660- and Rv2659-specific T cell responses. A direct *ex vivo* whole blood assay was initially assessed, however incubation with Rv2660 and Rv2659 resulted in a negligible antigen-specific T cell response. Soluble IL-2 and to a lesser extent, IFN-γ levels, were measureable with this short-term assay, however the low levels of soluble and intracellular cytokines measured, led to the exclusion of this assay.

Since cellular responses could not be reliably measured with a short-term assay, we assessed 6-day lymphoproliferation assays, which might facilitate the expansion of rare T cell populations. The BrdU whole blood assay, a routinely used lymphoproliferation assay (Dolbeare et al., 1983; Houck and Loken, 1985; Rosato et al., 2001), yielded reliable results and enabled the T cell-associated cytokine profiles to be dissected. Although cell death does occur after long-term incubation of whole blood with antigens, we demonstrated that inclusion of a viability dye in the analysis strategy resulted in a more reliable outcome. We conclude that the whole blood lymphoproliferation assay did result in the expansion of Rv2660 and Rv2659-specific T cells. The inclusion of a viability dye into long-term whole blood lymphoproliferation assays is vital for exclusion of dead cells. Dead cells bind non-specifically to antibodies making a clear delineation of positive and negative cell populations difficult.

Importantly, compared with the short-term assay, the long-term assay enabled the detection of soluble IFN-γ by ELISA, a classical screening tool for antigen recognition prior to further T cell characterisation. Unlike in the short-term assay, IL-2 was not detectable with the long-term assay, which may be due to

the consumption of IL-2 by antigen-specific proliferating T cells (Cheever et al., 1984). Since soluble IFN-γ measurement was proven to be the more reliable indication of antigen recognition, this outcome was chosen for the study.

During the optimisation period, we acquired cryopreserved PBMC samples from TB patients, which necessitated the optimisation of a PBMC–based long-term proliferation assay. Preliminary studies with a 6-day OG lymphoproliferation assay revealed that Rv2660- and Rv2659-specific T cell responses were measureable and consistently reliable with the small sample sizes that were use for optimisation. We finally selected the 6-day PBMC-based OG lymphoproliferation assay, which provides 2 independent readouts: (i) measurement of soluble IFN-γ for screening of T cell immune responsiveness to Rv2660 and Rv2659 (ii) flow cytometry characterisation of the Rv2660- and Rv2659-specific T cell responses.

2.4 Contributions

Lerisa Govender designed the experiments, conducted the laboratory and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B. Abel, Ms Jane Hughes and other members of SATVI laboratory.

Chapter 3: Higher human CD4 T cell response to novel *Mycobacterium tuberculosis* latency associated antigens Rv2660 and Rv2659 in latent infection compared with tuberculosis disease

Lerisa Govender[1], Brian Abel[1], E. Jane Hughes[1], Thomas J. Scriba[1], Benjamin M. N. Kagina[1], Marwou de Kock[1], Gerhard Walzl[2], Gillian Black[2], Ida Rosenkrands[3] Gregory D Hussey[1], Hassan Mahomed[1], Peter Andersen[3], Willem A. Hanekom[1*]

[1]South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine and School of Child and Adolescent Health, University of Cape Town, South Africa.

[2]Department of Biochemistry, University of Stellenbosch**,** Tygerberg, South Africa,

[3]Statens Serum Institut, Copenhagen, Denmark.

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3.1. Abstract

One third of the world's population is infected with *Mycobacterium tuberculosis* (*M.tb*). Only 10% of these persons will develop TB disease. A vaccine that would prevent progression to TB disease will have a dramatic impact on the worldwide TB burden. We propose that antigens of *M.tb* that are preferentially expressed during latent infection will be excellent candidates for post-exposure vaccination. We therefore assessed human T cell recognition of two antigens of *M.tb*, Rv2660 and Rv2659, expression of which have been shown to be associated with latency *in vitro*, and which show promise as post-infection vaccine candidates in animal models. In a pilot study IFN-γ responses to Rv2660 and Rv2659 were measured in the whole blood of 21 persons with latent tuberculosis infection (LTBI). Thereafter, PBMC from persons with LTBI and with tuberculosis (TB) disease were incubated with Rv2660 and Rv2659 for six days. Soluble IFN-γ levels in supernatants were quantified by ELISA, and $CD4^+$ and $CD8^+$ T cell proliferation, and cytokine producing capacity of proliferating cells, determined by flow cytometry. Rv2660 and Rv2659 induced IFN-γ production in a greater proportion of persons with LTBI, compared with patients with TB disease. Persons with LTBI also had increased viable $CD3^+$ lymphocytes and greater specific $CD4^+$ T cell proliferation and cytokine expression capacity, compared with patients with TB disease.

Persons with LTBI preferentially recognize Rv2659 and Rv2660, compared with patients with TB disease. These results suggest promise of these antigens for incorporation into post-exposure TB vaccines.

3.2. Introduction

The success of *M.tb* as a human pathogen may be ascribed to its ability to persist for long periods in asymptomatic people, a state known as latency (Andersen, 2007b). Healthy latently infected individuals have approximately 10% lifetime risk of developing active TB disease (Flynn and Chan, 2001). This risk increases to approximately 10% per year in HIV-infected persons not on antiretroviral therapy (Corbett et al., 2003). Approximately one third of the world's population is infected with *M.tb,* 9 million develop TB disease and 1.8 million die annually (Baumann et al., 2006; WHO Report., 2009). To control this pandemic, an effective vaccine is urgently needed; however, this requires extensive insight into the mechanisms underlying protective immunity against *M.tb.*

The low bacterial burden associated with a latent infection has proven to be a major obstacle in characterizing the mechanisms by which *M.tb* persists and reactivates in the host (Flynn and Chan, 2001). In latent granulomatous lesions, *M.tb* is successfully contained and has to adapt to a hypoxic and nutritionally compromised environment (Sherman et al., 2001). It has recently been hypothesized that this immune pressure drives *M.tb* into a different metabolic state, compared with actively replicating organisms in lesions that characterize early *M.tb* infection or active disease (Andersen, 2007b). This is supported by findings from *in vitro* studies which mimic the granuloma environment, where *M.tb* was shown to upregulate sets of genes that are distinct from those upregulated in actively replicating organisms; e.g., in nutrient deficient medium, a particular set of genes that were termed the "starvation stimulon" were upregulated (Betts et al., 2002). We focused on 2 proteins of the starvation stimulon, Rv2660 and Rv2659, and hypothesized that these antigens will be preferentially recognised by persons with latent *M.tb* infection (LTBI),, compared with persons with TB disease. Rv2660 and Rv2659 belong to the RD11 region encoded in *M.tb* (Gordon et al., 2001),

however the function of these proteins has not been determined (Betts et al., 2002).

The rationale for these studies is that the antigens might in the future be incorporated into novel post-exposure TB vaccines, which could protect infected individuals from developing TB disease (Andersen, 2007b).

3.3. Materials and Methods

3.3.1 Study participants

Participants were recruited from the Western Cape region of South Africa. Persons with latent TB infection (LTBI) were included if they had a positive QuantiFERON®-TB Gold In-Tube (QFT™) test, or a positive ELISPOT test for ESAT-6/CFP-10 (>17 spots/10^6 PBMC after background subtraction), or a positive Mantoux skin test (induration >10mm), for greater than 1 year at the time of recruitment. Any clinical signs or symptoms suggestive of TB disease, a history of TB treatment, HIV infection, any other acute or chronic medical conditions, receipt of immunosuppressive medication, or a haemoglobin level <9g/dL resulted in exclusion. Patients with TB disease were included if they had 2 positive sputum smears and/or a positive culture, with clinical features consistent with TB disease. Patients with extra-pulmonary TB disease were excluded, as were patients with exclusion criteria mentioned for LTBI above. Informed consent was obtained from all participants prior to study participation. The study was approved by the research ethics committees of the University of Cape Town and the University of Stellenbosch.

3.3.2 Whole blood incubation

Whole blood was collected in heparinised syringes. Blood was diluted in RPMI-1640 medium (Sigma) containing 1% L-glutamine (Sigma, final dilution 1:10), and incubated with an ESAT-6 peptide pool (17mers, overlapping by 10, final concentration 1μg/mL/peptide), CFP-10 peptide pool (17mers, overlapping by 10, final concentration 1μg/mL/peptide), TB10.4 protein (10μg/mL), Rv2660 peptide pool (20mers, overlapping by 10, final concentration 1μg/mL/peptide), or Rv2659 peptide pool (20mers, overlapping by 10, final concentration 1μg/mL/peptide), in 200μL volumes in 96-well U-bottom plates (Nunc, Cat No 163320), for 6 days at 37°C in 5% CO_2. All antigens were obtained from the Statens Serum Institut, Denmark. Blood incubated without antigen and blood incubated with phytohaemagglutinin (PHA, 5μg/mL) were used as negative and positive controls, respectively. After incubation, 150μL culture supernatant was

removed and stored at -80°C until further analysis.

3.3.3 Six-Day PBMC Oregon Green assay

Performed as described in chapter 2 (section 2.*1.5)* with the following inclusions: Duplicate wells (i.e. 400,000 PBMC per condition) containing cells were either stimulated with BCG (1331 Danish BCG) at an MOI of 0.01, PPD (0.5μg/mL), an ESAT-6/CFP10 peptide pool (2μg/mL/peptide); a combination of separate ESAT-6 and CFP10 peptide pools was used due to a limited number of PBMCs from TB patients, TB10.4 peptide pool (15mers, overlapping by 10, final concentration 1μg/mL/peptide), Rv2660 peptide pool (final concentration 1μg/mL/peptide), Rv2659 peptide pool (final concentration 1μg/mL/peptide), or SEB (0.05μg/mL, positive control, Sigma-Aldrich) or no antigen (negative control), for 6 days at 37°C with 5% CO_2.

3.3.4 Flow cytometric analysis of processed PBMC

Performed as described in chapter 2 (section *2.1.6)* with the following changes: PBMC were washed with PBS and stained with the following antibodies: anti-CD3 Qdot605 (clone UCHT1) anti-CD8 PerCPCy5.5 (SK-1), anti-IFN-γ AlexaFluor700 (B27), anti-IL2 APC (MQ1-17H12) and anti-TNF-a Cy7.PE (MAb11) (all from BD Biosciences) for 1 hour at 4°C. After washing, cells were acquired on a LSRII flow cytometer (BD Biosciences), configured with 3 lasers and 10 detectors, using the FACS Diva 6.1 software. Compensation settings were defined using anti-mouse kappa Comp Beads (BD Biosciences) stained with each fluorochrome–conjugated antibody. Because the cellular dyes Oregon Green and ViViD do not bind Comp Beads, compensation of these dyes was done using FITC and Pacific Blue conjugated antibody-bound Comp Beads, respectively. Data was analyzed using Flowjo 8.8.4 (Treestar). Frequencies of proliferating and cytokine-expressing CD4 or CD8 T cells were determined after exclusion of dead cells ($ViViD^{high}$ events). Since only a CD8 antibody was used, $CD4^+$ T cell expression was based on the absence of CD8 expression It is important to note that when detecting $CD4^+$ T cells based on the absence of CD8

expression, that BCG stimulation of PBMC or whole blood it is possible that responding gamma/delta (γ/δ) T cells can also be identified in the $CD8^-$ gate. If γ/ δ T cells are to be specifically excluded from analysis an additional anti-γ/δ T cell stain has to be incorporated. Boolean gating was applied to generate combinations of cytokine expressing $CD4^+$ and $CD8^+$ T cell subsets.

3.3.5 IFN-γ measurement in supernatants

Performed as described in chapter 2 (section *2.1.7).*

3.3.6 Data analysis

For soluble IFN-γ analysis, antigen-specific cytokine levels were determined by subtracting levels from unstimulated conditions. A positive IFN-γ response was defined as 31.2pg/mL (twice the limit of detection of 15.6pg/mL), after background subtraction.

For the flow cytometric assay, proliferation and cytokine expression levels from the unstimulated condition (restimulated with PMA and Ionomycin) were subtracted from levels obtained after antigen-specific stimulation. Individuals were excluded from analysis for samples with <100 viable $CD3^+$ T cells, or if the positive control yielded a negative result. Statistical analyses were calculated using GraphPad Prism v 5.0. The Mann-Whitney U test was used to assess differences between the two groups. The Fisher's exact test was used to assess differences between proportions of responders. P-values <0.05 were considered to be significant.

3.4. Results

3.4.1 Participant Characteristics

We examined immune recognition of Rv2660 and RV2659, measured by IFN-γ expression in whole blood in a pilot study on 21 adults with LTBI. The median age was 32 years (interquartile range: 28-40); 90% were female. All participants had been vaccinated with BCG at birth, and all were from a mixed ethnic background. Following the pilot study, PBMC proliferation and cytokine production were studied in 25 additional persons with LTBI, as well as 25 patients with TB disease. The median ages of the LTBI and TB diseased groups were 19 years (18.5-31.5) and 31 years (23.5-42), respectively (p=0.003); 64% of individuals with LTBI were female, compared with 24% in the TB group (p=0.044). Due to the difference in age and gender we compared the outcomes with respect to these co-variates. Only polyfunctional $CD4^+$ T cells, expressing IFN-γ, TNF-α and IL-2 were different in males and females. Participants excluded from analysis, with criteria for exclusion, are shown in Supplementary Figure 1. Twenty-eight % of TB patients were excluded due either culture contamination or <100 viable $CD3^+$ T cells; 8% of LTBI participants were excluded due to a negative SEB result.

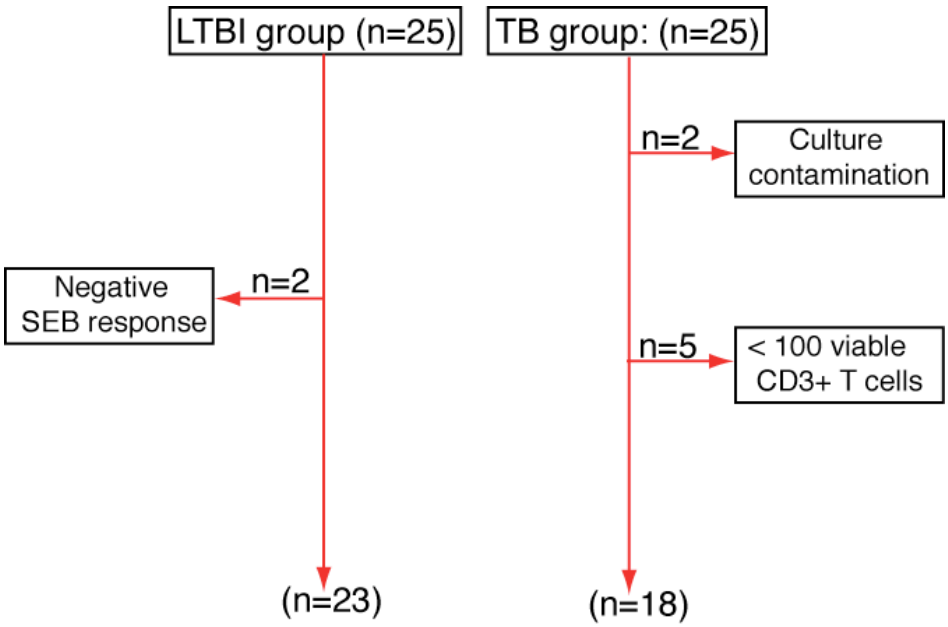

Figure 3.1. Participant exclusion during the study. Flow diagram indicating the number of individuals excluded from the study and criteria for exclusion.

3.4.2 Rv2660 and Rv2659 are recognized by T cells of persons with established latency

To assess whether Rv2660 and Rv2659 are recognised during established human latent *M.tb* infection, diluted whole blood was incubated with the antigens for 6 days, and IFN-γ measured in the supernatant. Rv2660 and Rv2659 induced detectable IFN-γ production in a high proportion of persons with LTBI (Figure 3.2). The immunodominant control antigen, TB10.4, present in both BCG and in *M.tb*, and the *M.tb*–specific antigens ESAT-6 and CFP-10, were recognized in substantially greater proportions of individuals than the two latency associated antigens (Figure 3.2). The positive control, SEB induced a response in all individuals (data not shown).

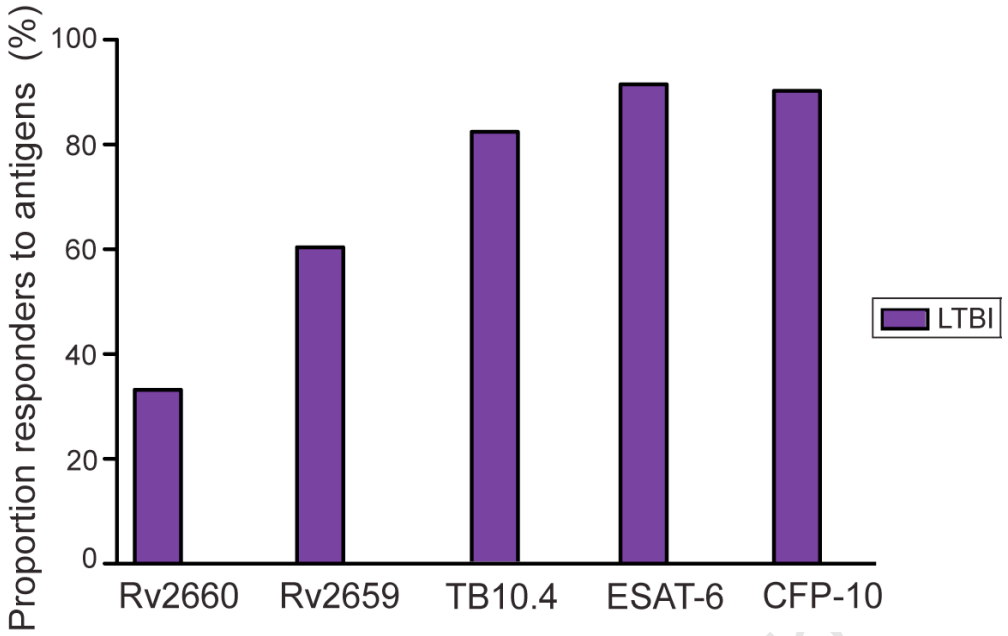

Figure 3.2. Recognition of novel *M.tb* antigens Rv2660 and Rv2659 in persons with LTBI. Whole blood from persons with LTBI was incubated for 6 days with mycobacterial antigens and IFN-γ levels in supernatants measured by ELISA. The proportion of persons with a positive response, defined as an IFN-γ level above 31.2pg/mL, following subtraction of the IFN-γ level from the unstimulated control, is shown. n = 21

To compare immune recognition of Rv2660 and Rv2659 during established latent infection and TB disease, we incubated PBMC with these antigens for 6 days. The optimum concentrations of Rv2660 and Rv2659 peptide pools were assessed for inducing proliferation of $CD4^+$ T cells. Two antigen peptide pool concentrations of 1μg/mL/peptide and 10μg/mL/peptide were compared. The peptide pool concentration of 1μg/mL was selected for both antigens because 1μg/mL induced higher frequencies of proliferating $CD4^+$ T cells in both cohorts (Figure 3.3A and B), with the exception of Rv2660 in the TB diseased group (Figure 3.3A).

