Characterisation of the T cell responses induced by BCG in infants over the first year of life

By

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Declaration

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Abbreviations

°C	Degrees Centigrade
α	Alpha
γδ	Gamma delta
β	Beta
γ	Gamma
Ag85A	Antigen 85 protein A
Ag85B	Antigen 85 protein B
APC	Allophycocyanin
APCs	Antigen presenting cells
BCG	Bacille Calmette Guerin
CCL	Chemokine ligand
CFP10	Culture filtrate protein 10
CO_2	Carbon dioxide
CXCL	Chemokine CXC motif ligand
Cy5.5-PerCP	Cyanine-5.5-peridin-chlorophyll
Cy7	Cyanine 7
DC	Dendritic cells
DC-SIGN	DC Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DosR	Dormancy regulon
EDTA	Ethylenediamine tetra-acetic acid
EHR	Enduring hypoxic response
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
EPI	Expanded programme on immunisation
ESAT6	Early secretory antigenic target 6
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
Fox	Forkhead box

FSC	Forward Scatter
HIV	Human Immunodeficiency virus
Hrs	Hours
Hsp	Heat shock protein
IL	Interleukin
L.major	Leishmania major
LPS	Lipopolyscharride
LTBI	Latent tuberculosis infection
ManLAM	Mannosylated lipoarabinomannan
MDR	Multidrug resistant
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
Mins	Minutes
mL	Milliliter
Mtb	Mycobacterium tuberculosis
MV	Measles vaccine
NO	Nitric oxide
OPV	Oral polio vaccine
PAMPS	Pathogen associated molecular patterns
PBMC	Peripheral blood monuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
PPD	Purified protein derivative
PRR	Pattern recognition receptor
Qdot	Quantum dot
R	Receptor
rBCG	Recombinant BCG
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates

Rpfs	Resuscitation promoting factors
SATVI	South African Tuberculosis Vaccine Initiative
SSC	Side Scatter
TB	Tuberculosis
TCM	Central memory T cell
TCR	T cell receptor
TEM	Effector memory T cell
TGF-β	Transforming growth factor beta
Th	T helper lymphocyte
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TST	Tuberculin skin test
mg	Microgram
µl	Microliter
WHO	World Health Organisation
XDR	Extremely drug resistant

Abstract

Mycobacterium bovis Bacille Calmette Guerin (BCG) is the only licensed tuberculosis (TB) vaccine. Despite the immunisation of 3 billion individuals with this vaccine, TB remains a major cause of mortality worldwide. Therefore, there is an urgent need for more effective TB vaccines. BCG is likely to remain central to future TB prevention strategies, which could include a BCG prime at birth, followed by boosts with novel TB vaccines within the first year of life, or at later ages. Therefore, a comprehensive understanding of BCG induced immunity is required for the successful design and implementation of novel TB vaccination strategies. This was addressed in the following two studies.

The aim of the first study was to characterise specific T cell immunity following BCG vaccination. These data are critical to determine when to optimally boost BCG induced immunity in infants. We enrolled infants routinely vaccinated with BCG at birth, and determined the frequency of T cells induced by immunisation, at various time points over the first year of life. The T cells were identified by binding of cell surface markers and characterised by cell-specific cytokine production, following 12 hr incubation of infant whole blood with BCG. Multiparameter flow cytometry was used for the analysis. We found that the peak vaccine induced CD4+ T cell response occurred at 10 weeks, followed by a contraction phase. BCG specific CD4+ T cells became more polyfunctional, and acquired the profile of long-lived T cells (measured by Bcl-2 expression), over the first year of life. The magnitude of the BCG specific CD8+ T cell response was lower than the CD4+ T cell response and a peak in the vaccine induced CD8+ T cell response was not observed.

The aim of the second study was to investigate T cell recognition of *Mycobacterium tuberculosis* (Mtb) latency and reactivation associated antigens in 10-week old infants, routinely vaccinated with BCG at birth. Immune responses to these antigens could contribute to protection against TB disease; therefore we propose that antigens, if recognised following BCG vaccination, could be included in new TB vaccines, to boost BCG induced

immunity. Supernatant IFN-γ was measured by ELISA following 6 days' incubation of PBMC with antigens. We found that no specific latency and reactivation antigens were not recognised by the majority of BCG vaccinated infants (0-38% recognition). Importantly, 58% of the BCG vaccinated infants recognise at least one antigen, suggesting that a combination of the most widely recognised antigens in a novel TB vaccine should boost an immune response in the majority of BCG vaccinated infants.

Taken together, our findings have important implications for future TB vaccine strategies. First, the boosting of BCG primed T cells is probably ideal at 14 weeks of age, or later, because boosting earlier during the peak effector phase is likely to result in suboptimal vaccine responses. Second, our data suggests that BCG vaccination induces suboptimal responses to latency and reactivation associated antigens. There is a need for novel TB vaccine strategies, other than using the current BCG, to target these antigens for prevention of latency, or reactivation of latent Mtb.

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Chapter 1. Literature review

In this thesis, I aim to address 2 key questions that are relevant to TB vaccinology:

(i) What is the optimal age to administer a boost vaccine after priming with BCG vaccine at birth? This question is fundamental as current evidence suggests that boosting the immune response too early after prime immunisation may result in suboptimal responses (Sallusto et al., 2010; Wherry et al., 2004; Wherry et al., 2007).

(ii) Are latency and reactivation associated antigens recognised by T cells, induced by BCG vaccination of infants? The rationale is to determine whether a response to these antigens was primed by BCG, which can then be boosted with follow-up vaccination.

In chapter 1, I describe an overview of TB immunology and vaccinology, focusing on the aims and hypotheses of this thesis.

1.1 Aims and hypotheses

1. To characterise the longitudinal changes in T cell immunity induced by BCG vaccination of newborns.

i. To determine the kinetics of specific T cell induction, as defined by BCG-specific T cell-associated cytokine production.

ii. To determine the kinetics of anti-apoptotic marker, Bcl-2 and activation marker, CD38 in BCG specific T cells.

We hypothesise that the peak effector response occurs at 4 weeks during which, BCG specific T cells become highly activated and express CD38 and that after the contraction of the immune response, BCG specific T cells downregulate CD38 and express Bcl-2 to become long-lived T cells.

2. To determine the recognition of latency and reactivation associated antigens by T cells, induced by BCG vaccination of newborns.

i. To determine which latency and reactivation associated antigens are recognised after BCG vaccination.

ii. To determine the proportion of BCG vaccinated infants who recognise latency and reactivation associated antigens.

We hypothesise that BCG vaccination of newborns induces an immune response to latency and reactivation associated antigens.

1.2 Tuberculosis

1.2.1 Epidemiology

Mycobacterium tuberculosis (Mtb), the etiological agent of TB, has infected approximately one third of the world's population. In 2009, 9.4 million incident cases of TB disease occurred worldwide, of whom 1.7 million died (WHO Global TB Control 2010 Report). South Africa has the second highest incidence in the world: 970 cases per 100,000 in 2009 (Figure 1.1).

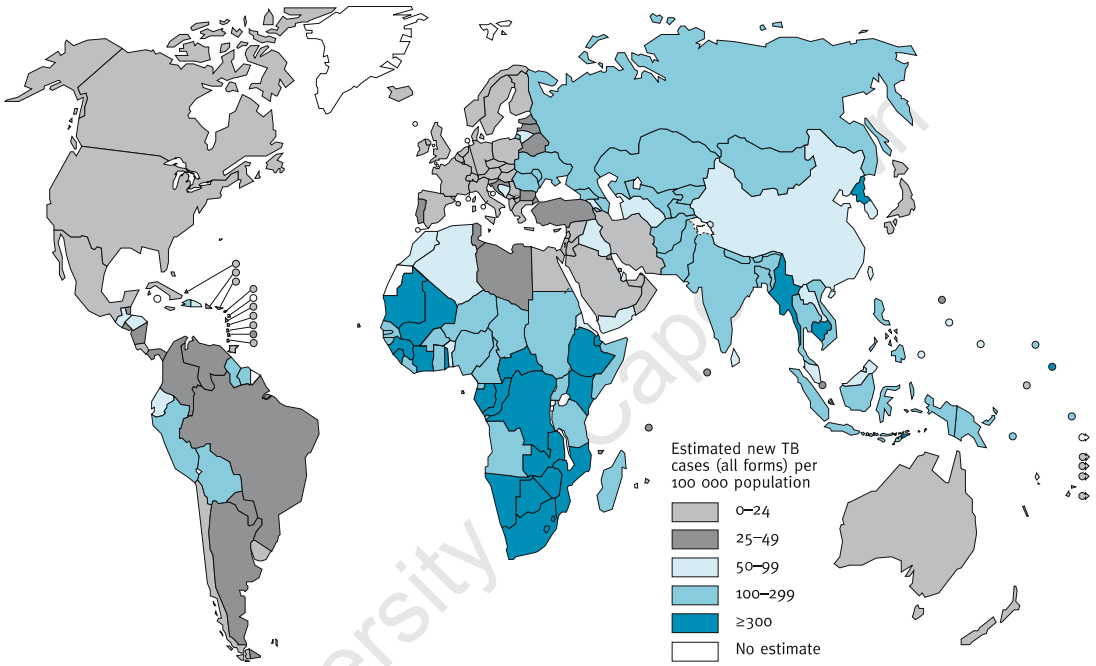

Figure 1.1. Worldwide incidence of TB (Source: WHO Global TB Control 2010 Report).

Adults who are infected with Mtb have a 10% chance of developing TB disease during their lifetime; 90% of latently infected individuals never develop disease. It is estimated that TB cases in children less than 15 years of age contribute up to 15% of the TB burden in highly endemic countries (Moyo et al., 2009). However, risk of developing TB disease following infection is much higher in younger children, with those less than 2 years old having up to 40-50% chance of progression (Hesseling et al., 2009). Hence, infants represent a subset of the population that are particularly vulnerable to TB, and will be the focus of studies presented here.

There are numerous drivers of the TB epidemic, including poor socio-economic status, poor nutrition and the human immunodeficiency virus (HIV).

Among other mechanisms, HIV depletes the host's CD4+ T cells, which are critical for successfully containing Mtb infection (Geldmacher et al., 2010). Recently, a study performed in the Western Cape showed a 24 and 17 fold higher risk of developing pulmonary TB and disseminated TB, respectively, in HIV infected infants, compared with HIV uninfected infants (Hesseling et al., 2009). TB is a major cause of mortality in HIV infected individuals.

The eradication of TB has become more challenging by the emergence of multidrug (MDR) and extensively drug resistant (XDR) Mtb strains. MDR strains are resistant to at least isoniazid and rifampicin, whereas XDR strains are resistant to isoniazid, rifampicin and at least 3 classes of second line drugs. MDR and XDR Mtb strains are associated with higher mortality rates, especially in HIV infected individuals (Gandhi et al., 2010). Moreover, in the past 45 years, no new TB drugs have been introduced, with the exception of fluoroquinolones (Caminero et al., 2006). Hence treatment options for MDR and XDR TB are limited. South Africa is classified as a global priority country by the World Health organization (WHO) for the improved management of MDR-TB. In 2009 alone, an alarming 7,343 cases of MDR TB were registered (WHO Global TB Control 2010 Report).

Given the burden caused by the TB pandemic, there is an urgent need for effective interventions. Vaccination remains the most sustainable solution to burden caused by infectious diseases (Moxon et al., 2011). Importantly, vaccination is likely to be as effective against MDR and XDR Mtb strains. Hence, our main focus here is on TB vaccines.

1.2.2 Clinical manifestations of TB

TB is primarily a disease of the lungs. Symptoms of pulmonary TB develop gradually, and include chest pain, prolonged coughing, fatigue, night sweats and weight loss (Harries and Dye, 2006). Chest radiography may show air space opacification and intrathoracic lymphadenopathy, pleural effusion, or even lung cavitations. Mtb can also disseminate to other organs. The most severe forms of disseminated TB are miliary TB, where multiple organs are involved, and TB meningitis. Other organs that may be involved include bone, the gastro-intestinal tract and kidneys.

The clinical manifestations of TB in infants and children differ from those in adults (Marais et al., 2004). Infants and children often manifest mild lung disease with intrathoracic adenopathy and the complications of the latter, such as airway compression. Cavitary lung lesions are crucial for TB disease transmission since these lesions allow dissemination of Mtb; however, patients from this age group rarely develop cavitary lung TB, which is characteristic in adults. Moreover, TB disease in infants and children is paucibacillary, compared with multibacillary disease in adults; therefore, infants and children do not contribute significantly to TB transmission (Marais et al., 2005a; Marais et al., 2005b). Adult-type disease classically develops after the onset of adolescence.

In our efforts to prevent TB through interrupting transmission, it is important to prioritise adolescents and adults for new TB vaccines. However, children and infants make up a significant proportion of the disease burden, and it is ethically correct to also target this population for new TB vaccines. Our focus in this study was therefore on infants.

1.2.3 Diagnosis of TB

TB can be diagnosed by smear or culture of acid-fast bacilli from sputum samples. Recently, the Xpert Mtb/RIF molecular test has been developed, which allows the direct detection of Mtb and resistance to rifampicin in sputum in an automated, closed system, through amplification of mycobacterial components (Boehme et al., 2010). Mutations in the *rpoB* gene are used to indicate resistance to rifampicin. The molecular test was able to identify 98.2% and 72.5% of Mtb culture positive TB patients who were routine smear positive and smear negative, respectively. Importantly, this test provides results within 2 hours.

The paucibacillary nature of TB in infants renders infant TB diagnosis difficult (Newton et al., 2008). Infants often produce less sputum than adults and swallow, rather than expectorate the sputum. Bacterial samples can be obtained by gastric washes early in the morning, although it requires the infant to be admitted to the hospital. Importantly, test results from acid-fast bacilli

cultures remain negative in about 70% of cases with probable TB (Zar et al., 2005).

Diagnosis of latent infection is classically performed using the tuberculin skin test (TST), which involves the intradermal injection of purified protein derivative (PPD) of tuberculin. Due to cross reactivity with other environmental mycobacterium species and with BCG, TST can give false positive results. However, it has been shown that a TST induration ≥ 10 mm is a good indicator of latent infection in high TB prevalence settings (Middelkoop et al., 2008; Wood et al., 2010). More recent diagnostic tests for the detection of latent TB include the QuantiFERON® TB Gold In-Tube (QFT^{TM}) and T-SPOT.TB (Mori et al., 2004). These commercial kits rely on the production of IFN-γ to specific Mtb antigens, including ESAT-6 and culture filtrate protein-10 (CFP-10).

1.3 Host response to Mtb

Extensive evidence suggests critical importance of T cells in the control of Mtb infection. Hence, we measured T cell outcomes to achieve the aims of this study. In this section, we first describe the innate immunity to Mtb, since the innate arm of the immune response plays a key role in mediating adaptive immunity.