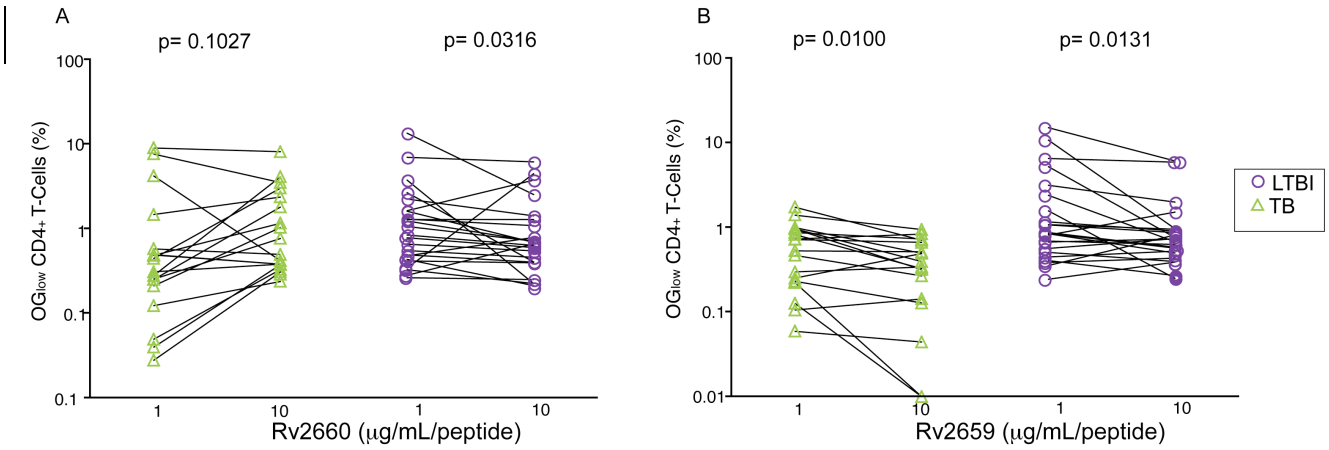

Figure 3.3. Comparison of Rv2660 and Rv2659 peptide pool concentrations. Comparison of Rv2660 (A) and Rv2659 (B) antigen concentrations in LTBI and TB diseased individuals. PBMC were stimulated with either 10μg/mL or 1μg/mL of (A) Rv2660 and (B) Rv2659, and the level of proliferation assessed by Oregon Green expression. The Wilcoxon signed rank test was used to calculate p-values of differences between the 2 groups. LTBI n = 23, TB n = 18

This was followed by measurement of IFN-γ in supernatants from PBMC of LTBI persons and TB diseased patients incubated with Rv2660 and Rv2659 for 6 days. A greater proportion of persons with LTBI recognised Rv2660 and Rv2659, compared with TB diseased patients (Figure 3.4A). A similar proportion of persons in the 2 study groups responded to BCG; however, more persons with LTBI responded to ESAT-6/CFP-10, compared with TB diseased patients. Further, median levels of IFN-γ in response to all antigens were higher in the LTBI group, compared with the TB diseased group (Figure 3.4B).

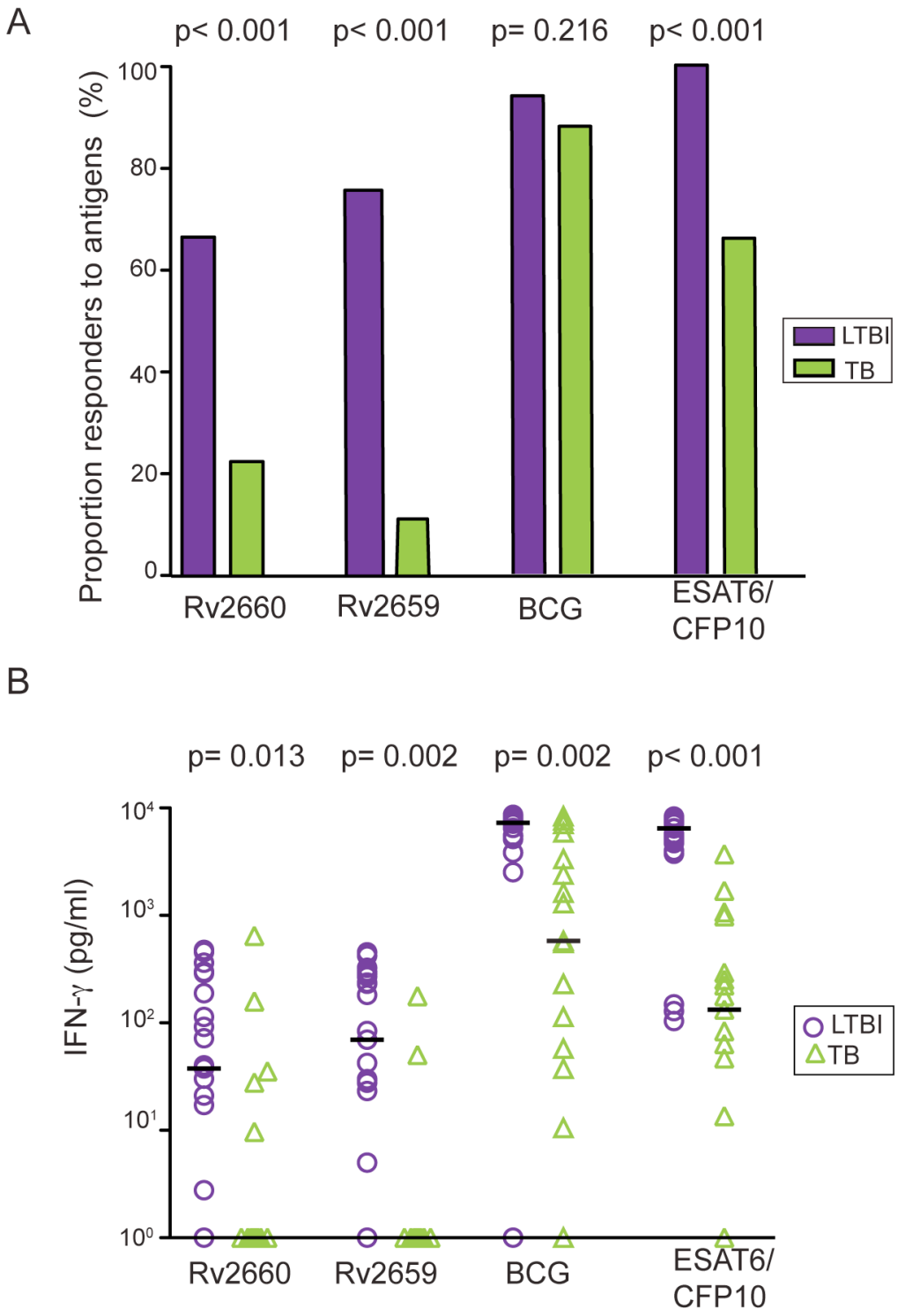

Figure 3.4. Recognition of novel TB antigens in persons with LTBI and with TB disease. (A) PBMC derived from persons with LTBI or TB disease were incubated with the indicated antigens for 6 days, and IFN-γ levels measured in supernatants by ELISA. Proportion of persons with a detectable response, as an IFN-γ level above 31.2pg/mL, following subtraction of the IFN-γ level from the unstimulated control, is shown. (B) IFN-γ response (pg/ml) to the antigens indicated. The medians are represented by the black horizontal bars. A positive response was defined as an IFN-γ level above 15.6pg/mL after background subtraction. The Mann-Whitney U test was used to assess differences between the 2 groups. LTBI n = 23, TB n = 18

3.4.3 Decreased viability of CD3⁺ T cells during TB disease

We hypothesized that lower Rv2660 and Rv2659 recognition during TB disease is due to lower survival capacity of T cells derived from TB diseased patients compared with LTBI persons. After 6 days of incubation of PBMC in conditions as aforementioned, cell viability was assessed with flow cytometry following staining with ViViD (Figure 3.5A). The LTBI group had significantly higher frequencies of viable CD3⁺ T cells (96.60%) compared to TB patients (65.95%), for all conditions tested (Figure 3.5B). The median number of viable CD3⁺ T cells was 15,771 (IQR, 4,912-99,654).

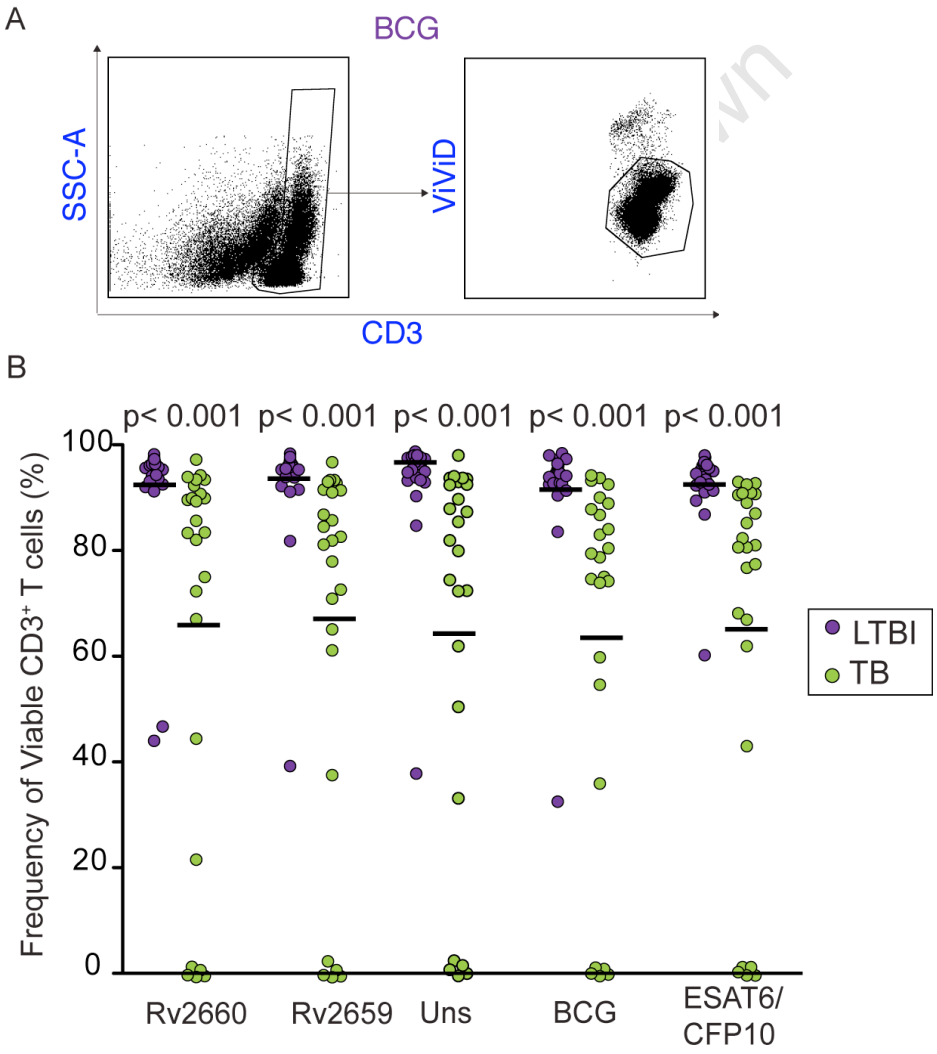

Figure 3.5. Comparison of CD3⁺ T cell Viability. Comparison of viable CD3⁺ T cells in PBMC of LTBI and TB individuals stimulated with BCG and *M.tb*-specific antigens for 6 days. (A) Viable T cells were selected by gating on CD3⁺ T cells against ViViD. (B) Frequency of viable CD3⁺ T cells detected by staining with ViViD. Viable cells were selected by gating on CD3⁺ T cells that stained ViViD^{low}. The medians are represented by the black horizontal bar. The Mann-Whitney U test was used to assess differences between the 2 groups. LTBI n = 23, TB n = 18

3.4.4 Increased frequency of proliferating Rv2660- and Rv2659-specific CD4⁺ T cells during established latency

We further characterized viable CD4⁺ T cell responses to Rv2660 and Rv2659 during established latent infection and TB disease by assessing viable antigen-specific proliferation with a dye dilution assay, and the cytokine producing capacity of proliferating cells (Figure 3.6A-B and C, respectively).

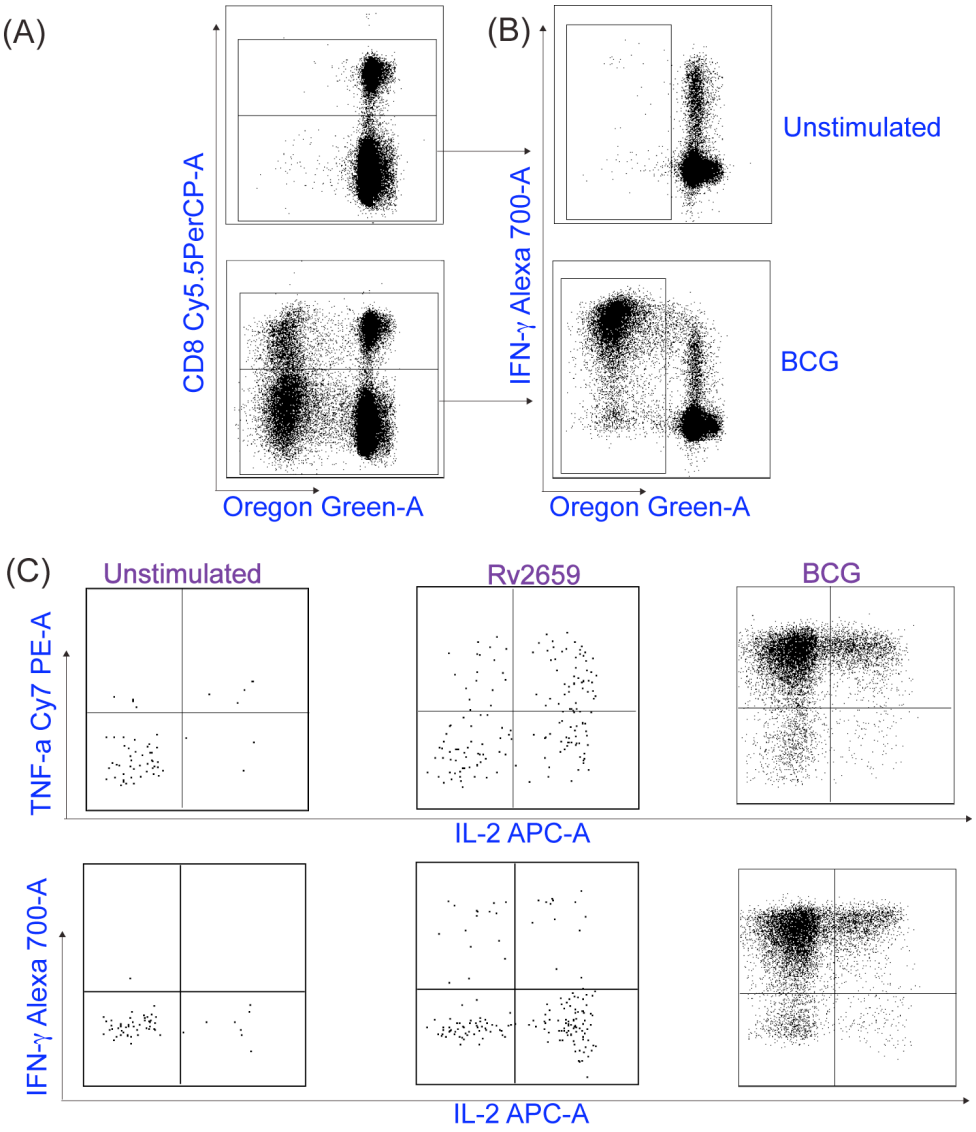

Figure 3.6. Gating strategy to analyze the cytokine expression profiles of proliferating CD4⁺ and CD8⁺ T cells. PBMC were stained with Oregon Green and stimulated with mycobacterial antigens, SEB, or were left unstimulated for 6 days, and analyzed by flow cytometry. Doublet cells were excluded by gating on forward scatter-height (FSC-H) against forward scatter-area (FSC-A) (not shown), and viable T cells were then selected by gating on $CD3^+$ against ViViD (Figure 3.5A). (A) $CD3^+$ T cells were further differentiated into $CD8^-$ and $CD8^+$ T cell subsets ($CD3^+CD8^-$ = $CD4^+$ T cells, $CD3^+CD8^+$ = $CD8^+$ T cells). (B) Proliferating (OG^{low}) $CD4^+$ (indicated by arrow) and $CD8^+$ T cell subsets were selected by gating on OG^{low} T cells. Cytokine expression patterns were then analyzed in proliferating T cells. (C) Representative dot plots of cytokine co-expression patterns in unstimulated or antigen-specific proliferating $CD4^+$ T cells. LTBI n = 23, TB n = 18.

Frequencies of proliferating antigen-specific CD4$^+$ T cells were higher in LTBI, compared with TB disease, for all antigens tested but the difference was clearly more pronounced for BCG and ESAT6/CFP10 (Figures 3.7A-D, left panels). The positive control, SEB induced a response in all individuals (data not shown).

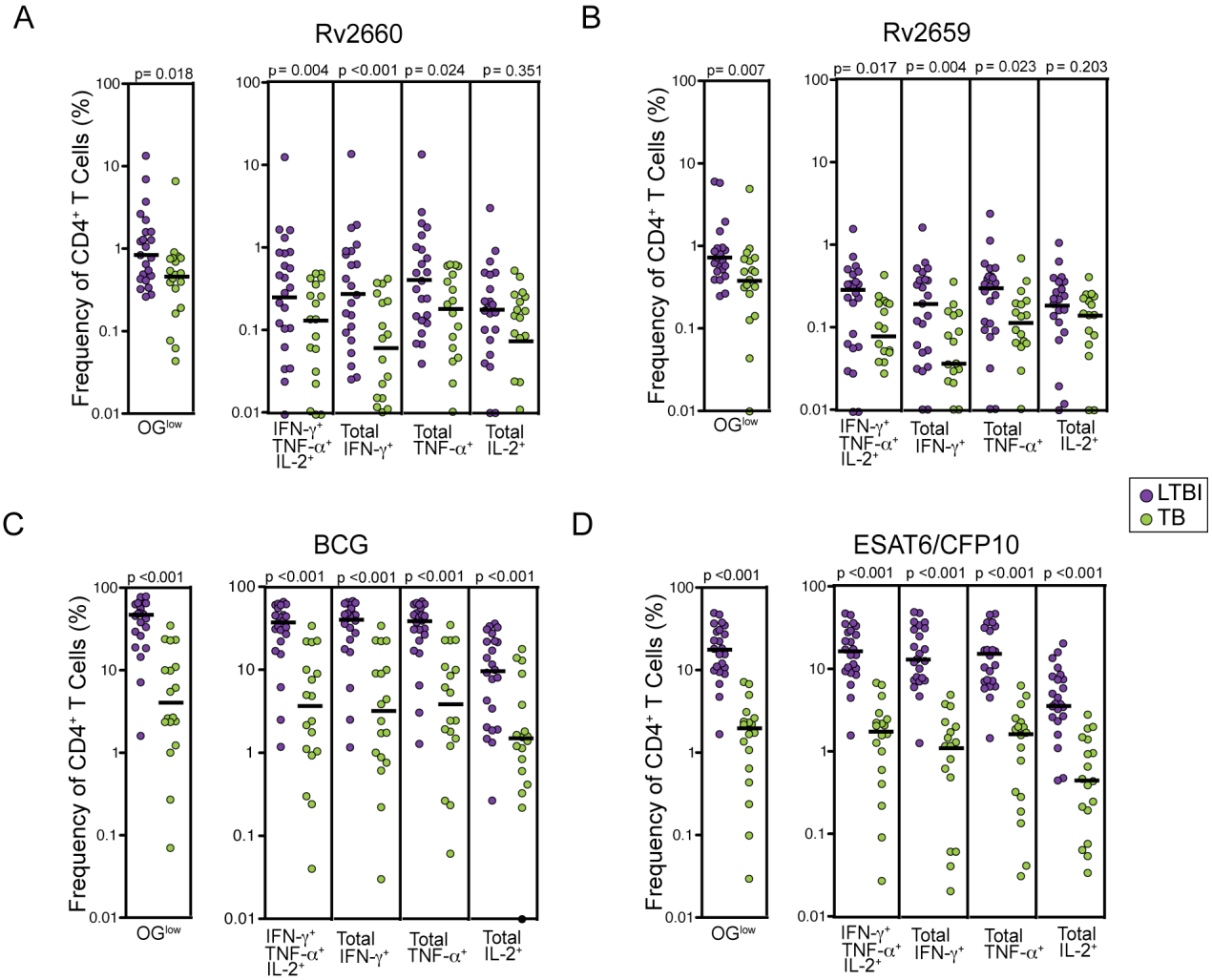

Figure 3.7. Comparison of CD4$^+$ T cell proliferation and intracellular cytokine expression in individuals with LTBI and TB disease. CD4$^+$ T cell antigen-specific proliferation upon incubation with Rv2660 (A), Rv2659 (B), BCG (C), and ESAT-6/ CFP-10 (D), detected by gating on Oregon Green^{low} CD4$^+$ T cells. Frequencies of CD4$^+$ T cell cytokine expression profiles; IFN-γ^+TNF-α^+IL2$^+$, total IFN-γ^+, total TNF-α^+ and total IL-2$^+$, of proliferating cells following incubation of PBMC with antigens (A-D, right panels), detected by intracellular cytokine staining. The medians are represented by the black horizontal bars. The Mann-Whitney U test was used to assess differences between the 2 groups. LTBI n = 23, TB n = 18

The cytokine expression profile of proliferating CD4$^+$ T cells was assessed by adding PMA and ionomycin, for the last 5 hours of incubation. Upon

stimulation with Rv2660 and Rv2659 antigens, frequencies of proliferating $CD4^+$ T cells were higher in the LTBI group for all cytokine-producing subsets, except for the total IL-2^+ population (Figures 3.7A-B, right panels, respectively). In addition, Rv2660 and Rv2659 induced more polyfunctional (co-expression of IFN-γ^+, TNF-α^+ and IL-2^+ in the same cell) cytokine expression in persons with LTBI, compared with TB patients. In response to BCG- and to ESAT-6/CFP-10, specific (proliferating) $CD4^+$ T cells were also more likely to be polyfunctional in persons with LTBI, compared with patients with TB disease (Figure 3.7C-D); total IFN-γ^+, total TNF-α^+ and total IL-2 were also higher in persons with latent infection (Figures 3.7C-D, right panels).

3.4.5 No difference in the proliferation and cytokine expression capacity of Rv2660- and Rv2659-specific $CD8^+$ T cells between the 2 groups

$CD8^+$ T cell proliferation and cytokine expression in response to Rv2660 and Rv2659 was very low, and no difference was demonstrated between the 2 groups (Figures 3.8A-B). However, persons with LTBI had greater frequencies of $CD8^+$ T cells that proliferated in response to ESAT-6/CFP10 and BCG, compared with frequencies in patients with TB disease; the proliferating cells were also more likely to be polyfunctional in persons with LTBI (Figures 3.8C-D).

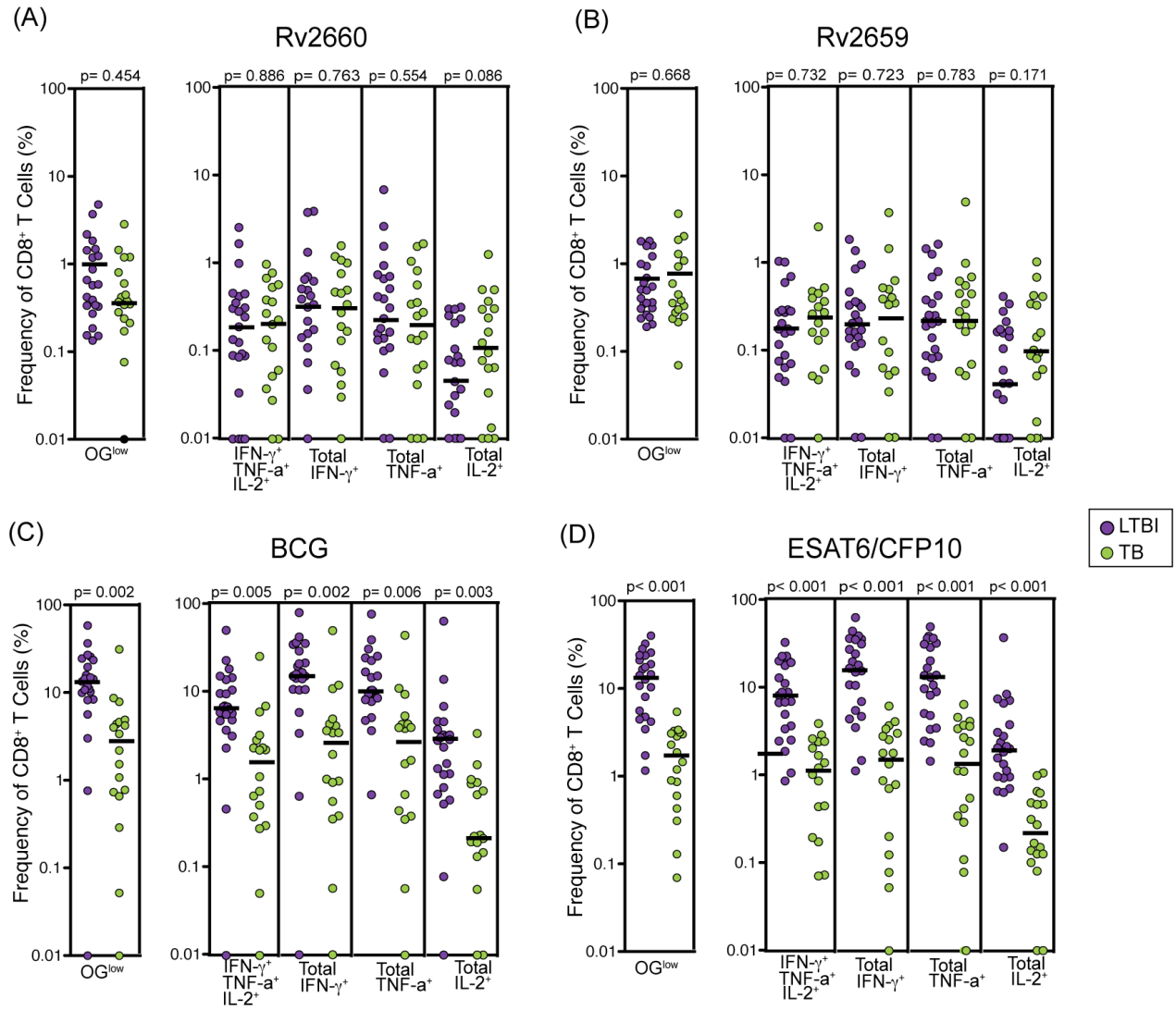

Figure 3.8. Comparison of CD8$^+$ T cell proliferation and intracellular cytokine expression in individuals with LTBI and TB disease. CD8$^+$ T cell antigen-specific proliferation upon stimulation with Rv2660 (A), Rv2659 (B), BCG (C) and ESAT-6/ CFP-10 (D), detected by gating on Oregon Green^{low} CD8$^+$ T cells. Frequencies of CD8$^+$ T cell cytokine expression profiles; IFN-γ^+TNF-α^+IL2$^+$, Total IFN-γ^+, Total TNF-α^+ and Total IL-2$^+$, of proliferating cells following incubation of PBMC with antigens (A-D), and detected by intracellular cytokine staining. The medians are represented by the black horizontal bars. The Mann-Whitney U test was used to assess between the 2 groups. LTBI n = 23, TB n = 18

3.4. Discussion

Mathematical modelling has indicated that an effective post-infection vaccine that targets latently infected individuals would have a dramatic impact on the TB epidemic (Abu-Raddad et al., 2009). Our study suggests that Rv2660 and Rv2659 are good antigenic candidates for inclusion in such a vaccine; we show that T cells of persons with LTBI preferentially recognise these two latency associated antigens, compared with T cells from patients with TB disease. This is the first clinical report of human immune recognition of the starvation stimulon gene products, Rv2660 and Rv2659.

Interestingly, the same pattern of preferential recognition in LTBI, compared with TB disease, was observed for the crude mycobacterial antigens BCG, PPD, and the immunodominant early antigens ESAT-6/ CFP-10 and TB10.4 (data for PPD and TB10.4 not shown). In agreement with our findings, Schuck *et al* recently reported higher T cell responses upon stimulation of PBMC with PPD and ESAT-6/ CFP10, and with other latency antigens such as Rv1733 and Rv0140 in individuals with LTBI, compared with TB patients (Schuck et al., 2009). This decreased T cell response in peripheral blood may result from migration of specific T cells to the lung during active disease (Tully et al., 2005) or may relate to a preferential expression of Rv2660 and Rv2659 during latency. Alternatively, aberrant immune regulation during disease, mediated by regulatory T cells (Schuck et al., 2009), anti-inflammatory cytokines such as IL-10 and TGF-β (Fiorenza et al., 2005; Guyot-Revol et al., 2006), or T_H2 cytokines like IL-4 and IL-13 may be responsible for the differences noted (Ordway et al., 2005). Our observation that T cells from patients with TB disease were strikingly less likely to survive in a 6-day culture, compared with T cells from persons with LTBI, suggest that T cell exhaustion may also be responsible for lower responses in the diseased group. In chronic infections such as HIV and CMV, a persistently high antigenic load drives specific T cell exhaustion and dysfunction (Barber et al., 2006; Day et al., 2006). These cells upregulate markers like PD-1, and are more prone to apoptosis (van Grevenynghe et al., 2008). We postulate that this also occurs during active TB

disease. We have recently shown that PD-1 expression is increased on *M.tb*-specific $CD4^+$ T cells in TB diseased patients, compared to persons with LTBI (C. Day, unpublished observations). The aforementioned differences in responses between LTBI and TB diseased individuals are depicted in a review by Kaufmann *et al,* and might partly explain the disparate clinical outcome (Figure 3.9) (Kaufmann and McMichael, 2005; Kaufmann, 2010).

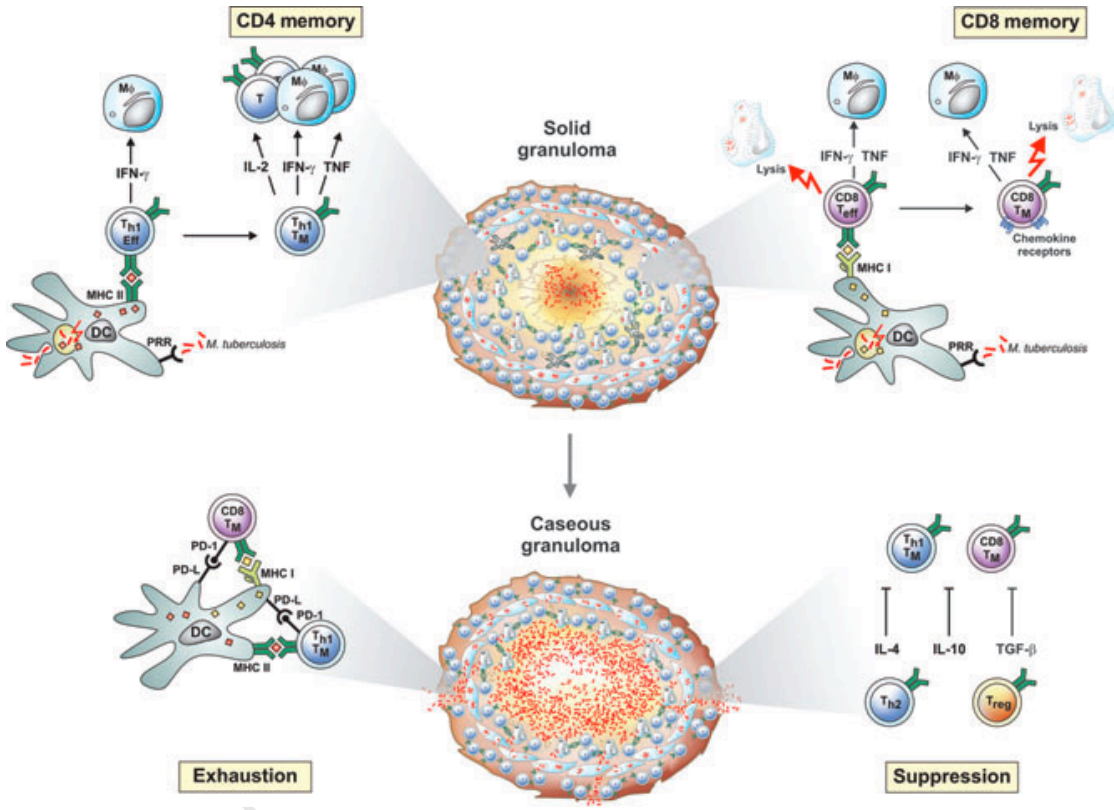

Figure 3.9. T cells in the context of granuloma formation and maintenance. Upper part: Immune responses that successfully contain *M.tb* within a granuloma. Lower part: Breakdown of protective immunity due to suppression or exhaustion leading to the development of caseous granuloma followed by bacterial dissemination. (From Kaufmann *et al* 2010, J. Internal Medicine)

Recent advances in imaging have led to the observation that granulomatous lesions in persons with LTBI reflect a spectrum of differential ability to support or suppress the persistence of viable bacteria (Barry et al., 2009). This spectrum is supported by our data of similar preferential recognition of latency-associated and immunodominant antigens in persons with LTBI, compared with active TB. A post-exposure vaccine would thus ideally contain both immunodominant and latency-associated antigens, respectively, to target

replicating or persisting bacteria in progressing lesions and dormant bacteria in successful lesions.

LTBI individuals had elevated frequencies of T_H1 cytokine expression among proliferating $CD4^+$ T cells, compared with those in TB patients. The pattern of cytokine production also differed: specific T cells from persons with LTBI were more likely to be "polyfunctional", i.e., able to co-express IFN-γ, TNF-α and IL-2. This characteristic has been proposed to be associated with long-term memory and efficient protection against intracellular infections like *M.tb* (Makedonas and Betts, 2006; Forbes et al., 2008; Seder et al., 2008; Lindenstrom et al., 2009). We therefore propose that robust polyfunctional responses to latency and immunodominant antigens may prevent LTBI individuals from progressing to TB disease. Therefore, because latency associated antigens would not be expressed in early stages of infection, T cells directed to these antigens may not be subjected to regulation by the established effector and/or regulatory T cells primed in the earlier stages of infection.

$CD8^+$ T cell responses to immunodominant antigens could be detected, and were higher in LTBI compared with TB disease; however, $CD8^+$ T cell responses to Rv2659 and Rv2660 were very low, which may be the reason why no difference could be demonstrated between the participant groups. We cannot exclude that differences in antigen presentation between $CD4^+$ and $CD8^+$ T cells could have affected these results.

A limitation of our study was the different age and gender ratios in the LTBI and TB groups. Ideally, participants in such groups should be matched for these co-variates. However, given the fact that only polyfunctional $CD4^+$ T cells and no other outcomes were different in males and females, the unequal gender ratio did not markedly confound our results.

The prophylactic TB subunit vaccines currently under development are almost exclusively based on early or immunodominant antigens secreted by replicating *M.tb*, which are recognized in the first stage of infection, and are designed as BCG boosters to prevent primary TB disease (Andersen, 2007a). The addition of latency associated antigens to well-established prophylactic vaccines (i.e., the vaccine candidates under clinical testing today) have been suggested as the basis for a future generation of multi-stage TB vaccines with activity against all stages of infection (Andersen, 2007a). Our finding of immune recognition of these novel antigens in persons with LTBI has contributed to the development of a new subunit vaccine, H56, by the Statens Serum Institut. This vaccine is based upon the H1 subunit vaccine currently in clinical trials, which contains Ag85B and ESAT-6 (van Dissel et al., 2010) and the Rv2660 antigen, and will be the first to enter clinical trials as a post-infection vaccine candidate. We hypothesize that inducing robust T cell responses to latency antigens may enhance immunological control of latent TB, and prevent reactivation. Much more research is needed to determine whether candidate latency-, starvation-, or resuscitation-associated antigens other than Rv2659 or Rv2660 would be needed for inclusion in an optimal post-infection vaccine.