1.3.1 Innate immunity

TB is transmitted via the aerosol route, through inhalation of infected microdroplets. Upon the arrival of the Mtb in the alveoli, the bacteria are phagocytosed by alveolar macrophages and dendritic cells (DCs), a process that involves specific receptors. These cells recognise Mtb as foreign through pattern recognition receptors (PRRs). PRRs are germ line encoded receptors that recognise pathogen molecular associated patterns (PAMPs), which are invariant molecular structures expressed by most microbes. PRR activation by Mtb ligands results in a signaling cascade via adaptor molecules MyD88 and Card9 (Dorhoi et al., 2010; Dorhoi and Kaufmann, 2009). This leads to the production of cytokines and chemokines, as well as the triggering of anti-microbial mechanisms.

The most well characterised PRRs in the context of Mtb recognition are the Toll like receptors (TLRs). The observation that mice deficient in MyD88, the main adaptor-signaling molecule for most TLRs, succumbed to Mtb infection more rapidly than control mice provided early evidence about the importance of TLRs (Fremond et al., 2004). TLR2 binds to peptidoglycan from Mtb whereas TLR4 recognise lipopolyscharride, and TLR9 recognise CpG motifs from Mtb DNA (Akira et al., 2006). TLR2 and TLR4 deficient mice have reduced ability to clear Mtb infection, compared with wild type mice and succumbed after 5 months post-infection (Abel et al., 2002; Drennan et al., 2004).

Other PRRs that have been shown to be involved in mycobacterial recognition include the nod like receptors (NOD) and C type lectin receptors (CLR). NOD2 recognises peptidoglycan, whereas CLR, DC-SIGN recognise mannosylated lipoarabinomannan (ManLAM) of Mtb (Ferwerda et al., 2005; Geijtenbeek et al., 2003).

Immune evasion mechanisms

In most cases, phagocytic cells are unable to eradicate Mtb. This is because the pathogen has developed an array of mechanisms to evade phagocyte clearance, and to evade other components of the immune system aimed at supporting phagocytic function.

After phagocytosis, intracellular bacteria are contained within a phagosome (Armstrong and Hart, 1971). Fusion of the phagosome with a lysosome leads to the formation of a phagolysosome. Classically, bacteria are killed inside the highly acidic and hypoxic environment of the phagolysosome, which contains many antibacterial molecules. However, Mtb prevents phagolysosome fusion, and prevents acidification of this compartment, allowing the pathogen to persist and replicate.

Mtb also prevents maturation of DCs (Geijtenbeek et al., 2003). Dendritic cells are key players in mediating the adaptive immune response to Mtb. Immature DCs are highly specialised at capturing and processing antigens whereas mature DCs are very efficient at activating naïve T cells, leading to the

initiation of the adaptive immune response. Mannosylated lipoarabinomannan (ManLAM) binding to C type lectin receptor, DC-SIGN blocks DC maturation, possibly by interfering with TLR signaling (Geijtenbeek et al., 2003). LPS, which binds to TLR4, is unable to activate DCs in the presence of manLAM. Blocking of DC-SIGN restores LPS mediated DC maturation. Therefore, mycobacteria interfere at an early phase of initiation of adaptive immunity, as mature DCs traffic to the mediastinal lymph node to present antigens to naïve T cells (Reiley et al., 2008; Wolf et al., 2008). Naïve T cells become activated and traffic back to the lungs to form granulomas, leading to the containment of infection.

The initiation of the adaptive immune response to Mtb is delayed, when compared with those caused by similar intracellular pathogens, such as *Listeria monocytogenes* (Kursar et al., 2002). In mice, the adaptive immune system is activated 8 to 11 days post Mtb infection only, a time when mycobacteria are detected in regional lymph nodes (Wolf et al., 2008). This delay results in an exponential expansion of mycobacteria in the lung, allowing a foothold for infection or disease. The exact mechanism of this delayed induction of adaptive immunity remains poorly understood, but is an example of how effectively Mtb evades host responses.

The granuloma

Successful containment of the bacilli inside granulomas occurs in 90% of infected individuals. Granulomas are pathological hallmarks of TB, and are organised structures that contain B cells, T cells and macrophages (Russell et al., 2010) (Figure 1.2). Inside the granuloma, macrophages can form giant multinucleated cells or foamy macrophages, which are filled with Mtb lipids. Progression to TB disease is characterised by increased cell death, and the accumulation of caseum within the centre of the granuloma. Rupture of the granuloma into airways can lead to local dissemination of bacilli, with more parts of the lung becoming involved.

Granuloma formation is generally accepted to be beneficial to the host, since these structures contain Mtb. However, recent evidence suggests that early granuloma formation may be beneficial to Mtb, since the recruitment of

macrophages enables Mtb to infect many other macrophages (Davis and Ramakrishnan, 2009). This leads to an expansion of bacterial numbers. Regardless, Mtb is able to persist inside the granulomas for years without causing any symptoms, leading to the establishment of a latent infection. Because latently infected persons represent a pool of people at risk of future TB disease, there is a need for new TB vaccines to also target Mtb during latency, and before reactivation of latent Mtb to cause TB disease. In our study, we are investigating the immune recognition of multiple antigens that are expressed during latency and reactivation of latent Mtb, for a potential inclusion in new TB vaccines.

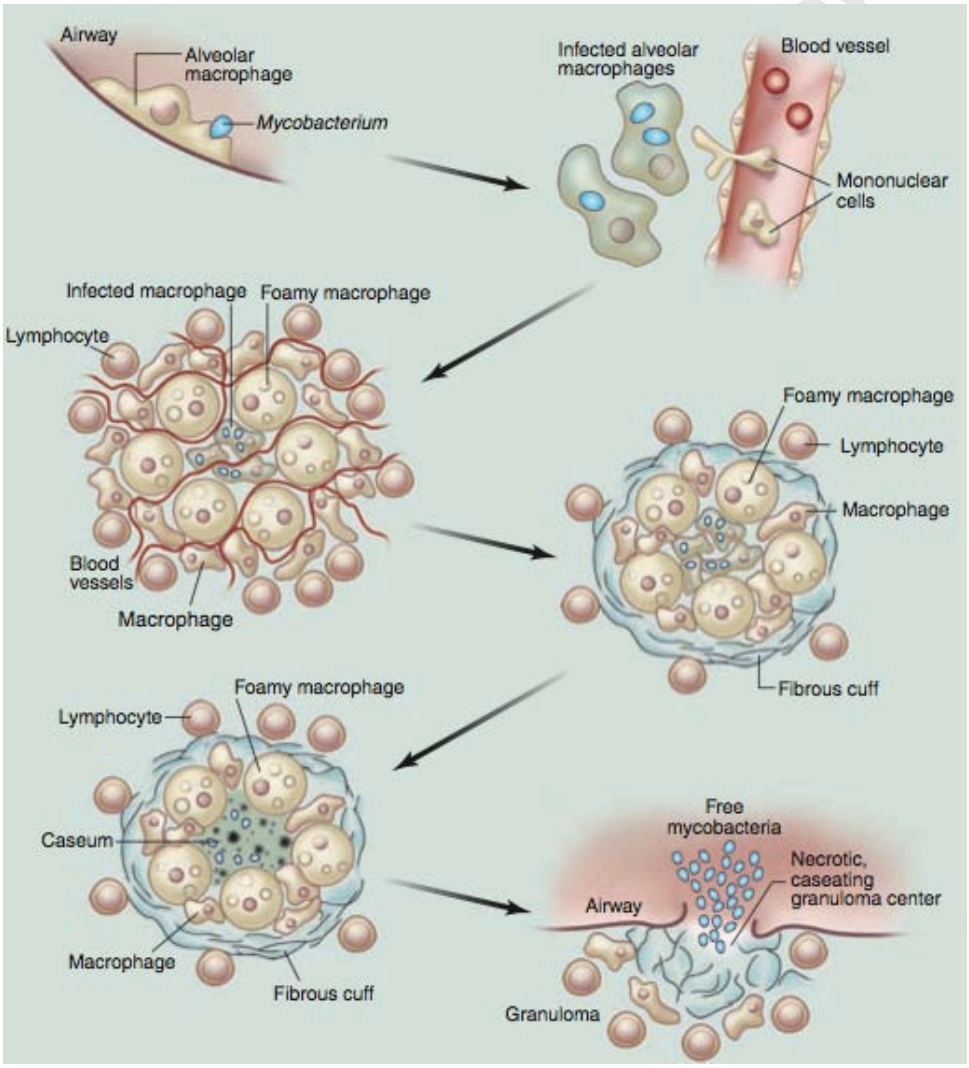

Figure 1.2. Granuloma formation during Mtb infection (Russell et al., 2010).

1.3.2 T cells

The adaptive immune response is required for the successful containment of Mtb within a granuloma, and is primarily mediated by different T cells subsets. Extensive evidence suggests the importance of CD4+ T cells in mediating protection against Mtb. Depletion of CD4+ T cells in HIV infection is one mechanism for increasing risk of developing active TB disease (Geldmacher et al., 2010). Mice devoid of MHC II expression, and therefore unable to prime CD4+ T cells fail to control Mtb infection, compared with wild type mice, resulting in their death by day 77 post infection (Mogues et al., 2001).

T helper 1 cells (Th1 cells)

Different subclasses of CD4+ T cells contribute to the immune responses to Mtb. The cytokine milieu dictates the differentiation of naïve CD4+ T cells into these different subsets (Abbas et al., 1996) (Figure 1.3). T helper 1 (Th1) CD4+ T cells are formed in the presence of IL-12; these cells produce IFN-γ, TNF-α and IL-2, cytokines that are critical in protective immunity against Mtb (Section 1.3.5). IFN-γ activates infected macrophages to produce NOS, whereas TNF-α interacts synergistically with IFN-γ to maintain granuloma formation (Flynn et al., 1995; Ray et al., 2009). In addition, IL-2 is required for the expansion of Mtb specific T cells. Hence, a Th1 phenotype is associated with protection against Mtb, whereas a Th2 phenotype, which is characterised by the expression of IL-4 and IL-13, blocks Th1 differentiation and correlates with increased susceptibility to Mtb in mice (Wangoo et al., 2001). Therefore, we propose that the measurement of Th1 cells is important in the assessment of the BCG induced immune response in infants.

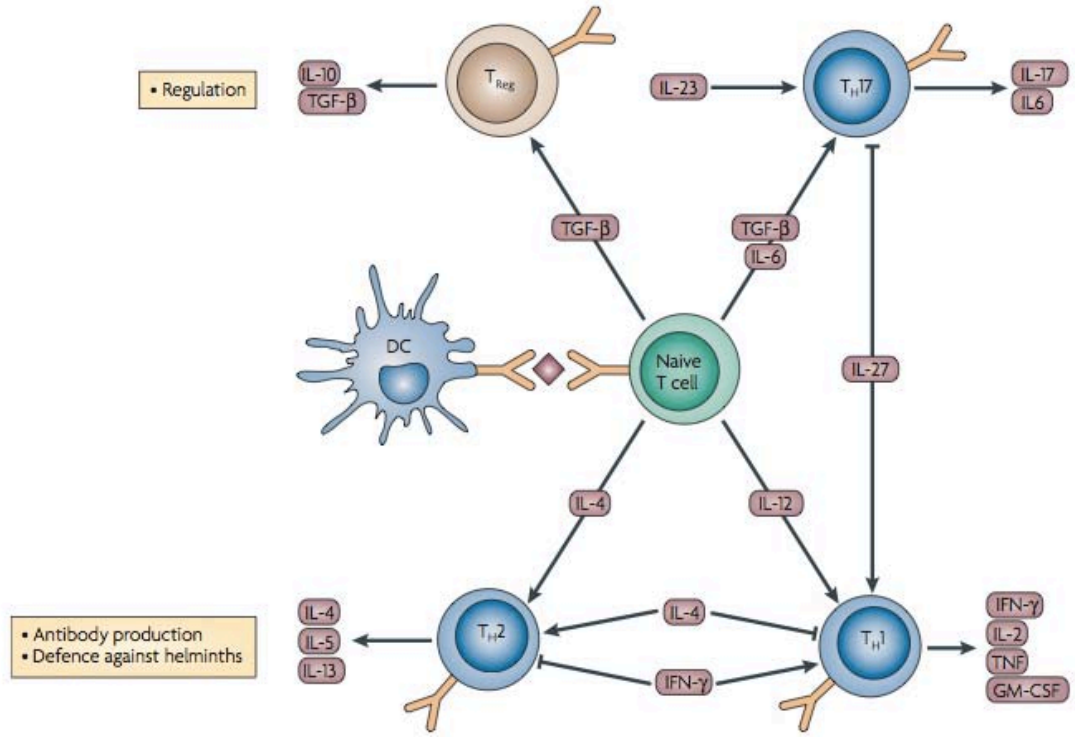

Figure 1.3. Differentiation of different subsets of CD4+ T cells (Kaufmann, 2007).

The presence of multifunctional Th1 cells, which produce multiple cytokines together, have been associated with protection in a murine model of another intracellular bacterium, *Leishmania major* (Darrah et al., 2007). Moreover, better clinical outcome have been associated with the presence of these polyfunctional CD8+ T cells in HIV infected individuals (Betts et al., 2006; Kannanganat et al., 2007a; Kannanganat et al., 2007b).

Surprisingly, higher frequencies of multifunctional CD4+ T cells have been found in TB patients compared to latently infected individuals, suggesting that this phenotype does not correlate with protection to Mtb (Caccamo et al., 2010; Young et al., 2010). Similarly, multifunctional CD4+ T cells do not correlate with protection against TB, after BCG vaccination of newborns (Kagina et al., 2009): there were no differences in multifunctional T cells producing IFN-γ, TNF-α and IL-2 in BCG vaccinated infants who developed TB disease and those who remained healthy after two years follow up. Recently, we have shown increases in the frequency of polyfunctional T cells in TB patients during anti-TB therapy, suggesting that presence of these T cells is associated with a recovering, and more optimal immune response (Day et al., 2011).

Interestingly, it has been shown that frequency of single-positive TNF-α Mtb specific CD4+ T cells was higher in people with active TB disease compared to latently infected individuals (Harari et al., 2011). Hence, the frequency of single TNF-α positive cells was used as an outcome of this study when determining the BCG induced immunity in infants.

T helper 17 cells (Th17 cells)

In humans, a relatively newly discovered subset of CD4+ T cells, Th17 subset, is formed in the presence of IL-1β and IL-6, among other cytokine combinations (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). IL-17 is a pro-inflammatory cytokine that has been implicated in pathogenesis of many autoimmune diseases. The cytokine's role in protection against TB has not been established. It has been reported that Th17 cells may contribute to the recall response against Mtb (Khader et al., 2007). Th17 cells are induced in mice vaccinated with an immunodominant epitope of ESAT-6. Upon challenge with Mtb, these cells produce chemokines CXCL9, CXCL10 and CXCL11, leading to the recruitment of Th1 cells to the site of infection. This results in the control of bacterial growth. Moreover, IL-17 induces the recruitment of neutrophils to the site of infection, and contributes to granuloma formation. Our group has demonstrated the presence of memory CD4+ T cells that produce IL-17 in Mtb-exposed individuals and in patients with TB, supporting a role for these cells in immunity to mycobacteria (Scriba et al., 2008). Hence, we investigated the ability of BCG specific CD4+ and CD8+ T cells to produce IL-17 in infants.