Ultimately, a combined strategy that includes primary vaccination with a live attenuated vaccine followed by a multi-stage subunit boost vaccine; may have maximum impact on the TB epidemic.

3.5 Contributions

Lerisa Govender designed the experiments, conducted the laboratory and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B. Abel, Jane Hughes and other members of SATVI laboratory. Gerhard Walz and others from Stellenbosch University provided the PBMC from the TB diseased group.

Chapter 4: Novel Application of Ki67 to Quantify Antigen-Specific *in vitro* Lymphoproliferation

Andreia Soares*, Lerisa Govender*, Jane Hughes, Wendy Mavakla, Marwou de Kock, Charlene Barnard, Bernadette Pienaar, Esme Janse van Rensburg, Gail Jacobs, Gloria Khomba, Lynnette Stone, Brian Abel, Thomas J. Scriba, Willem A. Hanekom.

South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine and School of Child and Adolescent Health, University of Cape Town, Cape Town, South Africa.

*Andreia Soares and Lerisa Govender contributed equally to this manuscript.

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4.1. Abstract

Antigen-specific proliferation is a critical function of memory T cells that is often utilized to measure vaccine immunogenicity and T cell function. We proposed that measurement of intracellular expression of the nuclear protein, Ki67, could reliably assess specific T cell proliferation *in vitro*.

Ki67 was expressed in $CD4^+$ and $CD8^+$ T cells that had undergone *in vitro* proliferation after 6-day culture of human whole blood or PBMC with antigens. T cells cultured with no antigen for 6 days did not express Ki67. When compared to current flow cytometry based proliferation assays, Ki67 detected proliferating cells with greater sensitivity than BrdU incorporation, whereas its sensitivity was similar to dye dilution of Oregon Green (OG), a CFSE derivative. Overall, the magnitude and cytokine expression profile of proliferating T cells detected by Ki67 expression correlated strongly with T cells detected with BrdU or OG. The intra-assay variability of Ki67 proliferation was 2-3% for $CD4^+$ T cells, and 10-16% for $CD8^+$ T cells. Finally, we demonstrate that the Ki67 assay detects tetanus toxoid-specific $CD4^+$ T cell proliferation after infant vaccination with tetanus toxoid (TT).

Overall our data suggest that intracellular Ki67 expression provides a specific, quantitative and reproducible measure of antigen-specific T cell proliferation *in vitro*.

4.2. Introduction

Proliferation and clonal expansion of antigen-specific T cells are critical functions for mediating protective immunity and immunological memory (Rosenberg et al., 1997; Combadiere et al., 2004). Previously, the most widely used method for detection of antigen-specific T cell proliferation has involved incorporation of ^{3}H-thymidine into DNA of dividing cells (Payan et al., 1983; Marchant et al., 1999). This technique has largely been replaced by flow cytometric assays of proliferation. Examples include fluorescent dye dilution assays, using CFSE or its derivative, Oregon Green (OG) (Magg and Albert, 2007; Wallace et al., 2008; MacMillan et al., 2009), and assays that detect the DNA intercalating agent, 5-bromo-2'-deoxyuridine (BrdU), detected by fluorochrome-conjugated antibody staining (Dolbeare et al., 1983; Houck and Loken, 1985; Rosato et al., 2001). The advantages of these assays are that they allow co-staining with other markers, enabling delineation of cellular sub-populations according to phenotype (Precopio et al., 2007) and functional characteristics, such as cytokine production (Lyons, 2000; Bachmann et al., 2005; Davids et al., 2006).

Ki67 is a nuclear protein that plays a role in the regulation of cell division. This marker has been used extensively in cancer biology to indicate tumour cell proliferation (Gerdes, 1990; Scholzen and Gerdes, 2000). The protein is expressed during all active phases of cell division, but is absent in quiescent cells and during DNA repair (Gerdes et al., 1984). Intracellular Ki67 expression directly *ex vivo*, or after *in vitro* cell culture, has been used to measure specific T cell responses induced by vaccination (Stubbe et al., 2006; Cellerai et al., 2007; Miller et al., 2008), or turnover of these cells in individuals with chronic viral infections, such as HIV infection (Sachsenberg et al., 1998; Doisne et al., 2004).

In this study, we show that Ki67 expression in T cells is a specific and quantitative indicator of proliferation, and that results are comparable to those when proliferation is measured by other methods. We also show that measurement of Ki67 may be applied to longitudinal monitoring of vaccine-

specific T cell responses. Overall, the Ki67 assay offers a reliable, versatile and simple method for detection of antigen-specific T cell proliferation.

4.3. Materials and methods

4.3.1 Study participants

Healthy adult donors were recruited at the Institute of Infectious Disease and Molecular Medicine, University of Cape Town. Healthy, 18-month old toddlers were recruited at the South African Tuberculosis Vaccine Initiative clinic sites in the Western Cape, South Africa, before, and 11-13 days after their routine 18-month vaccination against tetanus toxoid (TT). Enrolled toddlers had received all routine childhood vaccinations as set out by the WHO Expanded Programme on Immunisation. Heparinised venous blood from adults and toddlers was collected into BD Vacutainer CPT tubes (BD Biosciences) and immediately processed as outlined below. Participation of all participants was in accordance with the Declaration of Helsinki, the US Department of Health and Human Services guidelines, and good clinical practice guidelines. This included protocol approval by the Research Ethics Committee of the University of Cape Town, and written informed consent by all adults or parents of the toddlers.

4.3.2 Six-day Whole blood BrdU incorporation assay

Performed as described in chapter 2 (section 2.1.3) with the following changes: Antigens were used at the following final concentrations: 1 x 10^5cfu/mL Danish BCG (Danish strain 1331; Statens Serum Institut), 1μg/mL TB10.4 protein (kindly provided by Tom Ottenhoff, Leiden University, Leiden, Netherlands), 2μg/mL *M. tuberculosis* purified protein derivative (PPD, Statens Serum Institut) and 0.16IU tetanus toxoid (Tetavax, Sanofi Pasteur). On day 6, cells were harvested with 2mM EDTA (Sigma-Aldrich) and red blood cells lysed. White cells were stained with a viability dye (ViViD, LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen), fixed in BD FACS Lysing Solution (BD Biosciences) according to the manufacturer's instructions and cryopreserved until analysis.

4.3.3 PBMC isolation and the OG assay

Performed as described in chapter 2 (section *2.1.5*) with the following changes: Freshly isolated cells were either incubated with medium or stimulated with 1 x 10^5cfu/mL Danish BCG, 0.5μg/mL PPD, 1μg/mL TB10.4 protein or 0.05μg/mL *staphylococcal enterotoxin B* (SEB, positive control, Sigma-Aldrich), for 6 days at 37°C with 5% CO_2.

4.3.4 Antibodies and flow cytometry

Fixed, cryopreserved cells were thawed, washed with PBS and permeablised with Perm/Wash Solution for 10 minutes. The following monoclonal antibodies were used for phenotypic and/or intracellular cytokine staining: CD3-QDot 605 (UCHT1), CD4-PerCP (SK3), CD8-PerCPCy5.5 (SK1), Ki67-PE (B56), IFN-γ-Alexa Fluor 700 (B27), TNF-α-PECy7 (MAb11), IL-2-APC (MQ1-17H12), anti-BrdU-FITC (B44). All antibodies were from BD Biosciences except for CD3-QDot 605, which was from Invitrogen. Samples were acquired on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Boolean gating was applied to generate combinations of cytokine expressing $CD4^+$ and $CD8^+$ T cell subsets.

4.3.5 Data analysis

Data were analysed with FlowJo software v.8.8.6 (Treestar Inc.), Pestle v 1.6.2 and Spice v 4.3.2 software. Statistical analyses were calculated using GraphPad Prism v 5.0.

4.4. Results

4.4.1 Ki67 is a specific marker of in vitro lymphoproliferation

Ki67 is expressed by all cells undergoing cycling (Lopez et al., 1991; Scholzen and Gerdes, 2000). Since a subset of peripheral blood lymphocytes undergoes homeostatic proliferation *in vivo*, we investigated the kinetics of Ki67 expression by cultured T cells over 6 days. Whole blood was either cultured in the absence of antigen, or in the presence of purified protein derivative (PPD) or the co-stimulatory antibodies, anti-CD3 and anti-CD28 (αCD3/αCD28), and expression of Ki67 quantified each day. Ki67 expression by unstimulated $CD4^+$ T cells was low (median, 0.62%) on day 1 and, by day 6 had decreased to less than 0.1% of $CD4^+$ T cells (median, 0.08% on day 6, Figure 4.1A). Upon PPD stimulation, specific Ki67 expression, above levels in unstimulated cells, was detected between days 2 and 4, and this expression peaked on day 6 (Figure 4.1A and B). Markedly increased expression of Ki67 was already seen after 1 day upon polyclonal T cell stimulation with αCD3/αCD28. Ki67 expression peaked on day 3 after which the frequency of $Ki67^+$ $CD4^+$ T cells gradually decreased (Figure 4.1A and C).

Next, we quantified antigen-specific $CD4^+$ T cell proliferation by Ki67 detection in whole blood from 15 healthy donors after 6-day culture with no antigen, or with PPD. All donors had undetectable or very low frequencies (median, 0.07%) of $Ki67^+$ $CD4^+$ T cells in unstimulated blood. Upon PPD stimulation, however, frequencies of $Ki67^+$ $CD4^+$ T cells were readily detected in all donors at a median of 46.1% (Figure 4.1D).

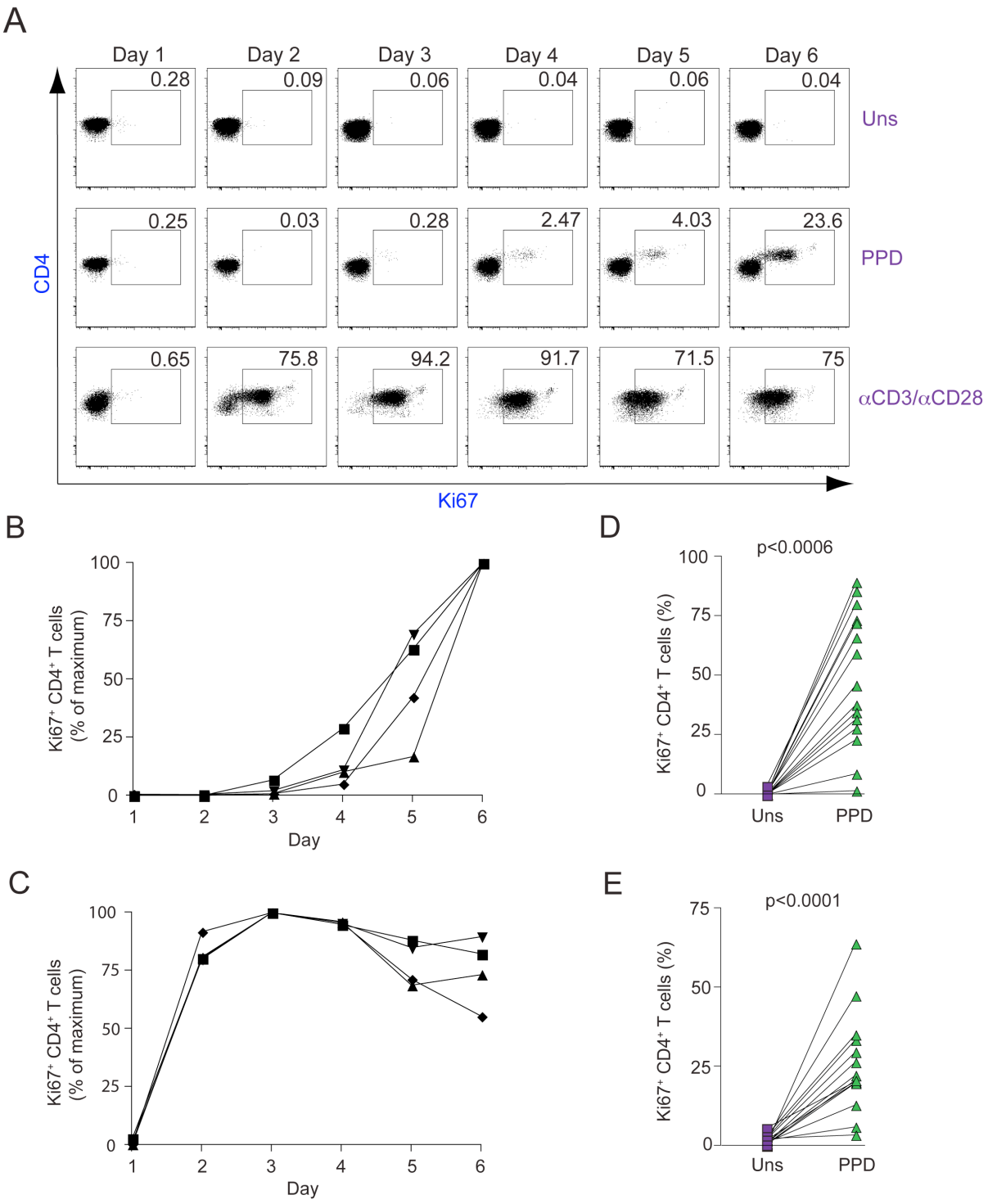


Figure 4.1. Ki67 is a specific marker of *in vitro* lymphoproliferation. Whole blood from healthy donors was incubated with the indicated antigens and Ki67 expression quantified on a daily basis over 6 days. (A) Representative example showing the frequencies of Ki67 expression by $CD4^+$ T cells after incubation of whole blood with medium only (Uns), PPD or αCD3/αCD28 over 6 days. Dotplots were gated on live, $CD3^+$ $CD4^+$ lymphocytes. $Ki67^+$ $CD4^+$ T cell frequencies after PPD stimulation (B) or αCD3/αCD28 stimulation (C) in 4 donors. Data are expressed as a percentage of the maximum response. The frequency of $Ki67^+$ $CD4^+$ T cells is indicated in each plot. (D) Frequencies of Ki67 expressing $CD4^+$ T cells in whole blood from 15 donors after 6-day culture with medium only (Uns) or PPD. (E) Frequencies of $Ki67^+$ $CD4^+$ T cells in PBMC from 14 donors. Differences were calculated using the Wilcoxon matched pairs test.

We also determined Ki67-based measurement of antigen-specific T cell proliferation in PBMC. Again, Ki67 expression identified *in vitro* $CD4^+$ T cell proliferation; frequencies of $Ki67^+$ cells after PPD stimulation exceeded those in unstimulated PBMC in all donors, at a median of 21.7% (Figure 4.1E).

These data suggest that in long-term PBMC or whole blood culture with antigen, Ki67 expression is upregulated in $CD4^+$ T cells undergoing *in vitro* proliferation.

4.4.2 Comparison of Ki67 expression with BrdU and Oregon Green assays

Next, we compared results obtained from a Ki67-based proliferation assay with those obtained using more traditional proliferation assays. We compared proliferation in whole blood measured by Ki67 expression with that in whole blood measured by BrdU incorporation, and in PBMC measured by Ki67 expression and dye dilution of OG (Figures. 4.2 and 4.3).

BrdU is incorporated into cells undergoing DNA synthesis, and is typically added during the last 2 to 24 hours of a proliferation assay; in this study we always added BrdU for the last 5 hours of the 6-day culture. Compared with the frequency of $BrdU^+$ cells the frequency of $Ki67^+$ $CD4^+$ T cells was higher after whole blood stimulation with PPD or TB10.4 protein (Figure. 4.2A-C). Importantly, all $BrdU^+$ cells co-expressed Ki67.

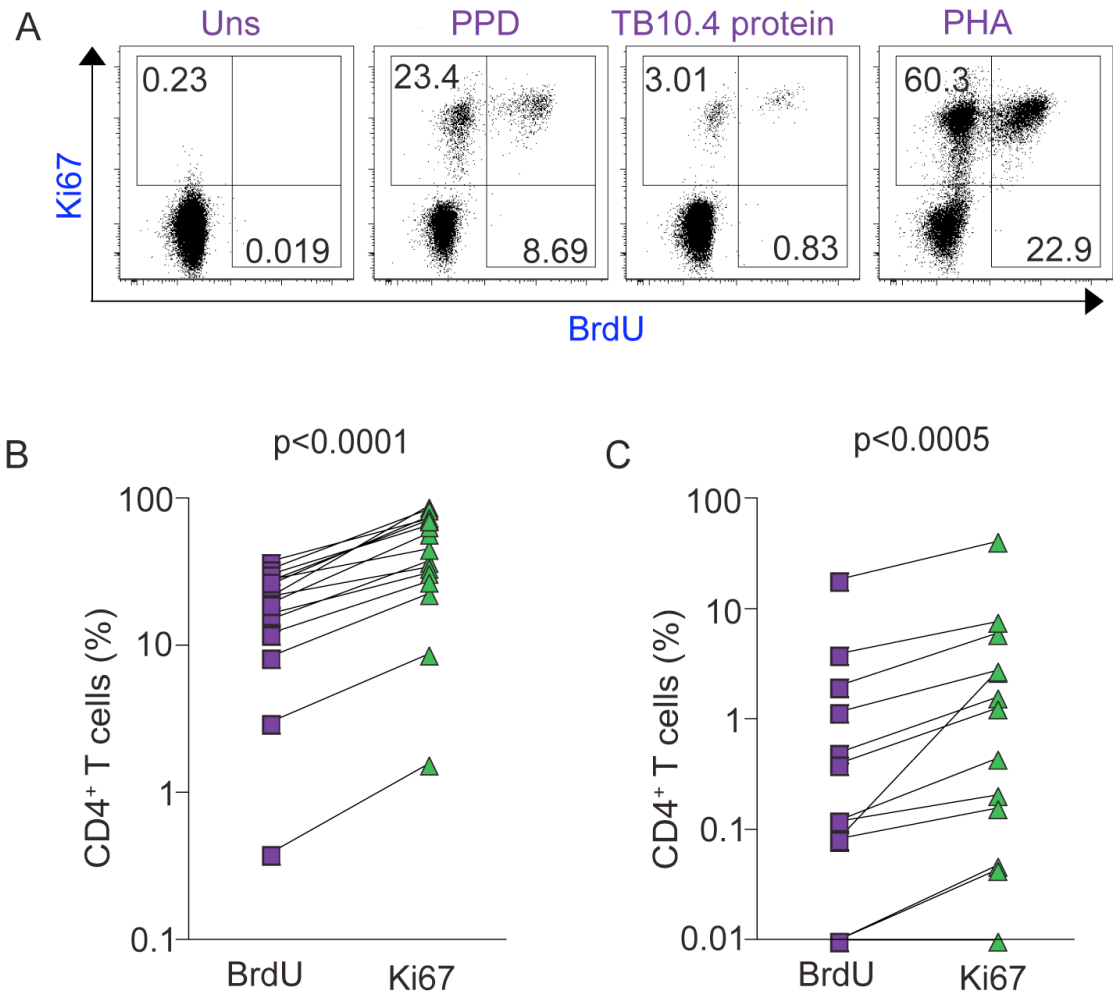


Figure 4.2. Comparison of the Ki67 proliferation assay with the BrdU proliferation assay. (A) Representative dotplots showing Ki67 versus BrdU expression by $CD4^+$ T cells in whole blood. Dotplots are gated on live, $CD3^+$ $CD8^-$ lymphocytes. Frequencies of (B) PPD and (C) TB10.4 protein specific $CD4^+$ T cell proliferation as detected by Ki67 expression (top left) or BrdU incorporation (bottom right) (n=15). $CD4^+$ T cells are defined as $CD3^+CD8^-$ T cells (see methods: Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

The OG assay requires uniform labelling of cells prior to long-term culture. In contrast to results from the BrdU assay, the OG and Ki67 assays yielded remarkably similar frequencies of proliferating, specific T cells; Ki67+ and OG^{low} CD4+ T cell frequencies were not different in PPD or TB10.4-stimulated PBMC (Figure. 4.3A-C).

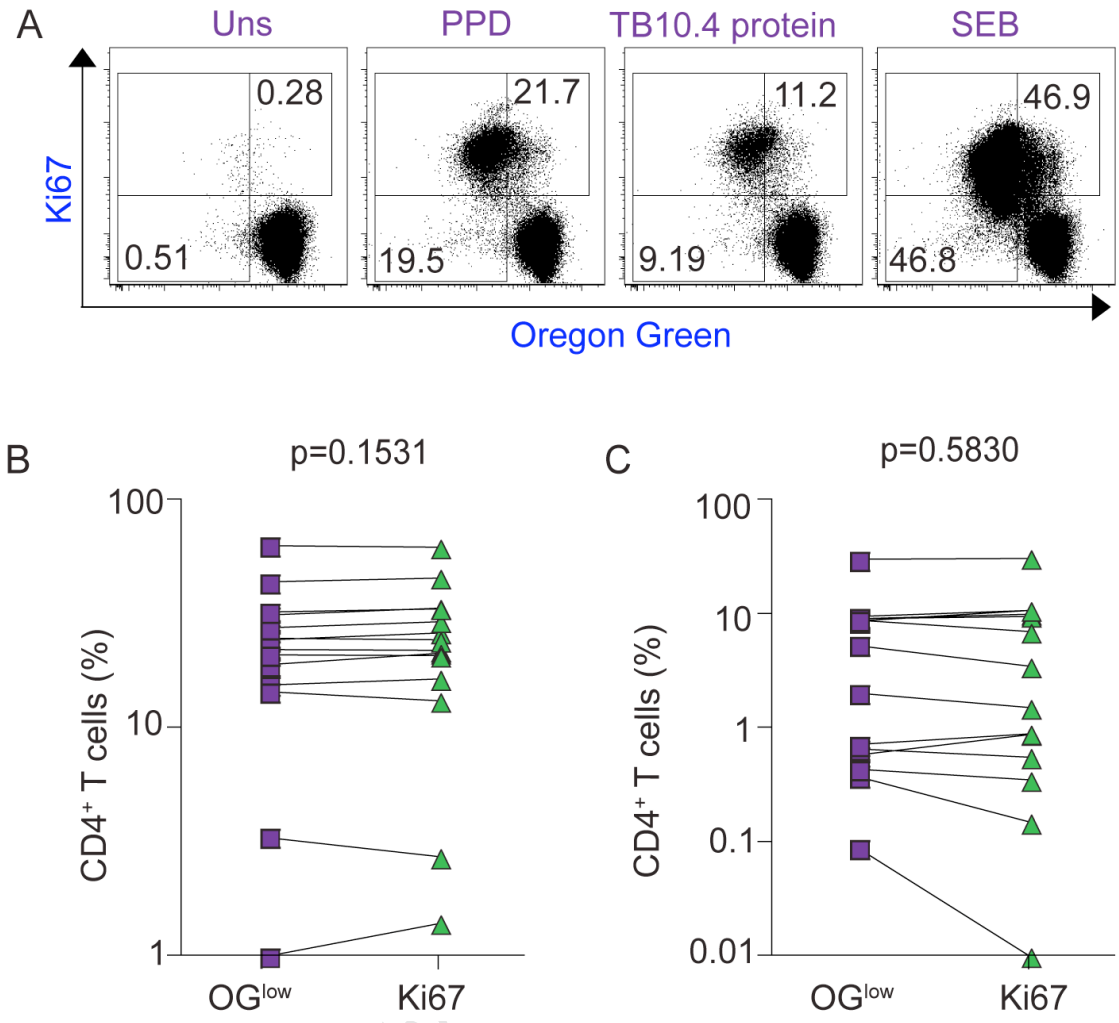


Figure 4.3. Comparison of the Ki67 proliferation assay with the Oregon Green proliferation assay. (A) Representative dotplots showing Ki67 and dye dilution of Oregon Green by $CD4^+$ T cells in PBMC. Dotplots are gated on live, $CD3^+$ $CD8^-$ lymphocytes. Frequencies of (B) PPD- and (C) TB10.4-specific $CD4^+$ T cell proliferation as detected by Ki67 expression (top right) or dye dilution of Oregon Green (OG^{low}) (bottom left) in 14 donors. $CD4^+$ T cells are defined as $CD3^+CD8^-$ T cells (see methods: Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

Frequencies of $Ki67^+$ $CD4^+$ T cells correlated strongly with $BrdU^+$ $CD4^+$ T cell frequencies (PPD: r = 0.8036, p=0.0003; TB10.4 protein: r=0.9308, p<0.0001) (Figure 4.4A and B). Similarly, a strong correlation was found between frequencies of antigen-specific $Ki67^+$ and OG^{low} $CD4^+$ T cells (PPD: r=0.9868, p<0.0001; TB10.4 protein: r=0.9473, p<0.0001) (Figure 4.4C and D).

These data show that intracellular Ki67 expression allows quantitative measurement of antigen-specific T cell proliferation, and that frequencies of

proliferating T cells detected by Ki67 expression correlate strongly with frequencies detected with conventional proliferation assays.

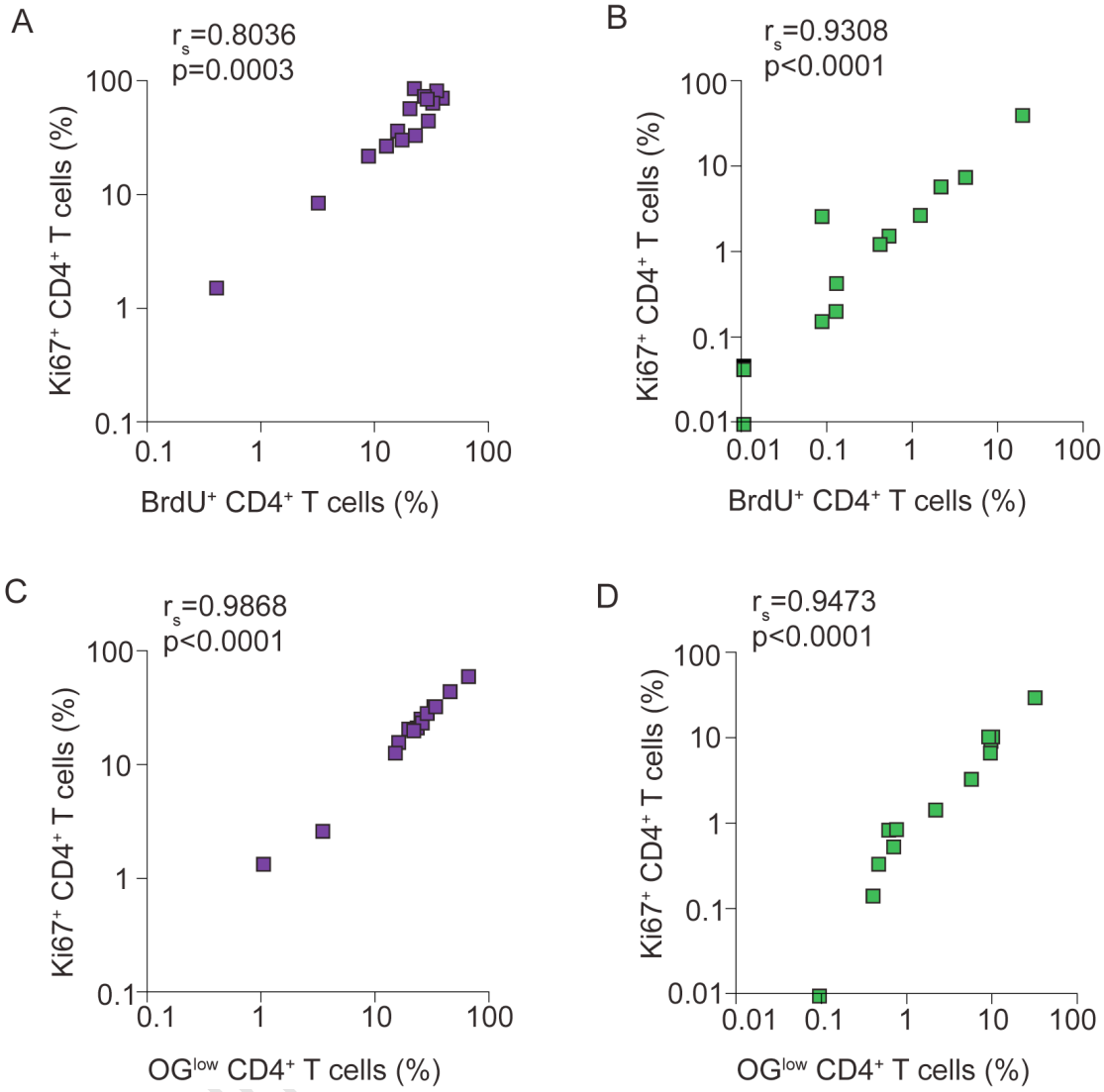
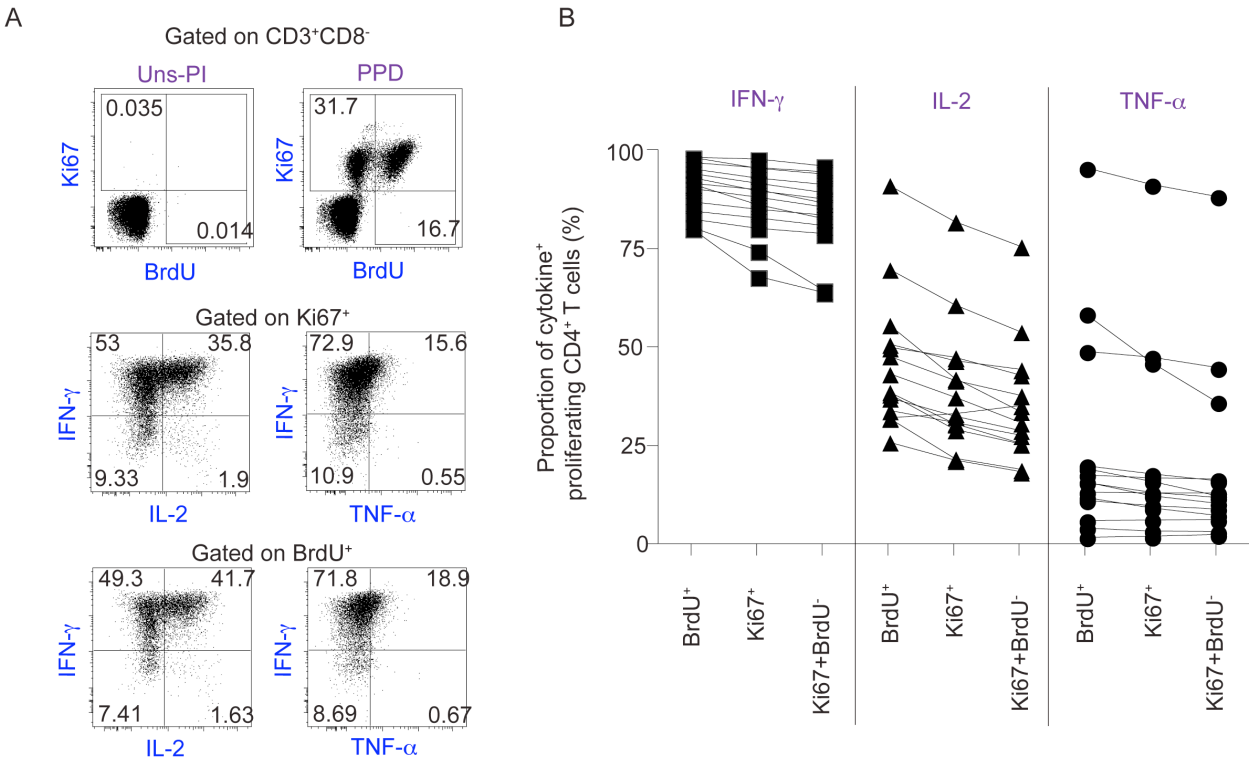


Figure 4.4 Correlation between Ki67$^+$ CD4$^+$ T cell expression and BrdU incorporation or dye dilution of Oregon Green. Whole blood was incubated with (A) PPD or (C) Tb10.4 protein for 6 days (n=15). PBMC were incubated with (B) PPD or (D) TB10.4 protein for 6 days (n=14).

4.4.3 Cytokine expression profiles of proliferating CD4$^+$ T cells

The functional capacity of T cells that have expanded during the 6-day culture may be assessed by short-term polyclonal re-stimulation with PMA and ionomycin on day 6. This induces cytokine production, which can be measured by intracellular staining. We compared expression of IFN-γ, IL-2 and TNF-α by Ki67$^+$ CD4$^+$ T cells with expression of these cytokines in BrdU$^+$

or OG^{low} $CD4^+$ T cells. When Ki67 and BrdU assay results were compared, similar expression of IFN-γ and TNF-α was observed in proliferating $CD4^+$ T cells. $BrdU^+$ $CD4^+$ T cells yielded higher proportions of $IL\text{-}2^+$ cells than $Ki67^+$ $CD4^+$ T cells but these differences were small (Figure 4.5A and B). Similar expression profiles of IFN-γ, IL-2 and TNF-α were observed when comparing Ki67+ and OG dilution (Figure 4.5C and D).



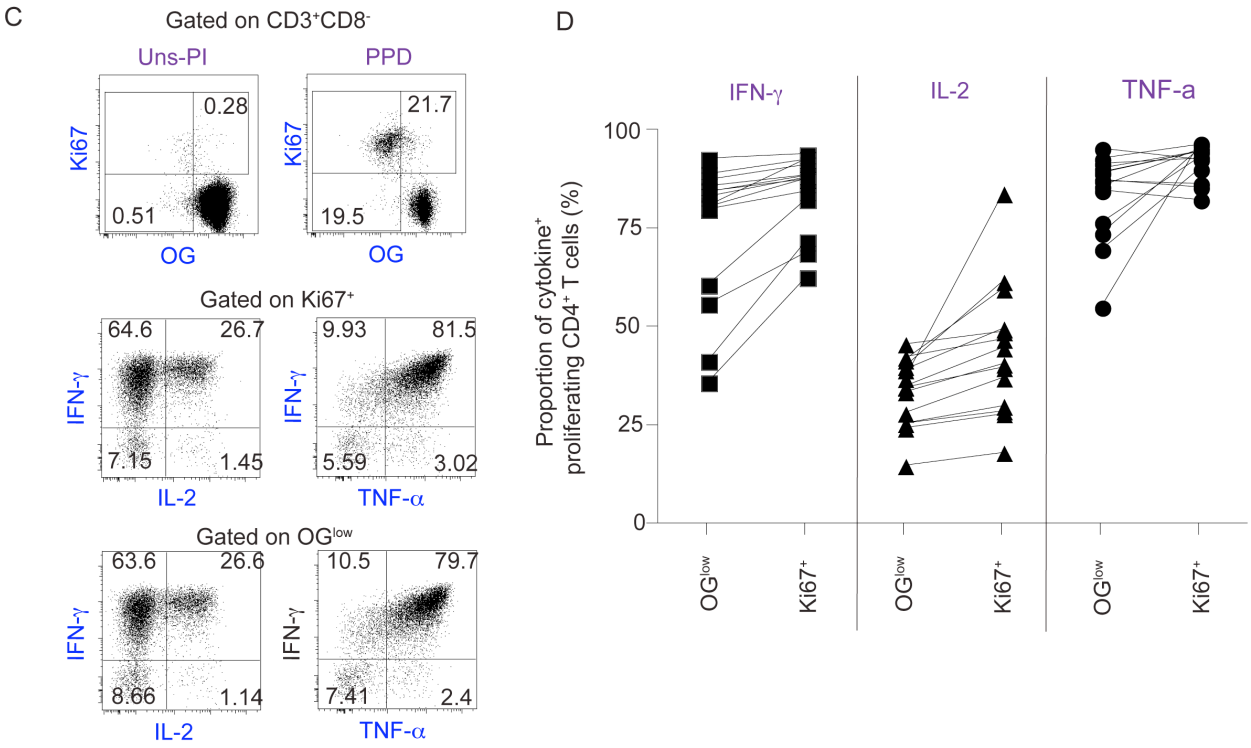


Figure 4.5. Cytokine expression profiles of proliferating CD4$^+$ T cells. Whole blood (A-B) or PBMC (C-D) were cultured for 6 days with no antigen or PPD. On day 6, cells were restimulated with PMA and ionomycin for 4 hours in the presence of Brefeldin A to detect cytokine expression by proliferating T cells. Representative dotplots of the cytokine expression profiles of (A) Ki67$^+$ (top left) or BrdU$^+$ (bottom right) CD4$^+$ T cells and (C) Ki67$^+$ (top right) or OG^{low} (bottom left) CD4$^+$ T cells. (B) Proportions of BrdU$^+$, Ki67$^+$ or Ki67$^+$BrdU$^-$ CD4$^+$ T cells expressing IFN-γ, IL-2 or TNF-α (n=15). (D) Proportions of Ki67$^+$ or OGlow CD4$^+$ T cells expressing IFN-γ, IL-2 or TNF-α (n=14).