Regulatory T cells

In presence of TGF-β, naïve T cells differentiate into regulatory T cells (Tregs), which express high levels of CD25 and of Foxp3 (Belkaid and Tarbell, 2009). Tregs regulate effector immune responses. This may be of particular importance for protection against tissue damage, by dampening the immune responses. On the other hand, excessive down regulation of effector responses can lead to lesser ability to clear the pathogen. It has been shown that TB patients have a higher frequency of Tregs in peripheral blood when compared with latently infected individuals, suggesting that Treg expansion

may have contributed to down regulation of Th1 responses during TB disease (Guyot-Revol et al., 2006). In addition, Tregs isolated from infected individuals have the ability to suppress the T cell responses to Mtb antigens in vitro (Hougardy et al., 2007). In mice, Shafiani and colleagues demonstrated that small numbers of Mtb specific Tregs are capable of delaying the recruitment and subsequent accumulation of effector T cells to the lungs (Shafiani et al., 2010). This mechanism may contribute to the delay in induction of adaptive immunity described above. In contrast, Green et al. showed that following Mtb infection, macaques that ultimately developed latent disease had a higher frequency of Tregs in broncho-alveolar lavage compared with macaques that developed disease (Green et al., 2010). Moreover, there was an increase in the frequency of Tregs during development of TB disease. Whether the increase in Tregs frequency was triggered by inflammation, or Tregs contributed to progression to TB disease is as yet unknown.

The identification of specific Tregs is difficult, as markers that are classically used to identify the cells, like CD25 and FoxP3, may also be upregulated by other cellular populations during functional assay. We therefore chose not to measure Tregs in our study.

CD8+ T cells

Protective immunity against TB may also rely on CD8+ T cells (Figure 1.4), as suggested by the rapid migration of CD8+ T cells to the sites of infection in the mouse lung (Serbina and Flynn, 1999) and their presence in murine granulomas, in mice (Gonzalez-Juarrero et al., 2001). The magnitude of the CD8+ T cell response to Mtb is often lower than that of CD4+ T cells, but mice deficient in β2-microglobulin (therefore, MHC class I expression) are more susceptible to Mtb challenge and have a higher bacterial load, compared with wild type mice (Flynn et al., 1992).

CD8+ T cells are capable of multiple functions. The cells produce IFN-γ and TNF-α, as well as cytotoxic molecules such as perforin, granulysin and granzymes, which can kill infected cells (Stenger et al., 1998; Stenger et al., 1997). Perforin creates pores in the membranes of infected cells allowing granulysin to penetrate the infected cells, causing apoptosis. This may reduce

the pool of infected cells, leading to the release of bacteria, which may be killed more efficiently by activated macrophages. Granzymes, on the other hand, can induce cell death by activating pro-caspases or molecules that can cause DNA damage (Russell and Ley, 2002)

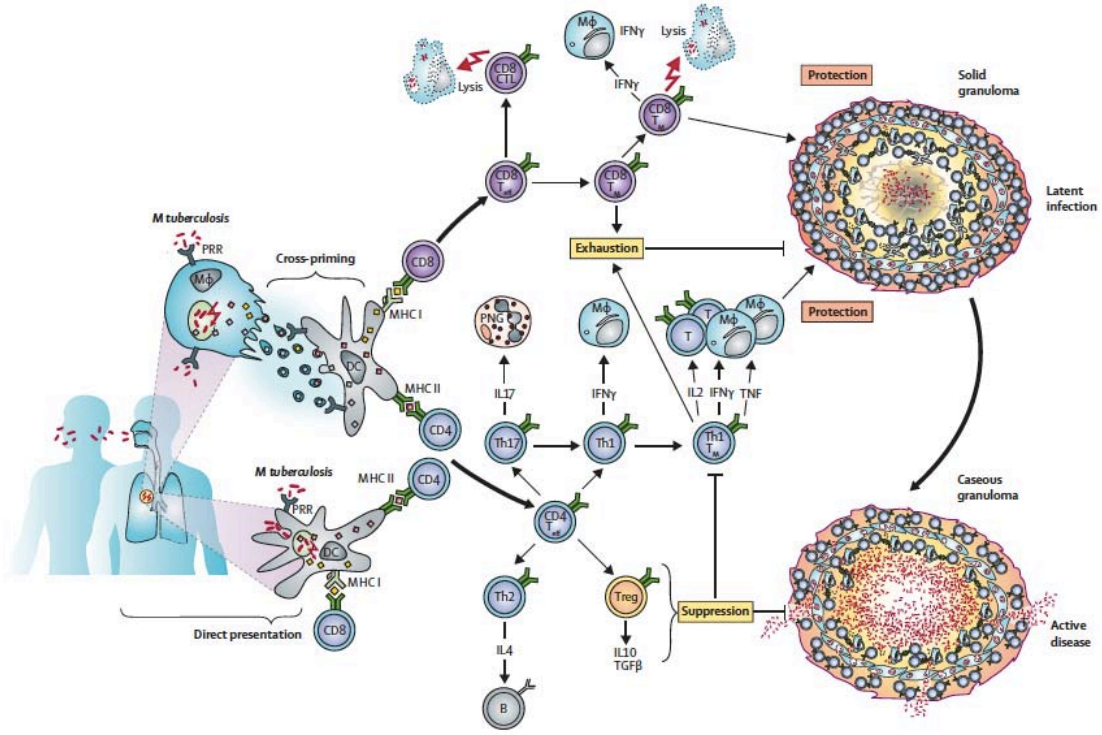

Figure 1.4. Overview of the immune response against Mtb (Kaufmann et al., 2010).

The importance of CD8+ T cells in the immune response to Mtb was initially controversial. This is because it was unclear how Mtb, which normally resides in phagosomes, could gain access to the cytosol leading to the presentation of mycobacterial peptides via the MHC class I pathway. Recently, it has been reported that Mtb is able to egress into the cytosol of infected DCs, allowing the direct presentation of Mtb antigens by MHC I molecules (van der Wel et al., 2007). Cross priming of CD8+ T cells can also occur, whereby apoptosis of infected macrophages causes the release of vesicles containing mycobacterial antigens; these vesicles are taken up by by-stander DCs that can then present the antigens through the MHC I pathway (Winau et al., 2006). Given the role of CD8+ T cells in protective immunity to Mtb, specific CD8+ T cells were assessed in this study, when investigating the immune responses induced by BCG in infants.

Unconventional T cells

Unconventional T cells constitute a small fraction of the total circulating T cells, but have been implicated in immunity against Mtb. CD1 restricted T cells recognise lipid and glycolipids presented on CD1 molecules (Park and Bendelac, 2000). Five isoforms of CD1 exist in humans; CD1a, b, c, d and e. Stimulation of PBMC with Mtb lipid extract resulted in significant proliferation of CD1 restricted T cells in Mtb infected individuals compared with Mtb uninfected individuals, suggesting that CD1 T cells contribute to immunity against Mtb (Ulrichs et al., 2003).

Gamma delta (γδ) T cells express a T cell receptor made up of γ and δ chains, and recognise phosphate-containing antigens. These unconventional T cells are significant IL-17 producers early after infection of mice with BCG (Umemura et al., 2007). In humans, expansion of γδ T cells occurs following BCG vaccination of newborns and adults (Lee et al., 2004). Re-stimulation of peripheral blood from BCG vaccinated adults with mycobacterial antigens induced a significant proliferation of γδ T cells (Hoft et al., 1998). Furthermore, a dramatic decrease in γδ T cells has been found in peripheral blood of TB patients, consistent with the notion that specific γδ T cells may have migrated to the disease site, and therefore could contribute to the immunity against Mtb (Li et al., 1996). Although unconventional T cells may play a role in protective immunity to Mtb, these cells are uncommon in peripheral blood, and our small infant blood volumes precluded accurate assessment of these populations.

1.3.3 Cytokines

Cytokines are crucial soluble mediators of the immune response to pathogens. It is well established that IFN-γ is required for protective immunity against TB, although IFN-γ alone is not sufficient for protection (Lalvani and Millington, 2008). Mice deficient in IFN-γ production have uncontrolled bacterial growth, necrotic granulomas and defective macrophage activation (Dalton et al., 1993; Flynn et al., 1993). Additionally, individuals with mutations in any of the IFN-γ receptor signaling pathways are high susceptible to TB and to other non-tuberculous mycobacteria (Casanova and Abel, 2002; Newport et al., 1996). IFN-γ is central in the activation of macrophages

(MacMicking et al., 1997). Macrophage activation promotes the maturation of the phagolysosome and the production of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). Importantly, IFN-γ acts synergistically with TNF-α to maintain granuloma formation (Flynn et al., 1995). IFN-γ is also able to induce autophagy in activated macrophages, which inhibits the survival of BCG and Mtb in infected macrophages (Gutierrez et al., 2004; Singh et al., 2006).

As stated above, TNF-α acts in synergy with IFN-γ in the formation of the granuloma (Flynn et al., 1995). Lack of granuloma formation, reduced RNI production and increased bacillary load are observed in TNF-α deficient mice (Botha and Ryffel, 2003). In humans, treatment with anti-TNF drugs often leads to reactivation of latent Mtb infection to TB disease (Keane, 2004). However, TB patients have higher frequencies of single TNF-α positive T cells compared with latently infected individuals, suggesting that TNF-α can also contribute to the increased inflammation, in addition to protection (Harari et al., 2011). This is dependent on the amount of TNF-α present; suboptimum level of TNF-α leads to the loss of protection against TB disease whereas, an excess of TNF-α results in excess inflammation and exacerbation of TB disease (Flynn and Chan, 2001; Flynn et al., 1995; Tsao et al., 2000).

While IL-2 is not exclusively associated with Th1 cells, the cytokine is critical for the expansion of Th1 cells and the formation of immunological memory (Dooms et al., 2004; Dooms et al., 2007). In the absence of IL-2, primed T cells are unable to trigger an effective recall response. The findings described above strongly support the measurement of these Th1 cytokines in our study in order to obtain a comprehensive understanding of BCG induced immune responses.

1.4 Latency

Latency is the result of the balance between the host immune response and the virulence of the pathogen (Lin and Flynn, 2010). On one hand, host defense mechanisms are induced to kill Mtb, on the other hand, the pathogen is able to evade the host immune system and persist, leading the incomplete eradication of bacilli, but to the absence of clinical symptoms in individuals

who are Mtb infected. The physical and physiological state of Mtb during latency is still a much-debated subject. It is generally accepted that during latency, the bacteria exist in an altered metabolic state, while subjected to oxygen and nutrient deprivation (Ehlers, 2009). Whether Mtb is in a non-replicating state or in a slow replicating state, is unclear. Perhaps, the fact that isoniazid, which targets only replicating mycobacteria, is effective in preventing TB reactivation, suggests that some organisms do replicate during latency and these organisms may be targeted by isoniazid upon reactivation (Diel et al., 2005). This highlights the need for new TB vaccines to target Mtb during latency. We therefore propose to assess the immune recognition of antigens that are expressed during latency following BCG vaccination, for potential inclusion in new TB vaccines.

1.4.1 Adaptation of Mtb to latency

Study of latency is fundamental for understanding how Mtb is able to persist in the human host for decades, but has been rendered difficult due the lack of animal models that truly represent human latency. Current animal models include the mouse, the guinea pig, the rabbit and the non-human primate. In mice, a high bacillary load is maintained throughout the infection (Flynn, 2006). Despite the formation of a granuloma, and cellular accumulation in the lungs, pathological consequences of TB such as caseation and cavitation, are rarely seen (Gill et al., 2009). Lesions seen in guinea pigs and rabbits show caseous necrosis similar to those observed in human lung granuloma (Via et al., 2008). However, guinea pigs are hypersusceptible and do not remain latently infected whereas rabbits are relatively resistant to Mtb; only specific clinical Mtb strains can be used to infect these animals. The use of the rabbit model also remains limited due to the lack of immunological reagents (Flynn, 2006; Tsenova et al., 2005). The non-human primate appears to be the only model that mimics human latent infection (Green et al., 2010). The use of this animal model remains expensive. Approximately 50% of cynomolgus macaques become latently infected after infection, whereas 50% develop active TB disease. In contrast, only 10% of immune competent humans develop TB disease after Mtb infection whereas 90% remain latently infected.

The availability of new techniques such as global gene expression assessment has enabled profiling of Mtb grown under hypoxic or nutrient starvation conditions, mimicking conditions which may be encountered by the bacilli during latency in the human host. Upregulation in a set of genes called the dormancy survival genes was shown, following hypoxia (Voskuil et al., 2003). Lesser hypoxia was accompanied by a decline in the expression of the *dos* regulon. The *dos* regulon consists of 48 genes and is regulated by the response regulator, the DosR protein (Rv3133c) (Kumar et al., 2007). Rv3133c is under the control of two sensor histidine kinases, DosT and DosS. Transient phosphorylation of either or both DosT and DosS, followed by the transfer of the phosphate to DosR, occurs during activation of DosR (Honaker et al., 2009). Comparison of the DosR mutant strains with wild type parental Mtb strains after growth of both strains for 50 days under hypoxic conditions, revealed that there is a 200-fold reduction in the viability of the mutant strains (Voskuil et al., 2003). Therefore, low oxygen levels induces DosR expression, which allows the pathogen to survive during long periods of *in vitro* dormancy.

Recently, Rustad et al. showed that the dos regulon is only transiently activated during conditions of hypoxia and that a second set of genes, called the enduring hypoxic response (EHR) genes, are upregulated in response to prolonged period of hypoxia (Rustad et al., 2008). This regulon consists of 230 genes and is regulated in a DosR-independent manner, although the exact function and regulation of these genes have yet to be identified.

Genes associated with nutrient starvation have also been shown to be expressed during latency. In an in vitro model of nutrient starvation, Betts and colleagues showed that there is a downregulation of several genes associated with aerobic respiration, cell division and lipid biosynthesis, as measured by microarray analysis (Betts et al., 2002). In this model, Mtb cultures were grown for 7 days in nutrient rich media and transferred thereafter into phosphate buffered saline (PBS). In addition, the transcription profile of Mtb in the lungs of infected mice showed that genes involved in carbon metabolism are downregulated whereas those involved in fatty acid metabolism are upregulated 4 and 9 weeks post infection (Timm et al., 2003). These

adaptations may ultimately allow the bacilli to survive long periods of nutrient starvation in the human host (Betts et al., 2002).

Recently, genes involved during Mtb reactivation have been characterised. Sherrid et al. developed an in vitro model of Mtb reactivation, in which Mtb were subjected to hypoxia and thereafter transferred into roller bottles to promote aeration, resulting in bacterial growth (Sherrid et al., 2010). Microarray analysis of Mtb cultures following aeration reavealed the upregulation of approximately 100 genes, among which are genes involved in transcription, translation and fatty acid synthesis. Another group of proteins called the resuscitation promoting factors (Rpfs) are involved during Mtb reactivation. Rpfs were initially identified in *Micrococcus luteus* and are important in promoting bacterial growth from a viable but non replicating state in vitro (Mukamolova et al., 2002). Five Rpfs genes expressed by Mtb have now been identified and have been shown to be expressed in vitro (Mukamolova et al., 2002; Tufariello et al., 2004). Mtb mutants lacking Rpfs are impaired in the ability to resusciate or grow following prolonged periods of nutrient starvation and hypoxia, compared with wild type, suggesting that Rpfs are important for Mtb reactivation (Downing et al., 2005; Kana et al., 2008).

Collectively, the data suggests that in order to adapt to environmental pressure, Mtb expresses different set of antigens. We therefore propose that antigens expressed during latency and reactivation may be important in the adaptation and survival of Mtb during the different life stages. These antigens could be potential candidates for new TB vaccines and highlights the need to investigate the T cell recogniton of these antigens following BCG vaccination.

1.4.2 Immune recognition of latency-associated antigens

Multiple reports have documented human immune recognition of latency-associated antigens, including DosR antigens and genes expressed during nutrient starvation. Leyten and colleagues tested 25 novel DosR antigens most highly expressed in Mtb cultures subjected to hypoxic conditions (Leyten et al., 2006). All 25 DosR antigens induced proliferation and IFN-γ production in T cell lines generated from TB patients and TST+ individuals. These results were confirmed using direct ex vivo experiments, when Mtb infected

individuals recognised 18 of the 25 antigens. These results suggest that the antigens are expressed during natural infection by Mtb. Antigens Rv1733c, Rv2029c, Rv2627c and Rv2628 were the most highly recognised in this study, with 61%, 61%, 52% and 35% responders, respectively. Importantly, DosR antigens were more highly recognised by TST+ individuals and induced higher IFN-γ production than TB patients, possibly indicating that immune responses to DosR antigens may contribute to the protection against TB disease. On the other hand, there is a higher recognition of immunodominant antigen culture filtrate protein 10 (CFP10) in TB patients, compared with latently infected individuals.

Overall, these results suggest that host responses to latency-associated antigens can be candidate biomarkers for latent infection, and therefore risk of disease reactivation (Schuck et al., 2009). It has been proposed that latency-associated antigens can be included in new TB vaccines to generate or boost responses expressed during latency, in order to reduce progression of latent infection to TB disease (Demissie et al., 2006). Therefore, in our study we are assessing immune responses to numerous latency-associated antigens in infants, following BCG vaccination.

1.5 Vaccination and memory

Immunological memory results in a more robust and rapid response upon re-infection with the same pathogen, and is the hallmark of a successful adaptive immune response (Figure 1.5) (Williams and Bevan, 2007). Following primary recognition of pathogen-derived peptides on MHC by naïve T cells in lymph nodes, these cells become activated, proliferate and migrate to the site of infection. After clearance of the infection, the effector immune response contracts; most pathogen specific T cells die by apoptosis but approximately 5-10% survive to form a population of memory cells. The survival of these memory cells appears to be antigen independent, but relies on the action of IL-7 and IL-15 (Surh et al., 2006). Upon secondary infection with the same pathogen, memory T cells undergo clonal expansion and differentiate into effector cells, leading to the rapid clearance of the pathogen. This is the basis of vaccination. Therefore, the ability of BCG to induce memory T cells that will

provide long-lived immunity is an important outcome when measuring BCG induced immunity in infants.

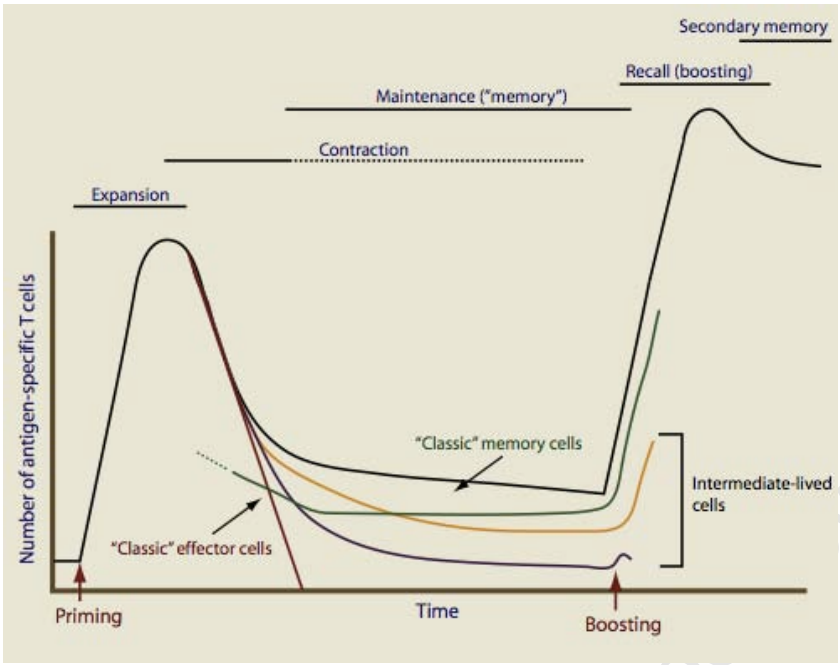

Figure 1.5. The formation of immunological memory. The black line represents the change in numbers of antigen specific cells following infection or vaccination. The red and green lines represent number of effector and memory T cells, respectively. Some cell populations have intermediate longevity and can contribute to the protection (maroon and gold lines) (Jameson and Masopust, 2009).

1.5.1 Diversity of memory T cell subsets

Classically, memory T cells have been classified according to the expression of CD45RA and CCR7 (Sallusto et al., 1999). Effector memory T cells have been described as being $CD45RA^{-}CCR7^{-}$ whereas central memory T cells are $CD45RA^{-}CCR7^{+}$. Terminally differentiated memory T cell, particularly among the CD8+ T cell subset, may re-express CD45RA. Effector memory T cells are located at the sites of inflammation where these cells have immediate effector functions. In contrast, central memory cells are long-lived T cells and are located in the lymph nodes, where these cells have better capacity than effector T cells to proliferate and differentiate into larger numbers of effector T cells. Therefore, the aim of vaccination is to induce central memory T cells to provide long-lived protection (Ahmed and Gray, 1996). This notion has been challenged recently in an HIV model. Vaccination of rhesus macaques with

SIV vaccine, which consisted of rhesus cytomegalovirus vectors, resulted in persistent effector memory T cell responses (Hansen et al., 2011). Rhesus macaques vaccinated with this vaccine controlled pathogenic SIV infection early following mucosal challenge. In contrast, in macaques vaccinated with a DNA prime/Ad5 boost vaccine, which induces a central memory based response, progressive SIV infection occurred, as characterised by the increased viral load.

Importantly, the delineation into central and effector memory T cell subsets may be reductionist. An array of phenotypic markers has been used to delineate different subsets of memory T cells, including CD127 (IL-7R) and CD62L (Cui and Kaech, 2010) (Figure 1.6). It may be better to describe memory T cells differentiation as a continuum. At one end of the spectrum, are T cells that possess a greater memory cell like properties and are long-lived, whereas at the other end, T cells display a terminally differentiated phenotype with greater effector functions. In between both extremes, are cells that display an intermediate differentiation phenotype, but possess memory like phenotypes and are capable of expanding upon secondary infection. Ultimately, vaccines aim to induce long-lived cells that are capable of expanding and clearing the infection. We therefore propose to assess whether BCG is able to induce the formation of long-lived memory T cells by measuring the expression of Bcl-2 in BCG specific T cells over the first year of life. Bcl-2 is an anti-apoptotic marker and identifies long-lived T cells (Cellerai et al., 2007).

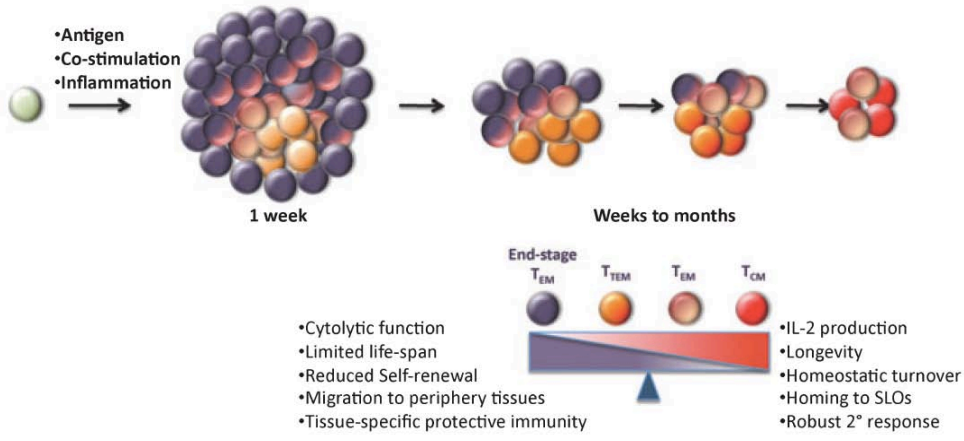

Figure 1.6. Differentiation of memory T cells (Cui and Kaech, 2010).

1.6 TB vaccines

1.6.1 BCG

BCG is the only available vaccine against TB. This vaccine is an attenuated strain of *Mycobacterium bovis*, generated in the early 20th century after several culture passages. BCG provides variable protection against pulmonary TB, ranging from 0 to 83% (Colditz et al., 1995; Fine, 1995). However, BCG is ~80% effective in protecting against TB meningitis and miliary TB. Several reasons have been proposed to explain the variable protection conferred by BCG, including the use of different BCG strains (Davids et al., 2006), difference in the dosage and route of vaccination (Power et al., 1998), human genetic factors (Newport et al., 2004) and exposure to non tuberculous mycobacteria, which may mask the protective effects of BCG (Burl et al., 2010; Stanford et al., 1981).

Given the variable efficacy of BCG, there is an urgent need for new TB vaccines. These vaccines will be described later in this section. First and foremost, it is fundamental to understand the immunity induced by BCG since most new TB vaccines that are in clinical trials, are designed to boost the immunity induced by BCG (Brennan et al., 2007). Therefore, one of the main objectives of this study is to characterise the longitudinal immunity induced by BCG, to determine when to optimally boost the BCG induced immune responses following vaccination.

BCG induced immunity

Even though BCG has been administered for almost 90 years, our knowledge of the immune responses induced by BCG remains incomplete. Earlier studies have used IFN-γ as the main immunological read-out, but measuring this cytokine alone does not reflect the comprehensive immune response to BCG (Soares et al., 2008). With the development of multiparameter flow cytometry, several markers can be measured simultaneously at a single cell level.

Detailed analysis, carried out by our group, of the cytokine profile of infants 10 weeks after BCG vaccination revealed the complexity of the BCG induced

immune response (Soares et al., 2008). Seven distinct CD4+ T cell population that produced Th1 cytokines were identified. Polyfunctional CD4+ T cells co-expressing IFN-γ, IL-2 and TNF-α, and CD4+ T cells that produced IFN-γ only, were the most pre-dominant. The frequency of BCG specific CD8+ T cells was much lower than CD4+ T cells, and these CD8+ T cells predominantly produced IFN-γ and/or IL-2, and less TNF-α.

Furthermore, an effector phenotype (CD45RA-, CCR7- and CD27+) was dominant in BCG specific T cells 10 weeks post BCG vaccination, which may suggested that BCG persisted for 10 weeks after vaccination, at 10 weeks of age (Soares et al., 2008). These results were confirmed by Kagina et al. who showed that an effector memory phenotype predominated in infants who were BCG vaccinated at birth. However, it was also shown that the same phenotype was dominant 1 year after newborn vaccination (Kagina et al., 2010). This raises the question whether BCG vaccination does not result in the induction of a central memory population, as suggested in the mouse model (Henao-Tamayo et al., 2010).

Since the studies by Soares et al. and Kagina et al. focused on a single time point post vaccination, we still do not know how this immune response changes over time. This knowledge is key to understand when to optimally boost the immune response in infants who were BCG vaccinated. Previous work in acute viral infection models, including yellow fever and smallpox, have shown that at the peak effector phase, T cells are highly activated and susceptible to apoptosis (Wherry et al., 2004; Wherry et al., 2007). Boosting an immune response during the peak effector phase can lead to T cell exhaustion and death. We propose that this may also happen if the BCG induced immune response, in this study, is boosted during the peak effector phase. Therefore, we investigated the kinetics of the BCG induced immune response to determine when to optimally boost the BCG induced immunity.

1.6.2 New TB vaccine strategies

Given that BCG is currently the only available TB vaccine, new TB vaccines should be at least as effective as BCG, or significantly improve on or enhance

the effect of BCG, for achieving global impact on the TB burden (Brennan et al., 2007). Several strategies have been put forward:

(i) Improving BCG through the inclusion and exclusion of relevant genes.

(ii) Boosting or redirecting the immune response induced by BCG in a prime-boost strategy. It has been shown that revaccination with BCG does not result in better protection, than when a single dose is administered (Dantas et al., 2006). Importantly, boosting an immune response with a vaccine vector that is different to the prime vaccine (heterologous prime boost) induces higher T cell responses than boosting with the same vector (homologous prime boost), due to the absence of pre-existing immunity to the vector (McConkey et al., 2003) (Figure 1.7).

(iii) Targeting individuals who are already infected with Mtb with a post infection vaccine. This vaccine could either eradicate the persistent latent bacteria and/or prevent the transition from infection to disease (Lambert et al., 2009).

Fourteen TB vaccine candidates have entered clinical trials, of which, 9 are pre-exposure vaccines that are given prior to Mtb infection (Kaufmann et al., 2010). Pre-exposure anti TB immunisations are primarily geared at containing Mtb, such that latency is maintained, thereby preventing or delaying the onset of TB disease. Two pre-exposure vaccine candidates are based on replacing the conventional BCG whereas the other vaccine candidates are booster vaccines. A current limitation in the implementation of these vaccines is the lack of BCG kinetic data to determine when to optimally administer new TB vaccines. This is therefore one of the main aims of this study.