4.4.4 Intra-assay variability of Ki67 proliferation assay

To test the reproducibility of the Ki67 proliferation assay, we performed 5 proliferation assays per donor on whole blood from 3 healthy adult volunteers. Intra-assay CV values for PPD-specific Ki67$^+$ CD4$^+$ T cells were ≤ 4,33% and for Ki67$^+$ CD8$^+$ T cells ≤16,14%. Even lower CV values were observed for PHA-stimulated blood (Table 1). This indicates that the Ki67 proliferation assay is highly reproducible.

Table 1. Intra-assay CV values for frequencies of Ki67 expression by T cells after PPD or PHA stimulation.

PPD stimulation						
	Donor 1		Donor 2		Donor 3	
subset	Ki67+CD4+	Ki67+CD8+	Ki67+CD4+	Ki67+CD8+	Ki67+CD4+	Ki67+CD8+
Mean	66.2	10.31	79.82	8.3	62.79	3.03
SD	1.88	1.66	1.46	0.86	1.33	0.34
CV	2.84	16.14	1.83	10.42	2.12	11.17

PHA stimulation						
	Donor 1		Donor 2		Donor 3	
subset	Ki67+CD4+	Ki67+CD8+	Ki67+CD4+	Ki67+CD8+	Ki67+CD4+	Ki67+CD8+
Mean	94.5	91.26	94.7	91.7	76.65	74.54
SD	0.87	1.14	0.81	1.11	3.32	2.4
CV	0.92	1.25	0.86	1.21	4.33	3.21

4.4.5 Monitoring of vaccination-induced T cell proliferation

It is well established that vaccination-induced T cell proliferation results in increased *in vivo* and, thus, *ex vivo* expression of Ki67 (Cellerai et al., 2007; Miller et al., 2008). To determine whether these "background" expression levels of Ki67 affect the specificity of detecting antigen-specific proliferation of T cells *in vitro*, we assessed 6-day antigen-specific T cell proliferation before and 11-13 days after tetanus toxoid (TT) vaccination of healthy, 18-month old toddlers (Figure 4.6). This post-vaccination time point was selected because it coincides with the peak TT-specific $CD4^+$ T cell response in healthy adults (Cellerai et al., 2007).

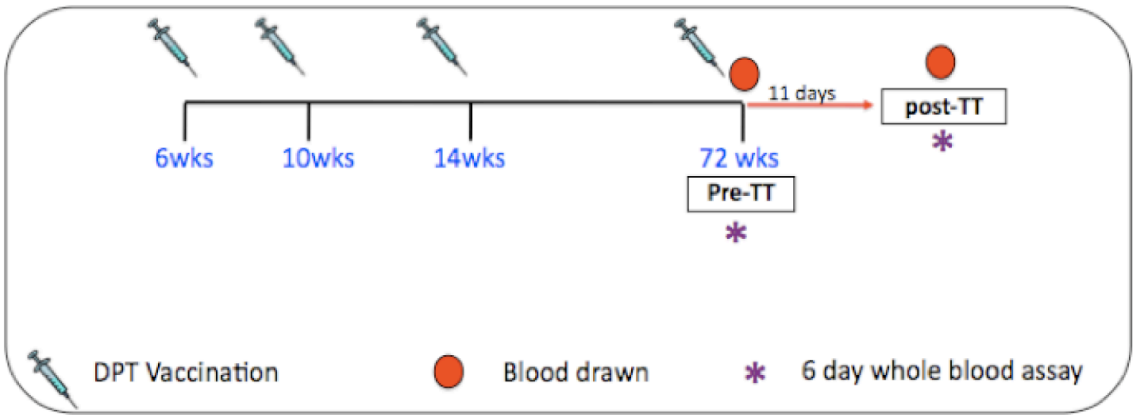


Figure 4.6. Diagram illustrating the strategy for monitoring of tetanus toxoid vaccine-specific responses by Ki67 whole blood assay.

The frequency of *in vitro* proliferating, Ki67$^+$ CD4$^+$ T cells observed pre-vaccination was low (median, 0.15%). After vaccination, TT-specific CD4$^+$ T cell proliferation increased markedly (median, 3.77%, Figure 4.7A and B). To control for possible non-specific up-regulation of Ki67 after TT vaccination, we also quantified BCG-specific T cell proliferation pre- and post- vaccination. Frequencies of BCG-specific Ki67$^+$ CD4$^+$ T cells before and after TT vaccination were not different (Figure 4.7A and C). We also compared the relative TT-induced increase in T cell proliferation between TT and BCG-specific Ki67$^+$ CD4$^+$ T cells. TT vaccine boosting of TT-specific Ki67$^+$ CD4$^+$ T cells was significantly higher than BCG-specific Ki67$^+$ CD4$^+$ T cells (Figure 7D).

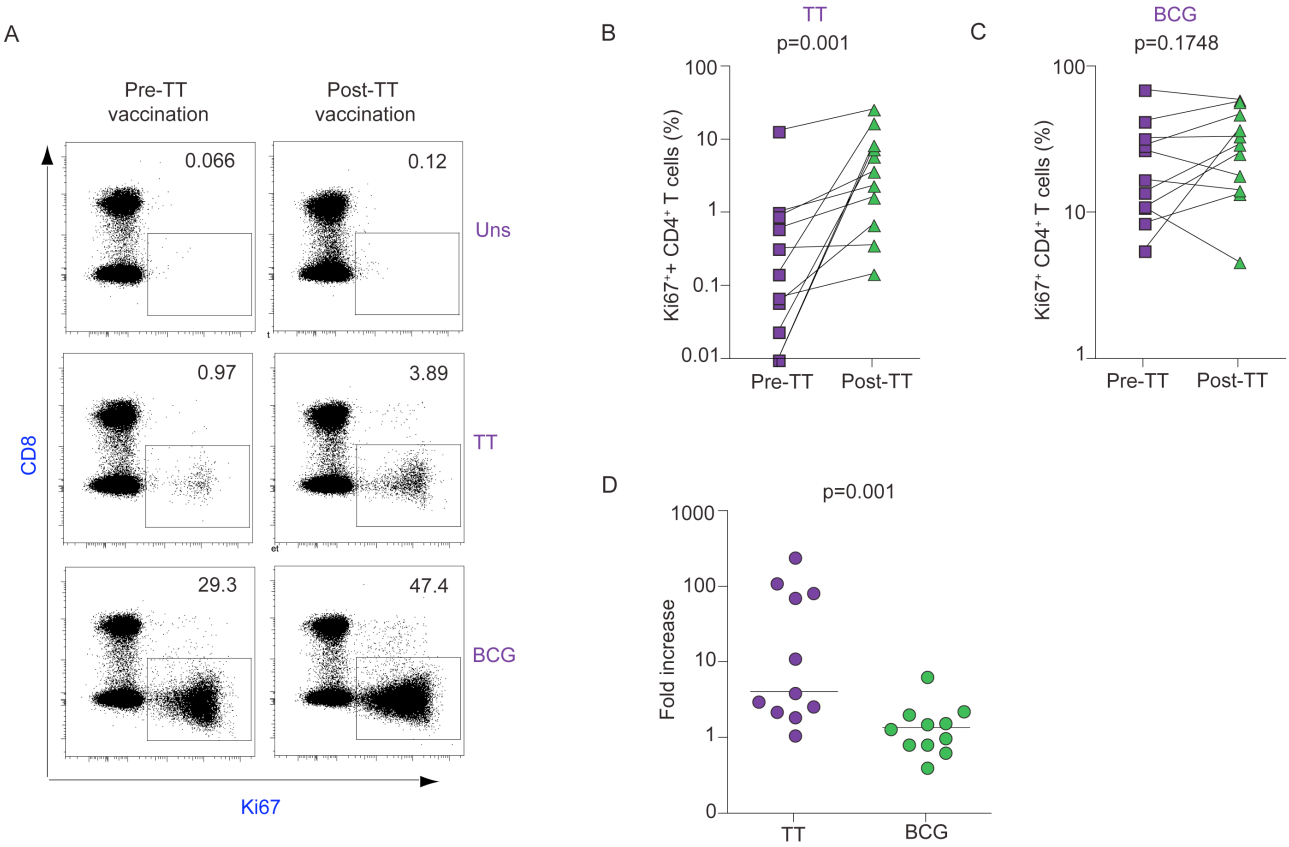


Figure 4.7. Monitoring of vaccination-induced T cell proliferation. (A) Dotplots showing Ki67 expression by $CD4^+$ T cells from a representative 18 month old toddler before (pre-TT) and after TT vaccination (post-TT). Dotplots are gated on live, $CD3^+$ lymphocytes. Values in each dotplot represent the frequency of $Ki67^+$ T cells within the $CD3^+CD8^-$ T cell population. Frequencies of (B) TT-specific and (C) BCG-specific $CD4^+$ T cells pre- and post-TT vaccination in 11 toddlers. $CD4^+$ T cells are defined as $CD3^+CD8^-$ T cells (see methods: Data analysis). (D) Relative increase in TT-specific or BCG-specific $CD4^+$ T cells pre- and post-TT. Lines represent the medians. Differences were calculated using the Wilcoxon matched pairs test.

To further examine the effects of vaccination-induced $CD4^+$ T cell proliferation on the Ki67 proliferation assay, we quantified Ki67 expression directly *ex vivo* in whole blood from toddlers before and 11-13 days after TT-vaccination. High *ex vivo* frequencies of $Ki67^+$ $CD4^+$ T cells were readily detected before and after vaccination in all toddlers (Figure 4.8A-B). Importantly, after 6 days of culture with no antigen, Ki67 expression decreased markedly in all toddlers to background levels (Figure 4.8A-B).

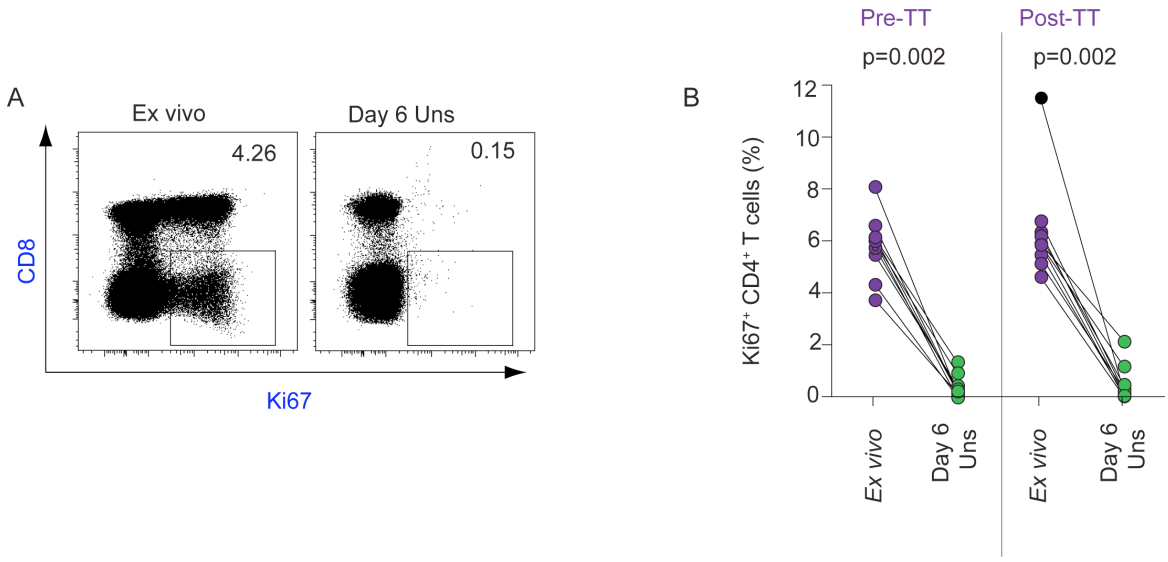


Figure 4.8. High *ex vivo* frequencies of Ki67+ cell are decreased after 6 days in culture without antigen. (A) Dotplots illustrating frequencies of $Ki67^+$ $CD4^+$ T cells in whole blood directly *ex vivo* or after culture in the absence of antigen (Uns) for 6 days. Values in each dotplot represent the frequency of $Ki67^+$ T cells within the $CD3^+CD8^-$ T cell population. (B) Frequencies of $Ki67^+$ $CD4^+$ T cells directly *ex vivo* or after culture for 6 days with medium (n=11). $CD4^+$ T cells are defined as $CD3^+CD8^-$ T cells (see methods: Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

These data suggest that *in vivo* $CD4^+$ T cell proliferation does not interfere with the specificity of the Ki67 proliferation assay. This assay is therefore sensitive for detecting antigen-specific *in vitro* $CD4^+$ T cell proliferation.

4.5. Discussion

Proliferation is a commonly measured indicator of T cell function. We assessed intracellular Ki67 expression as a marker of *in vitro* proliferation in whole blood or PBMC-based assays. We show that the Ki67 assay provides an alternative approach to measuring antigen-driven T cell proliferation, and found that results obtained were very similar to those generated by commonly used proliferation assay systems.

The development of fluorescent dyes and tracking markers has enabled combined analysis of antigen-specific T cell proliferation, phenotyping and cytokine expression by flow cytometry (Johannisson and Festin, 1995; Mehta and Maino, 1997; Lyons and Doherty, 2004; Wallace et al., 2008). To date, whole blood BrdU and PBMC dye dilution assays have been the preferred flow cytometry based methods to assess lymphocyte proliferation. In comparison, Ki67 expression identified approximately double the frequency of proliferating $CD4^+$ T cells detected by BrdU incorporation. Incubation of cells with BrdU is limited to 24 hours or less because incorporated BrdU inhibits cell cycle progression. Therefore a major limitation of the BrdU assay is that only cells that have progressed through the S-phase during this short incubation period may be detected. In contrast, cells express Ki67 in all active phases of the cell cycle. Therefore, Ki67 appears to be a more sensitive marker for the detection of rare T cell responses, and may reflect the extent of *in vitro* antigen-specific proliferation more accurately than BrdU incorporation.

Cellular proliferation in PBMC samples is routinely evaluated by dye dilution methods, using CFSE or derivatives such as OG (Robinson and Amara, 2005). A recent non-human primate study has proposed measurement of *in vitro* proliferation by the combined analysis of Ki67 and side scatter properties of cells (Shedlock et al., 2010). The authors demonstrate a correlation between this assay and the CFSE dilution assay. In this study, we show that the proliferation events detected by loss of OG dye are virtually identical to the Ki67+ events. From this we reasoned that Ki67 expression is an accurate measure of $CD4^+$ and $CD8^+$ T cell proliferation as only cells that have completed cycling display a decrease in OG fluorescence intensity.

Limitations of many protein reactive dye compounds include cellular toxicity (Last'ovicka et al., 2009; Shedlock et al., 2010) and sensitivity to pH and light (Wallace et al., 2008). The Ki67 proliferation assay requires no incubation or washing steps prior to or during the culture, and exposure of cells to toxic compounds is eliminated. Additionally, since labelling of cells is not required before antigen stimulation, detection of Ki67 by flow cytometry can be performed on antigen-stimulated cells after cryopreservation. A limitation of Ki67 as a proliferation marker is its inability to resolve the number of proliferation cycles that cells have undergone, as can be done with dye dilution assays (Parish, 1999; Lyons and Doherty, 2004). Enumeration of cell cycles enables calculation of the original precursor frequency of specific cells, since the number of cells and their respective number of divisions are known (Givan et al., 1999).

Monitoring vaccine-induced T cell proliferative potential is important for determining vaccine take, memory function and long-term persistence of vaccine-specific responses. Previous studies have quantified Ki67 expression directly *ex vivo* as a measure of the vaccine-induced proliferative response (Miller et al., 2008), or in combination with activation markers to identify antigen-specific T cells (Stubbe et al., 2006). To detect increases in the expression of Ki67, these studies relied on low-level Ki67 expression before vaccination in healthy adults. Direct *ex vivo* detection of antigen-specific Ki67 expression may thus be challenging in individuals with high levels of *in vivo* T cell proliferation – such as those resulting from recent vaccinations or infections. We observed high *ex vivo* frequencies of $Ki67^+$ $CD4^+$ T cells in toddlers, suggesting elevated levels of *in vivo* T cell turnover. This turnover is likely to be driven by routine childhood vaccinations and exposure to infections, common in this age group. Whole blood culture in the absence of antigen reduced Ki67 expression to barely detectable levels by day 6, presumably due to cells reverting to a quiescent state. Therefore, this 6-day assay proved to be sufficiently specific and sensitive for the identification of rare, antigen-specific $CD4^+$ T cells following vaccination in the context of high *ex vivo* frequencies of Ki67+ T cells.

Overall, our data show that outcomes of the Ki67 assay correlate strongly with current flow cytometry based whole blood and PBMC proliferation assays. This assay is highly reproducible, versatile, and presents several practical advantages over current techniques. We propose Ki67 as a marker for quantifying antigen-specific $CD4^+$ and $CD8^+$ T cell proliferation, and utilising this assay to monitor T cell responses in large field studies or paediatric studies based on limited blood volumes.

4.6 Contributions

Andreia Soares and Lerisa Govender designed the experiments, conducted the laboratory and data analysis, and wrote this chapter under the supervision of Prof. W.A. Hanekom, Dr T.J. Scriba and Dr. B. Abel, and other members of SATVI laboratory.

Chapter 5: General Discussion

Nearly 130 years have elapsed since the discovery of *M.tb,* yet TB disease still remains a major cause of morbidity and mortality in many developing countries. Research into new, safer and more effective vaccine regimes and strategies to combat the scourge of TB are ongoing. Since one third of the world's population is latently infected with *M.tb*, a post-infection vaccine that would prevent reactivation of *M.tb* infection in these people is urgently needed.

The selection of antigens for incorporation into novel vaccines is challenging, and much emphasis is placed on preliminary findings from *in vitro* models of *M.tb*, which attempt to simulate *in vivo* infection. However, prior to inclusion of potential antigens into a vaccine, antigen recognition should be assessed in appropriate clinical studies.