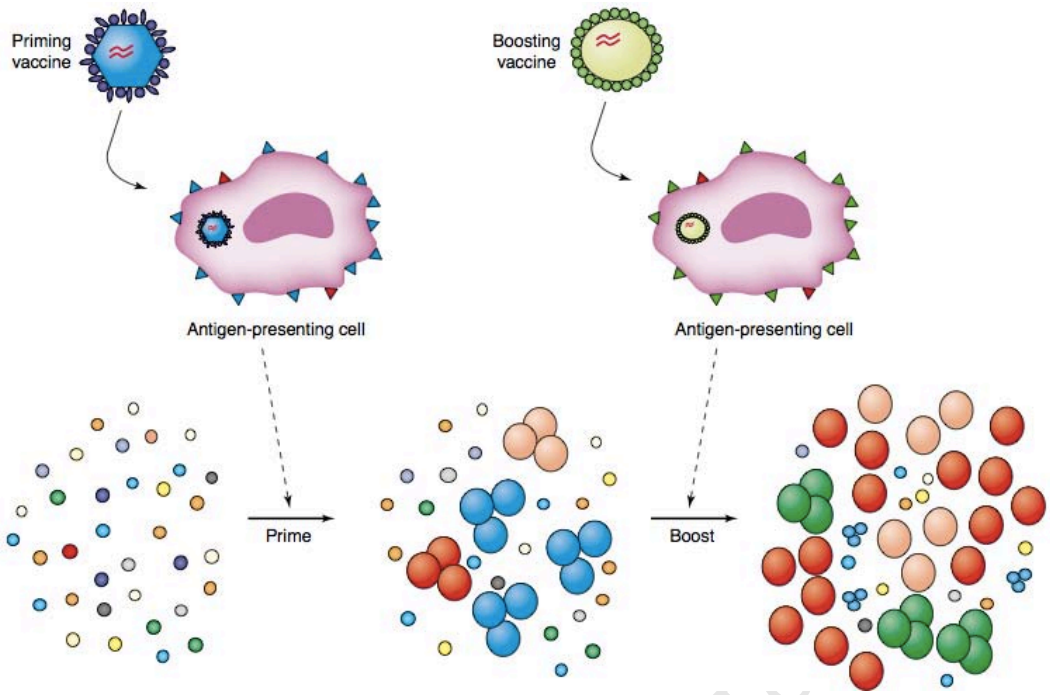

Figure 1.7. Boosting vaccine to improve immune responses. Priming with the first vaccine results in the expansion of antigen specific cells (in red) and vector specific T cells (in blue). Administration of a heterologous booster vaccine results in the presentation of the same antigen but on a different vector, leading to further expansion of antigen specific memory T cells (in red), as well as vector specific naïve T cells (in green) (Woodland, 2004).

1.6.3 Vaccine antigens

The need for a new TB vaccine has prompted the identification of relevant Mtb antigens and epitopes that are recognised in humans (Winslow et al., 2008). Currently, the most common vaccine antigens included in new TB vaccines are antigens that are secreted by Mtb, and were selected based on the premise that these antigens are readily recognised early during infection and disease (Hoft, 2008). These antigens include the antigen 85 complex, ESAT-6, CFP10 and Tb10.4. However, despite the huge repertoire of T cells, only certain epitopes of these antigens are efficiently recognised by specific T cells (Sant et al., 2007). These epitopes are described as being immunodominant.

Knowledge of immunodominant epitopes can be applied in the context of vaccination, such that the immune response is focused on only certain immunodominant epitopes to induce a robust immune response. The relevance of immunodominance to protection is not well understood. Recent

evidence suggests that focusing the immune response on such epitopes could be detrimental to the host (Friedrich et al., 2007). By directing the immune response to subdominant epitopes that are usually not well recognised during infection or during vaccination, it is possible to induce a broader immune response, which is associated with better disease outcome (Tobin et al., 2008). In this regard, Aagaard et al. showed a refocusing of the immune response to subdominant epitopes in mice immunised with a vaccine, consisting of a truncated ESAT6 protein (Aagaard et al., 2009). The latter contains a deletion in the immunodominant sequence of ESAT6 (Δ15 ESAT-6). Upon challenge with virulent Mtb, mice immunised with Δ15 ESAT-6 were better protected than mice vaccinated with the full-length protein.

Taken together, these data suggest that it is possible to induce a more protective immune response by refocusing the immune responses to the subdominant epitopes. This is of particular relevance to vaccination, since a potential problem with current TB immunisation strategies lies in the fact that the immune response is primed to epitopes that are already targeted by T cells during natural infection (Aagaard et al., 2009). Therefore, after the identification of highly recognised latency and reactivation associated antigens in infants following BCG vaccination, we will aim to determine which epitopes of these antigens are targeted following BCG vaccination in future studies at our lab.

1.6.4 New TB vaccines

Live mycobacterial vaccines

Live mycobacterial vaccines are designed to improve on BCG, through the inclusion of relevant genes that will improve the immunogenicity of the vaccine, and through deletion of genes that may cause interference in the immune response. One such recombinant vaccine, rBCG30, over expresses antigen 85B (Ag85b) (Horwitz and Harth, 2003; Horwitz et al., 2000). In a hypersusceptible model of TB, guinea pigs were immunised with rBCG30 and then challenged with virulent Mtb. Animals immunised with rBCG30 had lower bacterial load, increased survival time, smaller and fewer lesions in the lungs, spleen and liver, as well as reduced lung pathology, compared with animals

vaccinated with the conventional BCG. Furthermore, a phase I clinical trial of this vaccine demonstrated that rBCG30 induced a higher proliferation of Ag85b specific cells, greater IFN-γ secretion, and importantly enhanced number of Ag85b specific cells, compared to the parental BCG (Hoft et al., 2008).

A different approach involves the use of a mutant strain of BCG that secretes listeriolysin and is deficient in urease C expression (*ΔureC hly*+ rBCG) (Grode et al., 2005). Listeriolysin, which originates from *Listeria monocytogenes*, induces membrane pore formation, allowing the escape of the organism from the phagosome into the cytoplasm. This would be expected to result in better antigen presentation through the MHC class I pathway. However, listeriolysin is only active at low pH, hence deletion of urease C, which is involved in pH neutralisation, facilitates maintenance of a low pH environment. Membrane perforation is also known to induce apoptosis of host cells (Winau et al., 2006). Subsequent uptake of apoptotic vesicles containing Mtb antigen by dendritic cells allows for superior CD4+ and CD8+ priming. Mice vaccinated with *ΔureC hly*+ rBCG had a lower bacterial load in the lungs compared with mice immunised with the parental BCG strain, following challenge with virulent Mtb – both H37Rv and a clinical isolate (Grode et al., 2005). The clinical isolate used, the Beijing strain, is known for enhanced virulence and drug resistance (Tracevska et al., 2003).

Other live mycobacterial vaccines are expected to enter clinical trials in the coming years (Kaufmann et al., 2010). These vaccines are recombinant Mtb, in which virulence genes have been disrupted. The vaccine MTBVAC01 is a recombinant Mtb, which lacks the *phoP* gene. This gene is involved in Mtb virulence. (A new vaccine lacking *phoP* and *fad* genes have also been designed. The fad gene is involved in phthiocerol dimycocerosate synthesis). Testing of MTBVAC01 in mice and guinea pigs shows that the vaccine provides protection against TB (Martin et al., 2006). Mice vaccinated with the *phoP* deficient Mtb strain (SO2 strain) have a higher proportion of antigen specific CD4+ and CD8+ T cells compared with mice vaccinated with BCG. Vaccination of mice with BCG and the Mtb SO2 strain provided comparable level of protection in mice, as measured by the bacterial counts in the lung

and the spleen. In addition, guinea pigs vaccinated with SO2 strain had reduced pathology and increased survival time than guinea pigs immunised with BCG. Importantly, these vaccines are likely to be used as prime vaccines in the future to replace the current BCG vaccine.

Subunit vaccine

Subunit vaccines classically contain a fusion protein of two or three immunodominant Mtb antigens, together with an adjuvant. Certain antigens are weakly immunogenic and immunogenicity may be enhanced when antigens are used as fusion proteins, compared with individual proteins. An adjuvant is also included to promote robust Th1 responses. The H1 vaccine consists of a fusion protein of ESAT6 and Ag85b, delivered with the IC31 adjuvant (van Dissel et al., 2010). IC31 is a mixture of oligodeoxynucleotides and polycationic amino acids, which stimulate the innate immune responses via the TLR9 signaling pathway (Schellack et al., 2006). Result from a phase I clinical trial revealed that this vaccine is safe and well tolerated, as well as a strong inducer of Th1 responses (van Dissel et al., 2010). Responses to the H1 antigens still persisted after 2.5 years, suggesting that the vaccine induces memory responses in immunised recipients.

The M72F vaccine contains a fusion protein of Mtb32 and Mtb39, along with the ASO1E adjuvant (Skeiky et al., 1999). Both antigens Mtb32 and Mtb39 were identified in latently infected individuals by screening for T cell and antibody responses. The ASO1E adjuvant stimulates the innate immune system via activation of TLR4 signaling pathway (Skeiky et al., 2004). The M72F vaccine protects mice and guinea pigs against challenge with virulent Mtb (Von Eschen et al., 2009). Testing of this vaccine in healthy volunteers demonstrated robust induction of T cell and antibody responses. Importantly, responses to this vaccine were still detectable 6 months post immunisation.

Live viral vectors

The use of live mycobacterial vaccines to prime the host immune response can cause disease in HIV infected infants (Hatherill et al., 2010). A safe alternative for all infants, regardless of their HIV status, would involve the use

of viral vectors as prime vaccines, followed by a delayed delivery of BCG. Furthermore, pre-exposure to non-tuberculous mycobacteria can mask the effects of BCG (Doherty, 2005). Hence, the use of mycobacterial vectors would be less effective in prime boost vaccine strategies. Two major viral vectors have been engineered in the field of TB vaccines to express and deliver Mtb antigens: the modified vaccinia virus Ankara (MVA) and the replication-deficient Adenovirus (Ad) of serotype 35 (Abel et al., 2010; McShane et al., 2004).

The MVA85A vaccine is a non-replicating viral vector that expresses the Mtb antigen 85a (McShane et al., 2004). Phase I clinical trials, using this candidate, have been completed in numerous countries. Immunisation with this vaccine results in a potent Th1 cell responses. Importantly, the vaccine was able to boost immunity previously induced by BCG, even in individuals who had been vaccinated with BCG many years earlier. We have shown that MVA85A is safe and well tolerated in children and adolescents (Scriba et al., 2010). The vaccine induced a polyfunctional CD4+ T cell response, characterised by the co-expression of IFN-γ, IL-2 and TNF-α.

The vaccine Aeras 402 is comprised of an adenovirus 35 vector expressing Ag85a, Ag85b and Tb10.4 (Abel et al., 2010). This vaccine was safe and immunogenic in clinical trials, inducing a robust polyfunctional CD4+ T cell response in healthy Mtb uninfected but BCG vaccinated individuals. A CD8+ T cell response was also induced. These CD8+ T cells produced IFN-γ and/or TNF-α. Both CD4+ and CD8+ T cell responses persisted for at least 6 months. One concern is that immune responses to adenovirus-based vaccines can be hindered by previous natural exposure to cross-reactive strains of adenovirus (Lambert et al., 2009). In this regard, the Ad35 serotype was used in this vaccine, given that it has a low prevalence of infection in various populations, ranging from 3-5% in developed countries to 20% in Africa.

At SATVI, we are currently testing TB vaccines M72F, Aeras 402 and MVA85A. These vaccines are primarily designed as booster vaccines.

Successful implementation of these vaccines will require comprehensive understanding of the BCG induced immune responses after immunisation.

Post-infection vaccines

All the vaccines discussed above are prophylactic vaccines that contain antigens that are expressed early after Mtb infection. Early antigen specific T cells can persist after acute infection but are unlikely to contribute significantly to the eradication of Mtb during latency, given that a different antigenic profile is induced during latency (Andersen, 2007). Therefore, new TB vaccines should also be effective in a post infection setting given that high proportions of adults, who are the major source of TB transmission, are already latently infected with Mtb. This provides a rationale for the development of post-infection vaccines. Using mathematical modeling, it is predicted that using a combination of post-exposure and pre-exposure TB vaccines, would reduce the TB incidence by 92% by 2050 (Abu-Raddad et al., 2009).

The post-infection vaccine Hybrid 56 incorporates latency-associated antigen Rv2660, ESAT6 and Ag85b (Aagaard et al., 2011). Antigen Rv2660 is highly expressed by Mtb during nutrient starvation (Betts et al., 2002; Govender et al., 2010). This antigen is preferentially recognised by latently infected individuals compared with TB patients and is expressed during late stage of infection in mice. Immunisation with this vaccine before and after Mtb exposure protected mice against TB (Aagaard et al., 2011). Importantly, H56 was 10 times more effective at reducing the bacterial load than the H1 vaccine, which contains only the early antigens ESAT6 and Ag85b. The H56 vaccine is due to enter a phase I clinical trial soon.

Data generated from the pre-clinical testing of H56 vaccine supports the inclusion of latency-associated antigens in new TB vaccines. We are therefore determining the immune recognition of multiple latency-associated antigens following BCG vaccination in infants for potential inclusion in new TB vaccines.

1.7 Infant immunity

With the development of new TB vaccines, it is important to obtain a comprehensive understanding of infant immunity. This is because infants remain an important target of new TB vaccines and the infant immune system is different compared with adults (Levy, 2007). The transition at birth from a protective sterile environment to a hostile environment, where constant exposure to pathogen occurs, renders infant vaccination soon after birth a necessity (Lambert et al., 2005). However, infant immunisation presents its own set of challenges in the sense that the infant immune system is relatively immature, as compared with adults, this has considerable impact on vaccine induced responses (Siegrist and Aspinall, 2009).

Infant monocytes express less IFN-γ and IL-12p70, compared with adult cells (Corbett et al., 2010). In contrast, infant T cells have a greater capacity to produce IL-10 and IL-17 than adults T cells, which could, in part, account for the increased susceptibility of infants to intracellular pathogen such as Mtb (Kollmann et al., 2009; Prabhudas et al., 2011). Importantly, macrophages display reduced microbial killing capacities in infants compared to adults (Holt, 1995). Antigen presenting cells (APCs) from neonates are also inherently ineffective at polarising T cells to differentiate into Th1 cells, which can be attributed to low capacity of DCs to produce IL-12 during early childhood (Upham et al., 2006). Hence delaying BCG vaccination by 10 weeks, results in higher frequencies of polyfunctional CD4+ T cells that produce IFN-γ, IL-2 and TNF-α, at 1-year of age (Kagina et al., 2009).

Taken together, the reports suggest that the infant immune system is immature and impaired in Th1 functions that are known to be important in the protection against Mtb (Prabhudas et al., 2011). We propose that a more comprehensive understanding of infant immunity following BCG vaccination is warranted such that better interventions can be designed to target this vulnerable population.

Chapter 2. Optimisation of a polychromatic flow cytometry panel for the measurement of BCG induced T cell responses in infants.

2.1 Introduction

The ability to reliably and comprehensively measure an immune response is critical for the assessment of the host response to vaccines. In the TB vaccine field, many previous studies have used methods such as ELISA and ELISPOT, which measure the amount of IFN-γ produced or the number of cells that produce IFN-γ, respectively (McShane et al., 2004; van Dissel et al., 2010). However, measuring only one parameter provides limited information and does not reflect the magnitude and complexity of the immune response (De Rosa et al., 2003; Soares et al., 2008). The complexity of the immune system is demonstrated by the presence of highly heterogeneous T cell populations that are functionally and phenotypically distinct. To identify and evaluate the function of these distinct T cell subsets on a single cell level, simultaneous assessment of 4 or more markers is required (Roederer et al., 2004). This is possible with multi parameter flow cytometry.