The first study in this thesis provides insight into candidate antigens for a potential post-infection TB vaccine. We demonstrate that Rv2660 and Rv2659 are promising antigens for such a vaccine, since greater immune recognition and associated $CD4^+$ T cell effector function was observed in *M.tb* latently infected individuals compared with TB diseased patients. Indeed, based on initial findings at SATVI, Rv2660 was incorporated into a post-infection subunit vaccine, H56, which includes Ag85B and ESAT-6, and has already entered preclinical trials. Preclinical testing of this vaccine in a post-infection murine model has shown that vaccination with H56 decreases lung CFUs compared with only BCG vaccination, after challenge with *M.tb* (Peter Andersen, personal communication, 2010). Ultimately, this post-infection vaccine would only be considered a viable option if immune recognition to possible candidate antigens, such as Rv2660 and Rv2659 are shown in human studies. For this reason it is vital that candidate latency associated *M.tb* antigens initially be screened in human populations to assess the breadth of immune recognition and for potential inclusion in post-infection vaccines. Once antigens have been incorporated in a vaccine, the safety and

immunogenicity of the vaccine would be tested in preclinical settings. Multiple clinical trials have to be concluded before the vaccine can be introduced to the public. Phase I vaccine trials are designed to assess adverse advents and immunogenicity as a secondary outcome starting with healthy adults then adolescents and children. This will be followed by Phase II and III trials, in which the vaccine safety, immunogenicity and efficacy are assessed in latently infected individuals who are the target population for the post-infection vaccine.

To assess novel TB vaccines, it is imperative that appropriate assays are optimised to generate efficient, sensitive and reliable results. Hence the second study of this thesis addressed the optimal and efficient measurement of T cell vaccine-specific responses using Ki67 as marker of antigen-specific lymphoproliferation. We demonstrate that Ki67 expression in T cells is a specific and quantitative indicator of proliferation. Importantly, since this assay is not labour intensive and requires small volumes of blood, it can be used to study neonates where only limited blood volumes can be collected.

There were 3 major limitations of the study. Firstly, the study investigating Rv2660 and Rv2659 as candidate antigens for a potential post-infection vaccine could have been strengthened by LTBI and TB disease cohorts that were age and gender matched; this was not possible for this study due to the availability of cryopreserved PBMC from select TB patients at the time of study commencement. Secondly, the differential use of fresh and cryopreserved PBMC in the cohorts could have affected results, as freshly isolated PBMC allow for greater sensitivity in measuring immune responses. Finally, the Ki67 assay could be further optimised by using cryopreserved PBMCs, rather than fresh PBMC or whole blood. Preliminary results from our group have indicated that long-term cryopreservation may affect background levels of Ki67 expression – this needs to be evaluated formally.

There are many significant challenges in the field of TB vaccine research, including inadequate advocacy and funding, few and expensive optimal preclinical animal models, lack of validated immune correlates of vaccination-induced protection, difficulties in making accurate and reliable diagnosis of TB disease, the endpoint of phase IIb and III trials. In addition, there is a lack of standardisation of immunological assessment across trials, and no candidate vaccines in trials that are designed to specifically prevent progression of *M.tb* infection to TB disease. We propose that results presented in this thesis will address the latter 2 issues, through description of a practical assay that may be used in vaccine trials, and by confirming that 2 new latency-associated antigens are recognised in human populations,

6. Appendix

Alternative lysing solution (Ammonium chloride lysing solution)

All chemicals supplied by: Sigma Aldrich

150mM NH4Cl

Cat.No: A9434

10mM KHCO3

Cat.No: P9144

1mM EDTA

Cat. No: E6511

Ingredients per litre distilled water

BCG (Mycobacterium bovis bacillus Calmette-Guerin Danish 1331 strain)

Supplier: Statens Serum Institut

Cat. No.: 4203166

BrdU

Supplier: Sigma Aldrich

Cat. No.: 16880

Brefeldin-A

Supplier: Sigma Aldrich

Cat. No: B-7651

Cryo-solution

This will finally consist of 20% DMSO in FCS in RPMI.

-DMSO

Supplier: E Merck

Cat. No: BB103234L

FCS

Supplier: Adcock Ingram

Cat. No: 14-501AIH

DNase

Supplier: Sigma Aldrich

Cat. No: D4513

ELISA coating buffer

0.1M $NaHCO_3$

Supplier: Sigma Aldrich

Cat. No: S5761

Ingredient per litre 1X PBS, pH 8.2

ELISA dilution and blocking buffer (1% BSA)

10 g BSA

Supplier: Boehringer Mannheim

Cat No: 519 987

0.3 g NaN_3

Supplier: Sigma Aldrich

Cat. No: 08591

Ingredients per litre 1X PBS

FACS Lysing Solution

Supplier: Scientific Group

Cat No: B-D 349202

Heat inactivated AB serum

Supplier: Sigma Aldrich

Cat. No: H4522

Ionomycin

Supplier: Sigma Aldrich

Cat. No: I-0634

Oregon Green

Supplier: Invitrogen

Cat. No: 0344550

Perm/Wash Buffer:

Supplier: Scientific Group

Cat. No.: 554723

PHA

Supplier: Bioweb

Cat. No.: HA16

PBS

Supplier: BioWhittaker

Cat. No: 17-517Q

PMA

Supplier: Invitrogen

Cat. No: P-8139

PPD

Supplier: Statens Serum Institut

Cat. No.: 2390

RPMI

Supplier: Adcock Ingram

Cat. No: 12-702F

SEB

Supplier: Sigma Aldrich

Cat. No: S4881

ViViD

LIVE/DEAD Fixable Violet Dead Cell Stain kit

Supplier: Invitrogen

Cat. No: L34955

7. References

Aagaard, C., Dietrich, J., Doherty, M. and Andersen, P. (2009) TB vaccines: current status and future perspectives. Immunol Cell Biol 87, 279-86.

Aaron, L., Saadoun, D., Calatroni, I., Launay, O., Memain, N., Vincent, V., Marchal, G., Dupont, B., Bouchaud, O., Valeyre, D. and Lortholary, O. (2004) Tuberculosis in HIV-infected patients: a comprehensive review. Clin Microbiol Infect 10, 388-98.

Abel, B., Tameris, M., Mansoor, N., Gelderbloem, S., Hughes, J., Abrahams, D., Makhethe, L., Erasmus, M., de Kock, M., van der Merwe, L., Hawkridge, A., Veldsman, A., Hatherill, M., Schirru, G., Pau, M.G., Hendriks, J., Weverling, G.J., Goudsmit, J., Sizemore, D., McClain, J.B., Goetz, M., Gearhart, J., Mahomed, H., Hussey, G.D., Sadoff, J.C. and Hanekom, W.A. (2010) The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. Am J Respir Crit Care Med 181, 1407-17.

Abu-Raddad, L.J., Sabatelli, L., Achterberg, J.T., Sugimoto, J.D., Longini, I.M., Jr., Dye, C. and Halloran, M.E. (2009) Epidemiological benefits of more-effective tuberculosis vaccines, drugs, and diagnostics. Proc Natl Acad Sci U S A 106, 13980-5.

Aguirre-Blanco, A.M., Lukey, P.T., Cliff, J.M. and Dockrell, H.M. (2007) Strain-dependent variation in Mycobacterium bovis BCG-induced human T-cell activation and gamma interferon production in vitro. Infect Immun 75, 3197-201.

Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. Cell 124, 783-801.

Andersen, P. (2007a) Tuberculosis vaccines - an update. Nat Rev Microbiol 5, 484-7.

Andersen, P. (2007b) Vaccine strategies against latent tuberculosis infection. Trends Microbiol 15, 7-13.

Arend, S.M., Engelhard, A.C., Groot, G., de Boer, K., Andersen, P., Ottenhoff, T.H. and van Dissel, J.T. (2001) Tuberculin skin testing compared with T-cell responses to Mycobacterium tuberculosis-specific and nonspecific antigens for detection of latent infection in persons with recent tuberculosis contact. Clin Diagn Lab Immunol 8, 1089-96.

Bachmann, M.F., Wolint, P., Schwarz, K., Jager, P. and Oxenius, A. (2005) Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol 175, 4686-96.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J. and Ahmed, R. (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439, 682-7.

Barker, L.F., Brennan, M.J., Rosenstein, P.K. and Sadoff, J.C. (2009) Tuberculosis vaccine research: the impact of immunology. Curr Opin Immunol 21, 331-8.

Barry, C.E., 3rd, Boshoff, H.I., Dartois, V., Dick, T., Ehrt, S., Flynn, J., Schnappinger, D., Wilkinson, R.J. and Young, D. (2009) The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol 7, 845-55.

Baumann, S., Nasser Eddine, A. and Kaufmann, S.H. (2006) Progress in tuberculosis vaccine development. Curr Opin Immunol 18, 438-48.

Behr, M.A. (2002) BCG--different strains, different vaccines? Lancet Infect Dis 2, 86-92.

Belisle, J.T., Vissa, V.D., Sievert, T., Takayama, K., Brennan, P.J. and Besra, G.S. (1997) Role of the major antigen of Mycobacterium tuberculosis in cell wall biogenesis. Science 276, 1420-2.

Betts, J.C., Lukey, P.T., Robb, L.C., McAdam, R.A. and Duncan, K. (2002) Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol Microbiol 43, 717-31.

Bhatt, K. and Salgame, P. (2007) Host innate immune response to Mycobacterium tuberculosis. J Clin Immunol 27, 347-62.

Black, G.F., Thiel, B.A., Ota, M.O., Parida, S.K., Adegbola, R., Boom, W.H., Dockrell, H.M., Franken, K.L., Friggen, A.H., Hill, P.C., Klein, M.R., Lalor, M.K., Mayanja, H., Schoolnik, G., Stanley, K., Weldingh, K., Kaufmann, S.H., Walzl, G. and Ottenhoff, T.H. (2009) Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. Clin Vaccine Immunol 16, 1203-12.

Brandt, L., Feino Cunha, J., Weinreich Olsen, A., Chilima, B., Hirsch, P., Appelberg, R. and Andersen, P. (2002) Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. Infect Immun 70, 672-8.

Brennan, M.J., Fruth, U., Milstien, J., Tiernan, R., de Andrade Nishioka, S. and Chocarro, L. (2007) Development of new tuberculosis vaccines: a global perspective on regulatory issues. PLoS Med 4, e252.

Brosch, R., Gordon, S.V., Garnier, T., Eiglmeier, K., Frigui, W., Valenti, P., Dos Santos, S., Duthoy, S., Lacroix, C., Garcia-Pelayo, C., Inwald, J.K., Golby, P., Garcia, J.N., Hewinson, R.G., Behr, M.A., Quail, M.A., Churcher, C., Barrell, B.G., Parkhill, J. and Cole, S.T. (2007) Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci U S A 104, 5596-601.

Cellerai, C., Harari, A., Vallelian, F., Boyman, O. and Pantaleo, G. (2007) Functional and phenotypic characterization of tetanus toxoid-specific human CD4+ T cells following re-immunization. Eur J Immunol 37, 1129-38.

Chan, J. and Flynn, J. (2004) The immunological aspects of latency in tuberculosis. Clin Immunol 110, 2-12.

Chaplin, D.D. (2010) Overview of the immune response. J Allergy Clin Immunol 125, S3-23.

Cheever, M.A., Thompson, J.A., Kern, D.E. and Greenberg, P.D. (1984) Interleukin-2 administered in vivo induces the growth and augments the function of cultured T cells in vivo. J Biol Response Mod 3, 462-7.

Chen, C.Y., Huang, D., Wang, R.C., Shen, L., Zeng, G., Yao, S., Shen, Y., Halliday, L., Fortman, J., McAllister, M., Estep, J., Hunt, R., Vasconcelos, D., Du, G., Porcelli, S.A., Larsen, M.H., Jacobs, W.R., Jr., Haynes, B.F., Letvin, N.L. and Chen, Z.W. (2009) A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. PLoS Pathog 5, e1000392.

Colditz, G., Brewer, T.F., Berkey, C.S., Wilson, M.E., Burdick, E., Fineberg, H.V. and Mosteller, F. (1994) Efficacy of BCG vaccination in the prevention of tuberculosis. Meta-analysis of published literature. J of the American Medical Association 271, 698-702.

Combadiere, B., Boissonnas, A., Carcelain, G., Lefranc, E., Samri, A., Bricaire, F., Debre, P. and Autran, B. (2004) Distinct time effects of vaccination on long-term proliferative and IFN-gamma-producing T cell memory to smallpox in humans. J Exp Med 199, 1585-93.

Conradt, P., Hess, J. and Kaufmann, S.H. (1999) Cytolytic T-cell responses to human dendritic cells and macrophages infected with Mycobacterium bovis BCG and recombinant BCG secreting listeriolysin. Microbes Infect 1, 753-64.

Cooper, A.M., Dalton, D.K., Stewart, T.A., Griffin, J.P., Russell, D.G. and Orme, I.M. (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 178, 2243-7.

Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C. and Dye, C. (2003) The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med 163, 1009-21.

Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W., Davey, D.F., Flynn, B.J., Hoff, S.T., Andersen, P., Reed, S.G., Morris, S.L., Roederer, M. and Seder, R.A. (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 13, 843-50.

Davids, V., Hanekom, W.A., Mansoor, N., Gamieldien, H., Gelderbloem, S.J., Hawkridge, A., Hussey, G.D., Hughes, E.J., Soler, J., Murray, R.A.,

Ress, S.R. and Kaplan, G. (2006) The effect of bacille Calmette-Guerin vaccine strain and route of administration on induced immune responses in vaccinated infants. J Infect Dis 193, 531-6.

Day, C.L., Kaufmann, D.E., Kiepiela, P., Brown, J.A., Moodley, E.S., Reddy, S., Mackey, E.W., Miller, J.D., Leslie, A.J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E.J., Coovadia, H.M., Goulder, P.J., Klenerman, P., Ahmed, R., Freeman, G.J. and Walker, B.D. (2006) PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 443, 350-4.

de Valliere, S., Abate, G., Blazevic, A., Heuertz, R.M. and Hoft, D.F. (2005) Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. Infect Immun 73, 6711-20.

Doisne, J.M., Urrutia, A., Lacabaratz-Porret, C., Goujard, C., Meyer, L., Chaix, M.L., Sinet, M. and Venet, A. (2004) CD8+ T cells specific for EBV, cytomegalovirus, and influenza virus are activated during primary HIV infection. J Immunol 173, 2410-8.

Dolbeare, F., Gratzner, H., Pallavicini, M.G. and Gray, J.W. (1983) Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc Natl Acad Sci U S A 80, 5573-7.

Downing, K.J., Mischenko, V.V., Shleeva, M.O., Young, D.I., Young, M., Kaprelyants, A.S., Apt, A.S. and Mizrahi, V. (2005) Mutants of Mycobacterium tuberculosis lacking three of the five rpf-like genes are defective for growth in vivo and for resuscitation in vitro. Infect Immun 73, 3038-43.

Dulphy, N., Herrmann, J.L., Nigou, J., Rea, D., Boissel, N., Puzo, G., Charron, D., Lagrange, P.H. and Toubert, A. (2007) Intermediate maturation of Mycobacterium tuberculosis LAM-activated human dendritic cells. Cell Microbiol 9, 1412-25.

Ehlers, S. (2009) Lazy, dynamic or minimally recrudescent? On the elusive nature and location of the mycobacterium responsible for latent tuberculosis. Infection 37, 87-95.

Feng, C.G. and Britton, W.J. (2000) CD4+ and CD8+ T cells mediate adoptive immunity to aerosol infection of Mycobacterium bovis bacillus Calmette-Guerin. J Infect Dis 181, 1846-9.

Fine, P. (1998) Vaccines, genes and trials. Novartis Foundation Symposium 217, 57-69.

Fine, P.E. (1995) Variation in protection by BCG: implications of and for heterologous immunity. Lancet 346, 1339-45.

Fiorenza, G., Rateni, L., Farroni, M.A., Bogue, C. and Dlugovitzky, D.G. (2005) TNF-alpha, TGF-beta and NO relationship in sera from tuberculosis (TB) patients of different severity. Immunol Lett 98, 45-8.

Flynn, J.L. and Chan, J. (2001) Immunology of tuberculosis. Annu Rev Immunol 19, 93-129.

Flynn, J.L., Goldstein, M.M., Triebold, K.J., Koller, B. and Bloom, B.R. (1992) Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. Proc Natl Acad Sci U S A 89, 12013-7.

Forbes, E.K., Sander, C., Ronan, E.O., McShane, H., Hill, A.V., Beverley, P.C. and Tchilian, E.Z. (2008) Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. J Immunol 181, 4955-64.

Gallant, C.J., Cobat, A., Simkin, L., Black, G.F., Stanley, K., Hughes, J., Doherty, T.M., Hanekom, W.A., Eley, B., Beyers, N., Jais, J.P., van Helden, P., Abel, L., Alcais, A., Hoal, E.G. and Schurr, E. (2009) Tuberculin skin test and in vitro assays provide complementary measures of antimycobacterial immunity in children and adolescents. Chest 137, 1071-7.

Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Lalloo, U., Zeller, K., Andrews, J. and Friedland, G. (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 368, 1575-80.

Gerdes, J. (1990) Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. Semin Cancer Biol 1, 199-206.

Gerdes, J., Lemke, H., Baisch, H., Wacker, H.H., Schwab, U. and Stein, H. (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133, 1710-5.

Givan, A.L., Fisher, J.L., Waugh, M., Ernstoff, M.S. and Wallace, P.K. (1999) A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. J Immunol Methods 230, 99-112.

Gordon, S.V., Eiglmeier, K., Garnier, T., Brosch, R., Parkhill, J., Barrell, B., Cole, S.T. and Hewinson, R.G. (2001) Genomics of Mycobacterium bovis. Tuberculosis (Edinb) 81, 157-63.

Grode, L., Seiler, P., Baumann, S., Hess, J., Brinkmann, V., Nasser Eddine, A., Mann, P., Goosmann, C., Bandermann, S., Smith, D., Bancroft, G.J., Reyrat, J.M., van Soolingen, D., Raupach, B. and Kaufmann, S.H. (2005) Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest 115, 2472-9.

Guyot-Revol, V., Innes, J.A., Hackforth, S., Hinks, T. and Lalvani, A. (2006) Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med 173, 803-10.

Hanekom, W., ; and Ernst, J. (2008) Maintenance of Latent Infection, with Correlates of Protective Immunity. In: S.a.B.W. Kaufmann (Ed) Handbook of Tuberculosis: Immunuology and Cell Biology. Wiley-VCH, p. 283.

Hanekom, W.A. (2005) The immune response to BCG vaccination of newborns. Ann N Y Acad Sci 1062, 69-78.

Hanekom, W.A., Abel, B. and Scriba, T.J. (2007) Immunological protection against tuberculosis. S Afr Med J 97, 973-7.

Hanekom, W.A., Dockrell, H.M., Ottenhoff, T.H., Doherty, T.M., Fletcher, H., McShane, H., Weichold, F.F., Hoft, D.F., Parida, S.K. and Fruth, U.J. (2008) Immunological outcomes of new tuberculosis vaccine trials: WHO panel recommendations. PLoS Med 5, e145.