Newer flow cytometry instruments are able to measure up to 19 parameters (Perfetto et al., 2004). Several fluorochrome conjugates for antibodies against specific antigens are also commercially available, which may be used in several combinations. Complex antibody panels can result in fluorescence spillover from one detector to another, because of spectral overlap (Mahnke and Roederer, 2007). Different fluorochromes have different capacity for spillover, e.g., fluorochromes with broad emission spectra, such as phycoerythrin (PE), may be particularly problematic. Other important considerations in designing complex panels include target cell autofluorescence, brightness of the fluorochrome (therefore, whether rare events will be detectable) and antibody interactions.

Therefore, the design of multi-colour flow panels becomes difficult when multiple parameters are to be assessed (Mahnke and Roederer, 2007). Careful optimisation of these panels is then required. In this chapter, we describe the steps taken to optimise a 9-colour flow cytometry panel for the measurement of the BCG induced T cell responses in infants. The 9 markers,

chosen to address specific aims of this project, include: CD3, CD4 and CD8, IL-2, IFN-γ, IL-17, TNF-α, CD38 and Bcl-2; the choice of these specific markers is justified in chapter 3.

2.2 Aims

To reliably measure the changes in BCG induced T cell immunity over the first year of life, our objective was to optimise a 9-colour flow cytometry reagent panel by addressing the following aims:

i. To determine the optimum antibody titer of each antibody included in the panel.
ii. To determine the level of background fluorescence in each detector channel using fluorescence minus one.

The optimisation of flow cytometry panels often involves the assessment of other parameters including flow cytometry configurations, and optimal photomultiplier tubes (PMT) voltages. These parameters have already been optimised in other studies of our lab, as fluorochromes have been used for other projects. Here, we focused on the optimisation of the two parameters mentioned above.

2.3 Materials and Methods

2.3.1 Study participants

The development and optimisation of the 9-colour flow cytometry panel began with pilot studies performed using adult whole blood. Healthy adults who were BCG vaccinated at birth were recruited at the South African Tuberculosis Vaccine Initiative (SATVI) lab in Cape Town. After informed consent, blood was collected according to our University of Cape Town Research Ethics Committee approved Healthy Donor Protocol.

2.3.2 Whole blood intracellular cytokine assay

A whole blood intracellular cytokine assay has been optimised by our group, to measure specific T cell outcomes (Hanekom et al., 2004). After pheblotomy, 1 ml of whole blood was immediately added to 1.5 ml Sarstedt microtubes containing prepared antigens. Whole blood was either stimulated with $1.8x10^6$ cfu/ml BCG (Danish strain 1331; Statens Serum Institut) or with PHA (Sigma-Aldrich) at 5 µg/ml. Unstimulated blood was used as the negative control. The tubes were vortexed and were placed in a water bath at 37°C. Brefeldin-A (Sigma-Aldrich), which inhibits transport of intracellular proteins such as cytokines, was added after the first 7 hours (hrs) of stimulation, at 10 µg/ml. After an additional 5 hrs of stimulation, the waterbath switched off (pre-programmed) and the drop in temperature stopped the stimulation and cytokine production by the cells. Cells were harvested 8 hrs after the water bath was turned off.

To harvest the cells, EDTA (Sigma-Aldrich) at 20mM was added and incubated for 10 mins. EDTA is a chelating agent that binds to calcium and hence causes the cells to disadhere from the surface of the Sarstedt tubes. One ml of treated blood was then added to 9 ml of BD FACS lysing solution (BD Biosciences) to lyse the red blood cells and fix the white blood cells. After incubation for 10 mins, the tubes were centrifuged. The resulting pellet was resuspended in 1 ml of cryofreezing solution, which consists of 1 part RPMI (Lonza BioWhittaker) and 1 part 20% v/v DMSO (Sigma-Aldrich) in fetal calf serum (Gibco).

Resuspended cells were stored in a 1 ml cryo-vial tube. Cryo-vial tubes were placed in a Mr Frosty (Merck), which contains iso-propanol. This allows the stepwise freezing of the cell suspension at a rate of 1°C/min. Stepwise freezing is required for optimum cryopreservation of cells. The Mr Frosty was kept at -80°C for 24 hrs before the tubes were transferred to liquid nitrogen. Cells were stored in liquid nitrogen to prevent formation of ice crystals, which is restricted at a temperature below -130°C. This minimised the damage to cells caused by ice crystals and allowed maximum recovery of cells after thawing.

2.3.3 Staining protocol

Cryo-preserved cells were thawed rapidly in a waterbath at 37°C. Cells were washed twice in 2 ml PBS (Lonza BioWhittaker). After two washes, the pellet was incubated in 1 ml BD Perm/wash (BD Biosciences) for 10 mins at room temperature, resulting in the permeabilisation of the cells. Permeabilised cells were pelleted, the supernatant decanted, and the antibody cocktail was added to the remaining small volume. Cells were stained for 1 hr at 4°C in the dark. Stained cells were then washed in 1 ml of BD Perm/wash to remove any unbound antibody and then acquired on the BD Fortessa flow cytometer.

2.3.4 Allocation of fluorochrome to antibody conjugate

Antibody conjugates were chosen based on the following criteria:

(i) Laser, filter and mirror configurations of the flow cytometer.
(ii) Brightness of the antibody conjugate.
(iii) Spectral overlap of fluorochromes.

After assigning the antibody conjugate to the markers of interest, the putative antibody panels were assessed.

2.3.5 Antibody titration

All antibodies included in the panel were titrated to determine the optimum antibody titer for staining. The highest concentration used in each titration was twice the recommended concentration by the manufacturer. An unstained control was included to determine the level of background fluorescence.

Antibodies were titrated using 7 to 8 2-fold serial dilutions. This was done both for BCG stimulated and for unstimulated samples. After acquisition, dots plots and histograms were created to compare the performance of each antibody concentration. Different analysis strategies were used depending on the pattern of staining.

For discrete markers (expressed in a bimodal distribution), two outcomes were used to determine the optimum antibody concentration. First, the signal to noise ratio was calculated as the median fluorescent intensity (MFI) of the positive population divided by the MFI of the negative population. Second, the frequency of positive cells was used as an outcome. Both the signal to noise ratio and the frequency of positive cells were plotted against different antibody titers. The optimum antibody concentration was the one that provided the highest signal to noise ratio and highest frequency of positive events.

For continuous markers (expressed in a unimodal distribution), we used MFI as the main outcome. The MFI of the antigen specific T cells, defined by IFN-γ production after incubation of blood with specific antigens, was calculated. In addition, the signal to noise ratio was calculated as the MFI of IFN-γ positive T cells divided by the MFI of Ki67 positive T cells. The justification for this approach is described in the discussion.

2.3.6 Fluorescence minus one (FMO)

In FMO experiments, antibodies were added sequentially to the samples, while omitting one antibody at a time. The FMO and positive controls were stained using the optimised antibody concentrations. The same gating strategy that was used for the positive control was applied to the FMO controls. FMO controls were done for all antibodies included in the final antibody panel. The FMO controls were compared with a positive control that was stained using the complete set of antibodies. We considered a fluorescence spillover of less than 10%, compared with the fluorescence of the positively stained population, as acceptable.

2.4 Results

2.4.1 Allocation of the antibody conjugates to the markers of interest

The antibody conjugates were chosen based on the criteria described in section 2.3.4. As an example, Horizon V500, which has a low staining index, was assigned to the highly expressed CD8 marker. A bright fluorochrome, PE, was assigned to the marker Bcl-2, because this marker is not highly expressed. Members of our lab have tested several antibody conjugates including CD3 PacBlue, CD4 Qdot605, IFN-γ AlexaFlour 700, IL-17 AlexaFluor 700 and TNF-α PE-Cy7 (Scriba et al., 2008; Scriba et al., 2010; Soares et al., 2008). These antibodies were included in our panel. The initial antibody panel chosen for this study is listed in table 2.1.

Table 2.1. Initial antibody panel for the measurement of BCG specific immunity in infants.

Panel 1

Marker	Fluorochrome	Clone	Manufacturer
CD3	Pacific Blue	UCHT1	BD Biosciences
CD4	Qdot 605	S3.5	Invitrogen
CD8	Horizon V500	SK1	BD Biosciences
IL-2	PerCP-Cy5.5	MQ1-17H12	BD Biosciences
IFN-γ	Alexa Fluor 700	B27	BD Biosciences
IL-17	Alexa Fluor 647	SCPL 1362	eBiosciences
TNF-α	PE-Cy7	Mab 11	BD Biosciences
Bcl-2	PE	Bcl-2/100	BD Biosciences
CD38	FITC	HB7 clone	BD Biosciences

Testing of two antibody conjugates for CD38: CD38 FITC and CD38 PE-Cy7 revealed that staining and the resolution of the positive and negative populations was enhanced using CD38 PE-Cy7 conjugate (Figure 2.1A), compared with FITC (Figure 2.1B). Therefore, the fluorochrome PE-Cy7 was assigned for the detection of the CD38 marker, rather than TNF-α. The final antibody panel for the measurement of BCG induced immunity in infants is listed in Table 2.2.

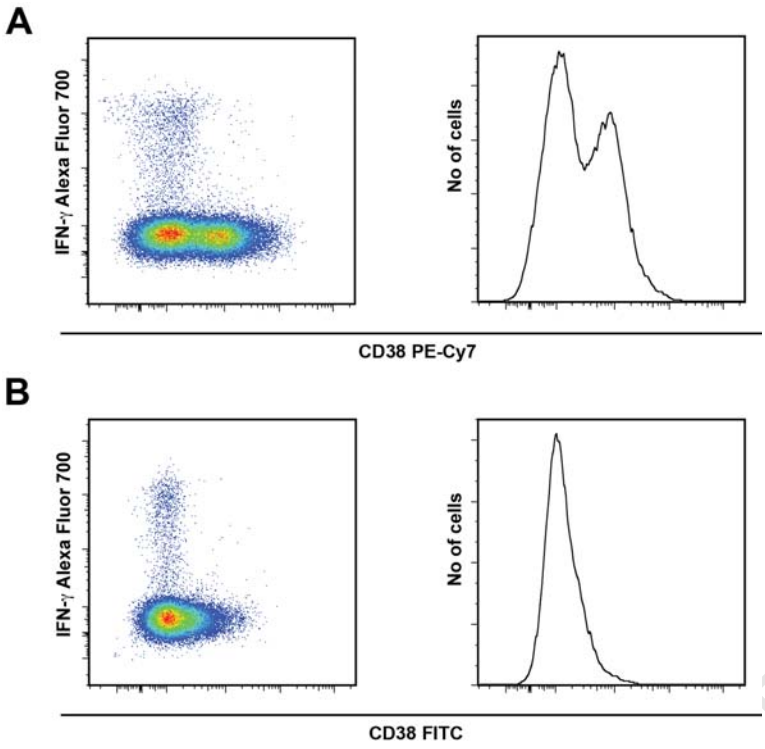

Figure 2.1. Staining of CD38. Cells obtained from whole blood incubated with BCG for 12 hours were stained with a (A) PE-Cy7 and (B) FITC conjugate.

Table 2.2. Final antibody panel for the measurement of BCG specific immunity in infants.

Panel 2

Marker	Fluorochrome	Clone	Manufacturer
CD3	Pacific Blue	UCHT1	BD Biosciences
CD4	Qdot 605	S3.5	Invitrogen
CD8	Horizon V500	SK1	BD Biosciences
IL-2	PerCP-Cy5.5	MQ1-17H12	BD Biosciences
IFN-γ	Alexa Fluor 700	B27	BD Biosciences
IL-17	Alexa Fluor 647	SCPL 1362	eBiosciences
TNF-α	FITC	Mab 11	BD Biosciences
Bcl-2	PE	Bcl-2/100	BD Biosciences
CD38	PE-Cy7	HB7 clone	BD Biosciences

2.4.2 Antibody titration

All antibody conjugates included in our final panel were titrated. TNF-α FITC (discrete expression) and Bcl-2 PE (continuous expression) are shown as representative examples since these 2 antibody conjugates have different staining patterns.

For the titration of TNF-α FITC (Figure 2.2), the highest signal to noise ratio, which indicates the resolution of the positive and negative populations, was at 0.64 µg/ml. However, a plateau between 0.32 µg/ml and 0.64 µg/ml was reached when measuring the frequency of CD3+ TNF-α+ T cells. Hence, 0.32 µg/ml was chosen as the optimum antibody titer for TNF-α FITC.

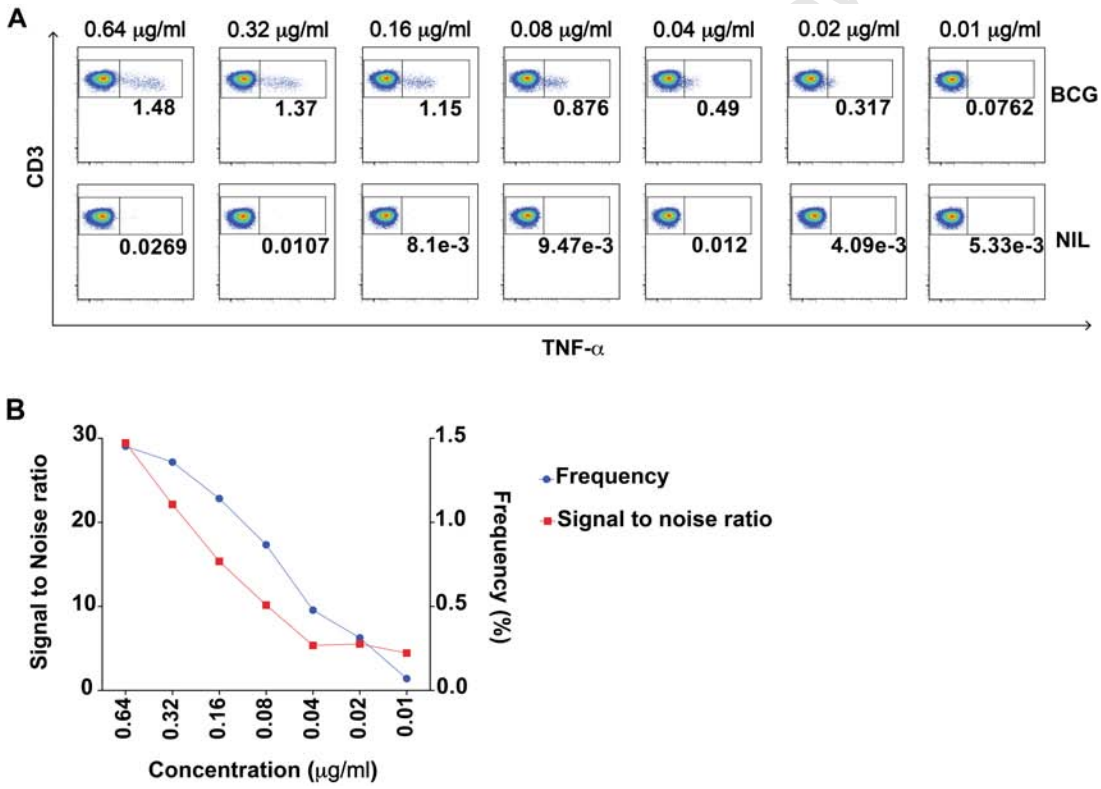

Figure 2.2. Titration of TNF-α FITC. (A) Cells obtained from 12 hrs incubation of whole blood with BCG (Top panel) or with no antigen (Bottom panel), were stained with shown concentrations of TNF-α FITC, in 50 µL of staining medium. The value shown is the frequency of TNF-α positive cells. (B) Signal to noise ratios and frequency of TNF-α positive events at different antibody concentrations, used in 50 µL of staining medium.