Hanekom, W.A., Hughes, J., Mavinkurve, M., Mendillo, M., Watkins, M., Gamieldien, H., Gelderbloem, S.J., Sidibana, M., Mansoor, N., Davids, V., Murray, R.A., Hawkridge, A., Haslett, P.A., Ress, S., Hussey, G.D. and Kaplan, G. (2004) Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies. J Immunol Methods 291, 185-95.

Harrington, L.E., Mangan, P.R. and Weaver, C.T. (2006) Expanding the effector CD4 T-cell repertoire: the Th17 lineage. Curr Opin Immunol 18, 349-56.

Horwitz, M.A. and Harth, G. (2003) A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. Infect Immun 71, 1672-9.

Horwitz, M.A., Harth, G., Dillon, B.J. and Maslesa-Galic, S. (2000) Recombinant bacillus calmette-guerin (BCG) vaccines expressing the Mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. Proc Natl Acad Sci U S A 97, 13853-8.

Houck, D.W. and Loken, M.R. (1985) Simultaneous analysis of cell surface antigens, bromodeoxyuridine incorporation and DNA content. Cytometry 6, 531-8.

Hussey, G., Hawkridge, T. and Hanekom, W. (2007) Childhood tuberculosis: old and new vaccines. Paediatr Respir Rev 8, 148-54.

Iwasaki, A. and Medzhitov, R. (2010) Regulation of adaptive immunity by the innate immune system. Science 327, 291-5.

Janeway CA, T.P.e.a. (2008) Immunobiology 6th Edition. Garland Science, New York.

Jaron, B., Maranghi, E., Leclerc, C. and Majlessi, L. (2008) Effect of attenuation of Treg during BCG immunization on anti-mycobacterial Th1 responses and protection against Mycobacterium tuberculosis. PLoS One 3, e2833.

Johannisson, A. and Festin, R. (1995) Phenotype transition of CD4+ T cells from CD45RA to CD45R0 is accompanied by cell activation and proliferation. Cytometry 19, 343-52.

Kariminia, A., Sharifnia, Z., Aghakhani, A., Banifazl, M., Eslamifar, A., Hazrati, M. and Ramezani, A. (2009) Comparison of QuantiFERON TB-G-test to TST for detecting latent tuberculosis infection in a high-incidence area containing BCG-vaccinated population. J Eval Clin Pract 15, 148-51.

Kaufmann, S.H. (2010) Novel tuberculosis vaccination strategies based on understanding the immune response. J Intern Med 267, 337-53.

Kaufmann, S.H., Cole, S.T., Mizrahi, V., Rubin, E. and Nathan, C. (2005) Mycobacterium tuberculosis and the host response. J Exp Med 201, 1693-7.

Kaufmann, S.H. and McMichael, A.J. (2005) Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. Nat Med 11, S33-44.

Khader, S.A., Bell, G.K., Pearl, J.E., Fountain, J.J., Rangel-Moreno, J., Cilley, G.E., Shen, F., Eaton, S.M., Gaffen, S.L., Swain, S.L., Locksley, R.M., Haynes, L., Randall, T.D. and Cooper, A.M. (2007) IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol 8, 369-77.

Kroger, L., Korppi, M., Brander, E., Kroger, H., Wasz-Hockert, O., Backman, A., Rapola, J., Launiala, K. and Katila, M.L. (1995) Osteitis caused by bacille Calmette-Guerin vaccination: a retrospective analysis of 222 cases. J Infect Dis 172, 574-6.

Lambert, P.H., Hawkridge, T. and Hanekom, W.A. (2009) New vaccines against tuberculosis. Clin Chest Med 30, 811-26, x.

Last'ovicka, J., Budinsky, V., Spisek, R. and Bartunkova, J. (2009) Assessment of lymphocyte proliferation: CFSE kills dividing cells and modulates expression of activation markers. Cell Immunol 256, 79-85.

Leyten, E.M., Lin, M.Y., Franken, K.L., Friggen, A.H., Prins, C., van Meijgaarden, K.E., Voskuil, M.I., Weldingh, K., Andersen, P., Schoolnik, G.K., Arend, S.M., Ottenhoff, T.H. and Klein, M.R. (2006) Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. Microbes Infect 8, 2052-60.

Lin, P.L. and Flynn, J.L. (2010) Understanding latent tuberculosis: a moving target. J Immunol 185, 15-22.

Lindenstrom, T., Agger, E.M., Korsholm, K.S., Darrah, P.A., Aagaard, C., Seder, R.A., Rosenkrands, I. and Andersen, P. (2009) Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 182, 8047-55.

Liu, P.T., Stenger, S., Tang, D.H. and Modlin, R.L. (2007) Cutting edge: vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin. J Immunol 179, 2060-3.

Lopez, F., Belloc, F., Lacombe, F., Dumain, P., Reiffers, J., Bernard, P. and Boisseau, M.R. (1991) Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. Cytometry 12, 42-9.

Lyons, A.B. (2000) Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods 243, 147-54.

Lyons, A.B. and Doherty, K.V. (2004) Flow cytometric analysis of cell division by dye dilution. Curr Protoc Cytom Chapter 9, Unit 9 11.

MacMillan, H.F., Lee, T. and Issekutz, A.C. (2009) Intravenous immunoglobulin G-mediated inhibition of T-cell proliferation reflects an endogenous mechanism by which IgG modulates T-cell activation. Clin Immunol 132, 222-33.

Magg, T. and Albert, M.H. (2007) Tracking cell proliferation using the far red fluorescent dye SNARF-1. Cytometry B Clin Cytom 72, 458-64.

Maglione, P.J. and Chan, J. (2009) How B cells shape the immune response against Mycobacterium tuberculosis. Eur J Immunol 39, 676-86.

Maglione, P.J., Xu, J. and Chan, J. (2007) B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis. J Immunol 178, 7222-34.

Makedonas, G. and Betts, M.R. (2006) Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. Springer Semin Immunopathol 28, 209-19.

Marchant, A., Goetghebuer, T., Ota, M.O., Wolfe, I., Ceesay, S.J., De Groote, D., Corrah, T., Bennett, S., Wheeler, J., Huygen, K., Aaby, P., McAdam, K.P. and Newport, M.J. (1999) Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. J Immunol 163, 2249-55.

Martin, C., Williams, A., Hernandez-Pando, R., Cardona, P.J., Gormley, E., Bordat, Y., Soto, C.Y., Clark, S.O., Hatch, G.J., Aguilar, D., Ausina, V. and Gicquel, B. (2006) The live Mycobacterium tuberculosis phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. Vaccine 24, 3408-19.

McCullough, K.C. and Summerfield, A. (2005) Basic concepts of immune response and defense development. ILAR J 46, 230-40.

McShane, H., Pathan, A.A., Sander, C.R., Keating, S.M., Gilbert, S.C., Huygen, K., Fletcher, H.A. and Hill, A.V. (2004) Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med 10, 1240-4.

Mehta, B.A. and Maino, V.C. (1997) Simultaneous detection of DNA synthesis and cytokine production in staphylococcal enterotoxin B activated CD4+ T lymphocytes by flow cytometry. J Immunol Methods 208, 49-59.

Miller, J.D., van der Most, R.G., Akondy, R.S., Glidewell, J.T., Albott, S., Masopust, D., Murali-Krishna, K., Mahar, P.L., Edupuganti, S., Lalor, S., Germon, S., Del Rio, C., Mulligan, M.J., Staprans, S.I., Altman, J.D., Feinberg, M.B. and Ahmed, R. (2008) Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. Immunity 28, 710-22.

Mori, T., Sakatani, M., Yamagishi, F., Takashima, T., Kawabe, Y., Nagao, K., Shigeto, E., Harada, N., Mitarai, S., Okada, M., Suzuki, K., Inoue, Y., Tsuyuguchi, K., Sasaki, Y., Mazurek, G.H. and Tsuyuguchi, I. (2004) Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am J Respir Crit Care Med 170, 59-64.

Myrvik, Q.N., Leake, E.S. and Wright, M.J. (1984) Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of Mycobacterium tuberculosis. A correlate of virulence. Am Rev Respir Dis 129, 322-8.

Ordway, D.J., Martins, M.S., Costa, L.M., Freire, M.S., Arroz, M.J., Dockrell, H.M. and Ventura, F.A. (2005) [Increased IL-4 production in response to virulent Mycobacterium tuberculosis in tuberculosis patients with advanced disease]. Acta Med Port 18, 27-36.

Parish, C.R. (1999) Fluorescent dyes for lymphocyte migration and proliferation studies. Immunol Cell Biol 77, 499-508.

Payan, D.G., Brewster, D.R. and Goetzl, E.J. (1983) Specific stimulation of human T lymphocytes by substance P. J Immunol 131, 1613-5.

Perez, E., Samper, S., Bordas, Y., Guilhot, C., Gicquel, B. and Martin, C. (2001) An essential role for phoP in Mycobacterium tuberculosis virulence. Mol Microbiol 41, 179-87.

Precopio, M.L., Betts, M.R., Parrino, J., Price, D.A., Gostick, E., Ambrozak, D.R., Asher, T.E., Douek, D.C., Harari, A., Pantaleo, G., Bailer, R., Graham, B.S., Roederer, M. and Koup, R.A. (2007) Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. J Exp Med 204, 1405-16.

Rao, V., Dhar, N. and Tyagi, A.K. (2003) Modulation of host immune responses by overexpression of immunodominant antigens of Mycobacterium tuberculosis in bacille Calmette-Guerin. Scand J Immunol 58, 449-61.

Reece, S.T. and Kaufmann, S.H. (2008) Rational design of vaccines against tuberculosis directed by basic immunology. Int J Med Microbiol 298, 143-50.

Robinson, H.L. and Amara, R.R. (2005) T cell vaccines for microbial infections. Nat Med 11, S25-32.

Rosato, M.T., Jabbour, A.J., Ponce, R.A., Kavanagh, T.J., Takaro, T.K., Hill, J.P., Poot, M., Rabinovitch, P.S. and Faustman, E.M. (2001) Simultaneous analysis of surface marker expression and cell cycle progression in human peripheral blood mononuclear cells. J Immunol Methods 256, 35-46.

Rosenberg, E.S., Billingsley, J.M., Caliendo, A.M., Boswell, S.L., Sax, P.E., Kalams, S.A. and Walker, B.D. (1997) Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science 278, 1447-50.

Russell, D.G. (2001) Mycobacterium tuberculosis: here today, and here tomorrow. Nat Rev Mol Cell Biol 2, 569-77.

Sachsenberg, N., Perelson, A.S., Yerly, S., Schockmel, G.A., Leduc, D., Hirschel, B. and Perrin, L. (1998) Turnover of CD4+ and CD8+ T lymphocytes in HIV-1 infection as measured by Ki-67 antigen. J Exp Med 187, 1295-303.

Sander, C.R., Pathan, A.A., Beveridge, N.E., Poulton, I., Minassian, A., Alder, N., Van Wijgerden, J., Hill, A.V., Gleeson, F.V., Davies, R.J., Pasvol, G. and McShane, H. (2009) Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in Mycobacterium tuberculosis-infected individuals. Am J Respir Crit Care Med 179, 724-33.

Schaible, U.E., Sturgill-Koszycki, S., Schlesinger, P.H. and Russell, D.G. (1998) Cytokine activation leads to acidification and increases

maturation of Mycobacterium avium-containing phagosomes in murine macrophages. J Immunol 160, 1290-6.

Schaible, U.E., Winau, F., Sieling, P.A., Fischer, K., Collins, H.L., Hagens, K., Modlin, R.L., Brinkmann, V. and Kaufmann, S.H. (2003) Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nat Med 9, 1039-46.

Scholzen, T. and Gerdes, J. (2000) The Ki-67 protein: from the known and the unknown. J Cell Physiol 182, 311-22.

Schuck, S.D., Mueller, H., Kunitz, F., Neher, A., Hoffmann, H., Franken, K.L., Repsilber, D., Ottenhoff, T.H., Kaufmann, S.H. and Jacobsen, M. (2009) Identification of T-cell antigens specific for latent mycobacterium tuberculosis infection. PLoS One 4, e5590.

Scriba, T.J., Kalsdorf, B., Abrahams, D.A., Isaacs, F., Hofmeister, J., Black, G., Hassan, H.Y., Wilkinson, R.J., Walzl, G., Gelderbloem, S.J., Mahomed, H., Hussey, G.D. and Hanekom, W.A. (2008) Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. J Immunol 180, 1962-70.

Seder, R.A., Darrah, P.A. and Roederer, M. (2008) T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 8, 247-58.

Shafiani, S., Tucker-Heard, G., Kariyone, A., Takatsu, K. and Urdahl, K.B. (2010) Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. J Exp Med 207, 1409-20.

Shedlock, D.J., Talbott, K.T., Morrow, M.P., Ferraro, B., Hokey, D.A., Muthumani, K. and Weiner, D.B. (2010) Ki-67 staining for determination of rhesus macaque T cell proliferative responses ex vivo. Cytometry A 77A, 275-284.

Sherman, D.R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M.I. and Schoolnik, G.K. (2001) Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. Proc Natl Acad Sci U S A 98, 7534-9.

Skeiky, Y.A., Alderson, M.R., Ovendale, P.J., Guderian, J.A., Brandt, L., Dillon, D.C., Campos-Neto, A., Lobet, Y., Dalemans, W., Orme, I.M. and Reed, S.G. (2004) Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 172, 7618-28.

Skeiky, Y.A. and Sadoff, J.C. (2006) Advances in tuberculosis vaccine strategies. Nat Rev Microbiol 4, 469-76.

Skjot, R.L., Brock, I., Arend, S.M., Munk, M.E., Theisen, M., Ottenhoff, T.H. and Andersen, P. (2002) Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. Infect Immun 70, 5446-53.

Soto, C.Y., Menendez, M.C., Perez, E., Samper, S., Gomez, A.B., Garcia, M.J. and Martin, C. (2004) IS6110 mediates increased transcription of the phoP virulence gene in a multidrug-resistant clinical isolate responsible for tuberculosis outbreaks. J Clin Microbiol 42, 212-9.

Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., Ganz, T., Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S.A., Bloom, B.R., Krensky, A.M. and Modlin, R.L. (1998) An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 282, 121-5.

Stenger, S., Mazzaccaro, R.J., Uyemura, K., Cho, S., Barnes, P.F., Rosat, J.P., Sette, A., Brenner, M.B., Porcelli, S.A., Bloom, B.R. and Modlin, R.L. (1997) Differential effects of cytolytic T cell subsets on intracellular infection. Science 276, 1684-7.

Stubbe, M., Vanderheyde, N., Goldman, M. and Marchant, A. (2006) Antigen-specific central memory CD4+ T lymphocytes produce multiple cytokines and proliferate in vivo in humans. J Immunol 177, 8185-90.

Teitelbaum, R., Glatman-Freedman, A., Chen, B., Robbins, J.B., Unanue, E., Casadevall, A. and Bloom, B.R. (1998) A mAb recognizing a surface

antigen of Mycobacterium tuberculosis enhances host survival. Proc Natl Acad Sci U S A 95, 15688-93.

Tullius, M.V., Harth, G., Maslesa-Galic, S., Dillon, B.J. and Horwitz, M.A. (2008) A Replication-Limited Recombinant Mycobacterium bovis BCG vaccine against tuberculosis designed for human immunodeficiency virus-positive persons is safer and more efficacious than BCG. Infect Immun 76, 5200-14.

Tully, G., Kortsik, C., Hohn, H., Zehbe, I., Hitzler, W.E., Neukirch, C., Freitag, K., Kayser, K. and Maeurer, M.J. (2005) Highly focused T cell responses in latent human pulmonary Mycobacterium tuberculosis infection. J Immunol 174, 2174-84.

van Dissel, J.T., Arend, S.M., Prins, C., Bang, P., Tingskov, P.N., Lingnau, K., Nouta, J., Klein, M.R., Rosenkrands, I., Ottenhoff, T.H., Kromann, I., Doherty, T.M. and Andersen, P. (2010) Ag85B-ESAT-6 adjuvanted with IC31((R)) promotes strong and long-lived Mycobacterium tuberculosis specific T cell responses in naive human volunteers. Vaccine.

van Grevenynghe, J., Procopio, F.A., He, Z., Chomont, N., Riou, C., Zhang, Y., Gimmig, S., Boucher, G., Wilkinson, P., Shi, Y., Yassine-Diab, B., Said, E.A., Trautmann, L., El Far, M., Balderas, R.S., Boulassel, M.R., Routy, J.P., Haddad, E.K. and Sekaly, R.P. (2008) Transcription factor FOXO3a controls the persistence of memory CD4(+) T cells during HIV infection. Nat Med 14, 266-74.

Verreck, F.A., Vervenne, R.A., Kondova, I., van Kralingen, K.W., Remarque, E.J., Braskamp, G., van der Werff, N.M., Kersbergen, A., Ottenhoff, T.H., Heidt, P.J., Gilbert, S.C., Gicquel, B., Hill, A.V., Martin, C., McShane, H. and Thomas, A.W. (2009) MVA.85A boosting of BCG and an attenuated, phoP deficient M. tuberculosis vaccine both show protective efficacy against tuberculosis in rhesus macaques. PLoS One 4, e5264.

Von Eschen, K., Morrison, R., Braun, M., Ofori-Anyinam, O., De Kock, E., Pavithran, P., Koutsoukos, M., Moris, P., Cain, D., Dubois, M.C., Cohen, J. and Ballou, W.R. (2009) The candidate tuberculosis vaccine

Mtb72F/AS02A: Tolerability and immunogenicity in humans. Hum Vaccin 5, 475-82.

Wallace, P.K., Tario, J.D., Jr., Fisher, J.L., Wallace, S.S., Ernstoff, M.S. and Muirhead, K.A. (2008) Tracking antigen-driven responses by flow cytometry: monitoring proliferation by dye dilution. Cytometry A 73, 1019-34.

Wan, Y.Y. (2010) Regulatory T cells: immune suppression and beyond. Cell Mol Immunol 7, 204-10.

Weaver, C.T., Hatton, R.D., Mangan, P.R. and Harrington, L.E. (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 25, 821-52.

Weinrich Olsen, A., van Pinxteren, L.A., Meng Okkels, L., Birk Rasmussen, P. and Andersen, P. (2001) Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6. Infect Immun 69, 2773-8.

Wozniak, T.M., Ryan, A.A. and Britton, W.J. (2006) Interleukin-23 restores immunity to Mycobacterium tuberculosis infection in IL-12p40-deficient mice and is not required for the development of IL-17-secreting T cell responses. J Immunol 177, 8684-92.