For the titration of Bcl-2 antibody conjugate, we used MFI as the main outcome. The MFI of Bcl-2 PE in CD3+IFN-γ+ T cells increased exponentially

with increasing antibody titers (Figure 2.3A), but no plateau was reached (Figure 2.3B).

CD4+ T cells that are IFN-γ+Ki67- express higher levels of Bcl-2 compared with CD4+ T cells are IFN-γ-Ki67+ (Figure 2.3C). The MFI of Bcl-2 positive events in IFN-γ positive cells over Ki67 positive cells, was used as the signal to noise ratio. The signal to noise ratio peaked at 0.1 µg/ml and a plateau was reached between 0.1 µg/ml and 0.4 µg/ml (Figure 2.3D). Hence, 0.1 µg/ml was chosen as the optimum titer for Bcl-2 PE. The final antibody titers used in the final antibody reagent panel is shown in Table 2.3.

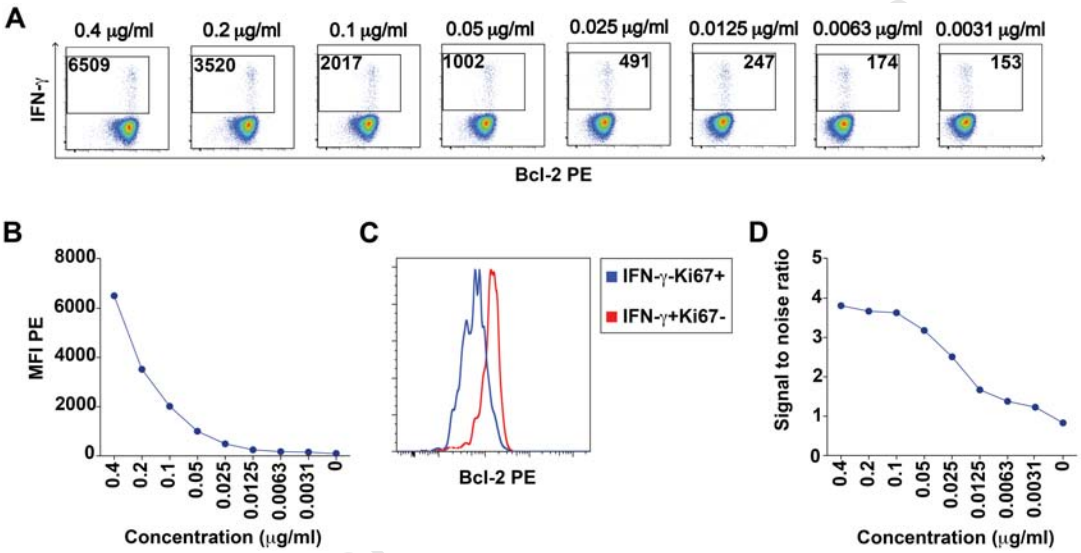

Figure 2.3. Titration of Bcl-2 PE. (A) Cells obtained from incubation of whole blood with BCG for 12 hrs, were stained with shown concentrations of Bcl-2 PE, in 50uL staining medium. The value shown is the MFI of Bcl-2 positive events. (B) MFI of Bcl-2 positive events at different antibody concentrations, used in 50uL of staining medium. (C) Bcl-2 expression by IFN-γ+ and Ki67+ CD4 T cells, and (D) Signal to noise ratio of Bcl-2 positive events at different antibody concentrations, used in 50uL of staining medium.

Table 2.3. Optimum concentrations of antibodies included in the final panel

Marker	Fluorochrome	Clone	Optimal concentration per test
CD3	Pacific Blue	UCHT1	2 μg/ml
CD4	Qdot 605	S3.5	1 nM
CD8	Horizon V500	SK1	1.6 μg/ml
IL2	PerCP-Cy5.5	MQ1-17H12	1 μg/ml
IFN-γ	Alexa Fluor 700	B27	2 μg/ml
IL17	Alexa Fluor 647	SCPL 1362	0.375 μg/ml
TNF-α	FITC	Mab 11	0.32 μg/ml
Bcl-2	PE	Bcl-2/100	0.1 μg/ml
CD38	PE-Cy7	HB7 clone	0.125 μg/ml

2.4.3 Fluorescence minus one

Fluorescence minus one experiment was performed for each antibody conjugate included in the final panel. The detection of fluorescence remained low in all channels, that is, less than 10% of the positively stained population (Figure 2.4). The highest spillover was detected in the PerCP-Cy5.5 channel, which was allocated for the detection of the marker IL-2. Importantly, the frequency or MFI of each positive population remained consistent across each FMO controls and was comparable to the control sample, stained with the complete set of antibodies (Table 2.4).

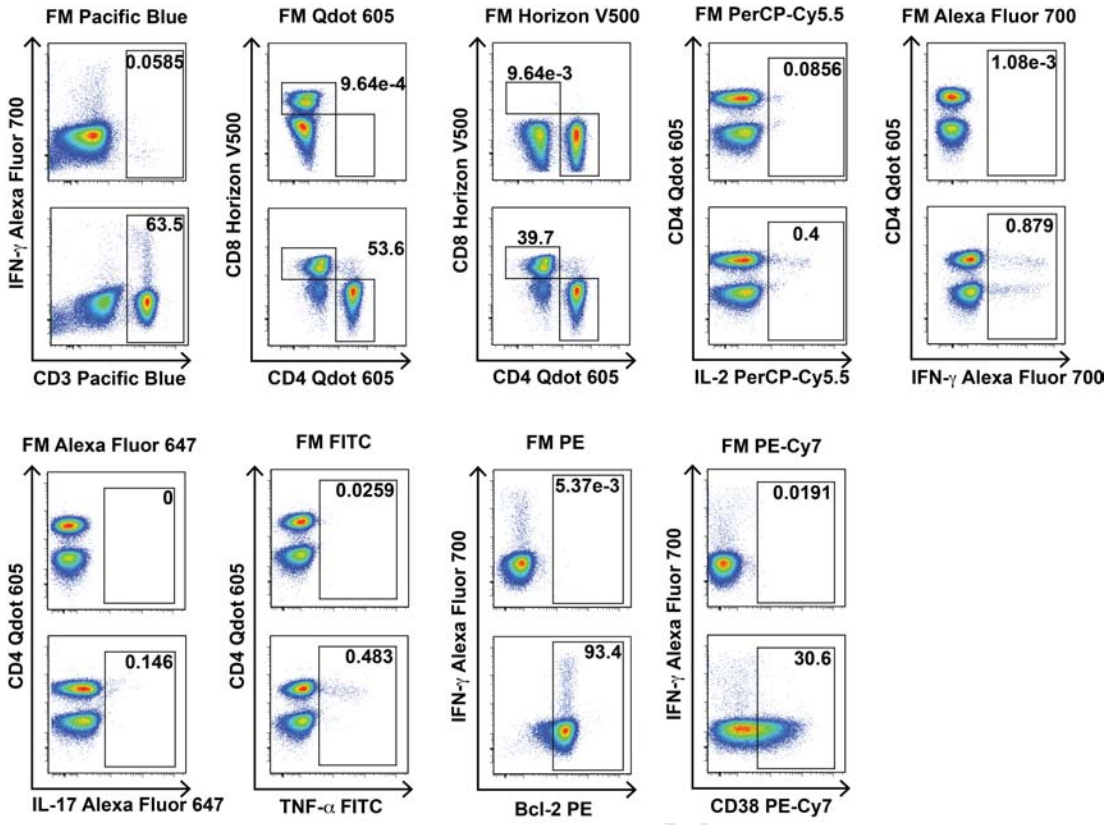

Figure 2.4. Examples of FMO controls for each antibody included in the final reagent panel. Cells obtained after 12 hrs incubation with BCG, were stained with the optimised antibody concentrations, while omitting one antibody at a time for the FMO control. The label above the FMO control represents the antibody that was omitted. Cells stained with the complete set of antibodies were used as the positive control.

Table 2.4. FMO results for the complete reagent panel. Results in blue indicate the amount of fluorescence spillover in a specific channel when the fluorochrome detected in that channel was omitted. Values are reported as frequency of CD4+ T cells. For fluorescence minus Pacific Blue, the values show the percentage of fluorescence spillover as the frequency of lymphocytes. For fluorescence minus PE and FM PE-Cy7, the amounts of fluorescence spillover are reported as MFI.

FM	Pacific Blue	Horizon V500	Qdot 605	PerCP-Cy5.5	Alexa Flour 700	Alexa Flour 647	FITC	PE	PE-Cy7
Pacific Blue	**0.0585**	27.1	34	2.91	0.841	0.185	1.07	1277	350
Horizon V500	63	**9.64e-3**	53.7	0.316	0.769	0.153	0.342	1622	182
Qdot 605	63.4	40	**9.64e-4**	0.415	0.859	0.13	0.453	1624	225
PerCP-Cy5.5	64	37.1	53.7	**0.0856**	0.895	0.176	0.468	1626	240
Alexa Flour 700	63.7	39.6	53.6	0.348	**1.08e-3**	0.127	0.58	1769	205
Alexa Flour 647	63.4	39.2	53.7	0.335	0.829	**0**	0.391	1523	219
FITC	62.8	39.5	53.5	0.39	0.839	0.169	**0.0259**	1688	203
PE	63.1	39.6	53.4	0.377	0.849	0.174	0.41	**74.8**	199
PE-Cy7	63.3	39.7	53.5	0.347	0.835	0.127	0.427	1655	**12.4**
Full panel	63.5	39.7	53.6	0.4	0.879	0.146	0.483	1718	202

2.5 Discussion

We report here the steps taken towards the optimisation of a 9-colour flow cytometry panel for the measurement of BCG induced immune responses in infants. Although numerous antibody conjugates are available for the detection of a specific marker, the performance of these antibodies can differ. CD38 PE-Cy7, rather than CD38 FITC was chosen in our final panel since staining with the former antibody resulted in an enhanced resolution of the positive and negative populations. The fluorochrome PE-Cy7 has a higher staining index than FITC, which could account for these differences.

Antibody titration is critical for optimum cell staining and ensures that reliable results are generated (Mahnke and Roederer, 2007). We found that the optimum titer of each antibody in our panel was consistently lower than the concentration recommended by the manufacturer. This highlights the need to titrate each antibody, given that the addition of excessive antibody results in non-specific binding to cells and higher background, and greater expense. The determination of the optimum antibody titer for the measurement of continuous markers was challenging. Continuous markers are expressed in a unimodal distribution and the cutoff between the negative and positive population is not distinct. Therefore, the frequency of positive cells was not used as an outcome. Since activated T cells that are proliferating, express high levels of nuclear protein Ki67 but low levels of Bcl-2 (Miller et al., 2008), Ki67 was used to identify Bcl-2 low and Bcl-2 high populations. We found that IFN-γ+Ki67- CD4+ T cells expressed higher levels of Bcl-2 than IFN-γ-Ki67+ CD4+ T cells. Hence, the ratio of MFI of Bcl-2 positive events in IFN-γ+Ki67- CD4+ T cells over that of IFN-γ-Ki67+ CD4+ T cells was used to determine the optimum antibody titer for Bcl-2.

Fluorescence spillover is common when using tandem dyes since these dyes are more susceptible to light and heat than single dyes (Maecker and Trotter, 2006). Upon dissociation, tandem dyes cause fluorescence spillover in the channel of the parent fluorochrome. Using FMO experiments, we showed that fluorescence spillover remained low in all channels despite the inclusion of tandem dyes in our panel. Overall, these results show that the antibody

conjugates selected in our final panel can be used simultaneously to measure all markers of interest.

While the optimisation of flow cytometry reagent panel is time consuming and costly, this process remains critical for the accurate and sensitive detection of all markers of interest. We show that 9 different markers can be measured simultaneously, which allows significant amounts data to be collected from limited amounts of sample. This approach supports the use of this technology to investigate the changes in T cell immunity induced by BCG in infants.

Chapter 3. Functional and phenotypic characterisation of mycobacteria-specific T cells over the first year of life, following BCG vaccination at birth.

3.1 Introduction

Our understanding of the kinetics of an immune response is mostly based on findings from acute viral infection models (Miller et al., 2008). At the peak of an immune response, effector T cells display an activated phenotype, characterised by the high expression of CD38 and HLA-DR. These activated T cells proliferate, have effector functions like cytokine production, and are highly prone to apoptosis. The immune response contracts after clearance of the pathogen; during this phase most antigen specific T cells die by apoptosis. The remaining T cells differentiate into long-lived memory T cells that express markers like Bcl-2. Bcl-2 is a mitochondrial membrane protein which protects the cell from apoptosis by blocking export of cytochrome C from the mitochondria; cytochrome C is responsible for the activation of capases, involved in apoptosis (Riedl and Salvesen, 2007).

The kinetics of T cell responses may differ in chronic infection. Constant exposure to antigens may lead to exhaustion of T cells, and impairment of memory formation (Barber et al., 2006). T cells from BCG vaccinated infants exhibit a predominantly effector memory phenotype at 10 weeks of age, possibly indicating that BCG is able to persist to this age, resulting in chronic immune stimulation (Kagina et al., 2009; Soares et al., 2008). It is possible that the formation of long-lived memory T cells is impaired in BCG vaccinated individuals; in murine models of BCG infection. absence of a transition to central memory phenotypes has been shown (Henao-Tamayo et al., 2010).

To date, two studies have investigated the kinetics of the BCG induced immune responses (McShane et al., 2004; Nabeshima et al., 2005). When TST negative adults were vaccinated with BCG, the peak of the vaccine response occurred 4 weeks after vaccination (McShane et al., 2004). This was determined using IFN-γ ELISPOT on PBMC stimulated with PPD for 10 days. In contrast, Nabeshima et al. found that in TST negative adults vaccinated with BCG, the peak effector phase occurs at 8 weeks. This was

done by measuring the IFN-γ production on PBMC stimulated with PPD for 4 days.

In determining the kinetics of the BCG-induced response, expression of markers such as CD38 and Bcl-2 on specific cells may be helpful. For identification of specific cells, we propose evaluation of mycobacteria-induced T cell expression of IFN-γ, IL-2 and TNF-α. Protective immunity against Mtb involves production of these Th1 cytokines. It is well established that individuals with mutations in the IFN-γ receptor are highly susceptible to severe mycobacterial disease (Ottenhoff et al., 2003; van de Vosse et al., 2003), while patients with rheumatoid arthritis treated with TNF-α blockers are more prone to reactivation of latent Mtb infection (Saliu et al., 2006). Further, IL-2 is required for the expansion of Mtb specific T cells, and is important for sustaining long term T cell memory (Bouneaud et al., 2005). BCG vaccination of newborns does result in the production of Th1 cytokines in various combinations (Soares et al., 2008); however, longitudinal changes in this response have not been described, which is the focus of this chapter.

Other cytokines may also contribute to immunity against mycobacteria. Recently, it has been shown that memory CD4 T cells that produce IL-17 are present in mycobacteria-exposed individuals, and in patients with TB (Scriba et al., 2008). We therefore also propose to measure the specific production of this cytokine.

3.2 Aims

In order to characterise the changes in mycobacteria-specific T cell immunity following newborn vaccination with BCG, we aimed to determine:

i. The kinetics of the specific CD4+ and CD8+ T cell-associated cytokine response.
ii. The kinetics of Bcl-2 and CD38 expression of specific CD4+ and CD8+ T cells.

We proposed to use these results to determine an optimum time to boost BCG induced immunity with a second vaccine.

3.3 Materials and Methods

3.3.1 Infant participants

The study described in this chapter was part of a larger project aimed at comprehensively determining the changes in BCG induced immunity over the first year of life. For the latter project, we collected a longitudinal cohort of infants vaccinated with BCG (Danish strain 1331; Statens Serum Institut) at birth. Infants were followed up at 7 time points over the first year of life, when blood was collected and processed. These time points mostly coincided with the standard Expanded Programme on Immunisation (EPI) schedule of South Africa (Figure 3.1). Blood samples were collected from 42 BCG vaccinated infants at 3 to 4 time points, at most – different schedules were designed for each infant, to allow at least 19 participants' blood to be collected for each time point. This was done because we regarded more frequent phlebotomy as unacceptable, and because only 3 ml of blood per kg can safely be taken over an 8-week period.

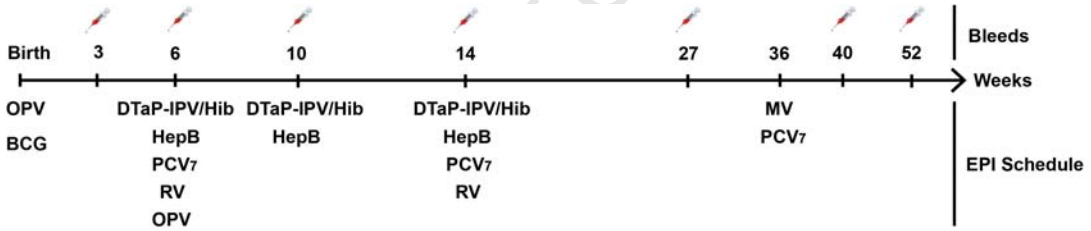

Figure 3.1 Phlebotomy schedule of the BCG vaccinated infant cohort. OPV: Oral polio vaccine, BCG: Bacille Calmette Guerin, DTaP-IPV/Hib: Diphtheria, Tetanus, acellular Pertussis, Inactivated Polio vaccine and *Haemophilus influenza* type b combined vaccine, HepB: Hepatitis B vaccine, PCV_7: Pneumococcal conjugated vaccine, RV: Rotavirus vaccine and MV: Measles vaccine.

Inclusion criteria were:

i. Receipt of the BCG vaccine within 48 hours after birth. Delayed BCG vaccination may have an effect on the infant immune responses to BCG.
ii. Birth weight > 2.5 kg. Infants. The immune system of low birth weight (<2.5 kg) infant may be different to infants of normal birth weight (>2.5 kg; Dube, Day and Hanekom, unpublished results). Moreover, the amount of blood

samples that could be collected from infants of low birth weight may be insufficient for the completion of the assay.

iii. Healthy, with no chronic disease, or no acute disease at a time of blood draw, since these could alter the immune responses to BCG.

iv. Absence of a T cell response to ESAT6/CFP10 measured by T cell proliferation following 6 day incubation of infant whole blood with ESAT6/CFP10 fusion protein, at each time point, indicating absence of infection with Mtb, which could have confounded results.

3.3.2 Sample processing

Infant whole blood was either stimulated with $1.8x10^6$ CFU/ml BCG, or 5µg/ml PHA or 10µg/ml Staphylococcal enterotoxin B (SEB; Sigma Aldrich) in the 12 hr whole blood assay described in section 2.3.2. The co-stimulants anti-CD28 and anti-CD49d (0.5 µg/ml each; BD Biosciences) were added to all conditions. Pilot studies at SATVI have shown that addition of co-stimulants results in an enhanced specific T cell cytokine response, without affecting background responses (Hanekom et al., 2004; Waldrop et al., 1997). Whole blood incubated with co-stimulants only served as a negative control. Following 12 hrs incubation, cells were harvested. Fixed white blood cells were cryopreserved, and stored in liquid nitrogen.

3.3.3 Cell staining and acquisition

Cryo-preserved infant cells were thawed and stained as described in section 2.3.3, with optimised concentrations of CD3 PacBlue (UCHT1), CD4 Qdot605 (S3.5), CD8 Horizon V500 (SK1), IFN-γ Alexa Fluor 700 (B27), IL-2 PerCp-Cy5.5 (MQ1-17H12), IL-17 Alexa Fluor 647 (SCPL 1362), TNF-α FITC (Mab 11), Bcl-2 PE (Bcl-2/100), and CD38 PE-Cy7 (HB7). All antibodies were from BD Biosciences, except for CD4 Qdot605 and IL-17 Alexa Fluor 647, which were from Invitrogen and eBiosciences, respectively. Stained cells were acquired on a BD Fortessa flow cytometer (BD Biosciences). Compensation settings were set using anti-mouse anti-kappa compensation beads stained with each antibody conjugate. To reduce inherent assay variations, samples

for each infant at all time points were stained with a master mix of antibodies and acquired on the same day using the BD Fortessa flow cytometer.

3.3.4 Flow cytometry analysis

For flow cytometry data analysis, the first gate was a time gate to exclude cell populations that were not acquired uniformly over time. Because Qdot antibodies are prone at forming antibody aggregates, antibody aggregates were then excluded using a keeper gate. In the third gate, single cells, which had a diagonal distribution with forward scatter height (FSC-H) over forward scatter area (FSC-A), were included.

Lymphocytes were selected using side scatter area (SSC-A) over forward scatter area (FSC-A). CD3+ T cells were then selected. Cell debris, which can accumulate in cryo-preserved samples were then excluded using IL-2 PerCP-Cy5.5 over TNF-α FITC. We selected CD8- and CD4- T cell populations using CD8 Horizon V500 over IFN-γ Alexa Fluor 700, and CD4 Qdot605 over IFN-γ Alexa Fluor 700, respectively. CD4+ T cells were selected from the CD8- T cells whereas CD8+ T cells were selected from the CD4- T cell populations. The gates for CD4+ and CD8+ T cells were established using CD4 Qdot605 over IFN-γ Alexa Fluor 700, and CD8 Horizon V500 over IFN-γ Alexa Fluor 700, respectively. This stringent gating allowed specific selection of CD4+ and CD8+ T cells.

The cytokine expression within the CD4+ and CD8+ T cell populations were analysed. To select for IFN-γ+ and IL-2+ T cell populations, we used IFN-γ Alexa Fluor 700 over IL-2 PerCP-Cy5.5. Similarly, to select for TNF-α+ and IL-17+ T cell populations, we used TNF-α FITC over IL-17 Alexa Fluor 647. The cytokine gates were determined based on the cytokine expression in whole blood stimulated with co-stimulants only (negative control). The same gates were applied to all the different conditions. Boolean gating analysis was done once the gates for cytokine positive T cell populations were established. This analysis enables the measurement of different combinations of cytokine positive cell populations.

All analyses were performed using FlowJo v9.2 (Treestar). Pestle v1.6.2, and Spice v5.0 (provided by M.Roederer, National Institutes of Health).

3.3.5 Statistical analysis

A "background" response was defined as that in the unstimulated sample. For the analysis of the BCG induced T cell cytokine responses, the background was subtracted from the response in the BCG stimulated sample.

Bcl-2 and CD38 marker expression were assessed by determining MFI in specific T cells. The latter T cells were identified based on production of IFN-γ, IL-2, IL-17 and TNF-α, following incubation of whole blood with BCG. Infants' results had to make the following criteria for Bcl-2 and CD38 expression to be analysed:

i. Frequencies of BCG-specific cells greater than the median plus 3 times the median absolute deviation (median + 3 MADs) of all unstimulated samples at each particular time point.
ii. Frequencies of BCG-specific cells that were at least twice the unstimulated sample.
iii. At least over 20 BCG-specific cells, following background subtraction.

Cross-sectional analysis of the T cell cytokine responses, and the Bcl-2 and CD38 expression in BCG specific T cells at different time points, was performed. We first used a non-parametric Kruskal-Wallis test to determine whether there were any significant differences between all possible pairs of data. If a difference between any 2 groups was found to be significant, a Mann-Whitney test was then applied to compare the differences between the 2 groups. Differences were considered to be significant if the p value was less than 0.05. Statistical tests were performed using GraphPad Prism v5.0a (GraphPad).

3.4 Results

3.4.1 BCG specific T cell cytokine production

Incubation of infant whole blood after 12 hrs with BCG and PHA resulted in detectable levels of cytokines IFN-γ, IL-2, IL-17 and TNF-α (Figure 3.2). The levels of expression of these cytokines in the unstimulated samples were uniformly low, relative to the stimulated samples. All infants responded to the positive controls PHA or SEB.

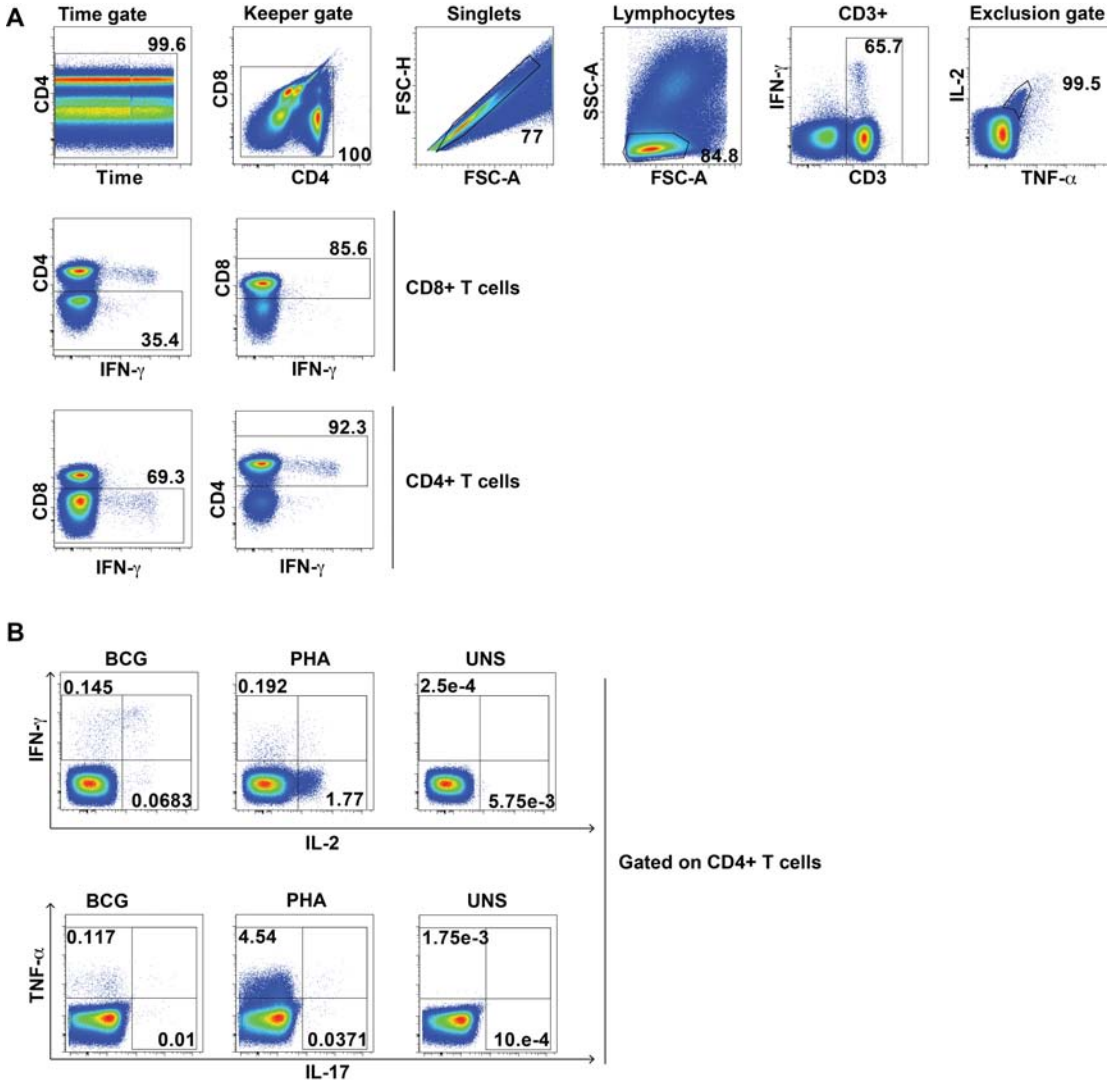

Figure 3.2. Cytokine responses of a 6-week BCG vaccinated infant. (A) Flow cytometry gating strategy to select for CD4+ and CD8+ T cells after 12 hrs stimulation of infant whole blood with BCG. (B) Frequencies of CD4+ T cells expressing IFN-γ, IL-2, IL-17 or TNF-α.

3.4.2 Kinetics of the BCG induced immune response

To assess the changes in BCG induced T cell immunity over the first year of life, we determined specific IFN-γ, IL-2, TNF-α, and IL-17 production by CD4+ and CD8+ T cells in 42 infants, at various time points over the first year of life. Specific production of IFN-γ, IL-2 and TNF-α by CD4+ T cells followed the same kinetics (Figure 3.3A). At 3 weeks, the frequencies of specific IFN-γ, IL-2 and TNF-α secreting CD4+ T cells were low. Substantial expansion of these cells had occurred by 6 weeks. The peak of the Th1 cytokine response occurred at 10 weeks, followed by a contraction phase. The specific IL-17 response was the lowest among the 4 cytokines assessed; the peak of this response could not be determined.

In infants for whom blood was not collected at 10 weeks, the peak CD4+ specific Th1 cytokine response was observed at 6 weeks. Comparison of the specific cytokine expression of 4 infants who had blood collected at both 6 and at 10 weeks revealed that the cytokine responses were highest at 10 weeks.

The magnitude of the specific CD8+ T cell response was lower than that of specific CD4+ T cells, for all 4 cytokines (Figure 3.3B). The frequency of specific CD8+IFN-γ+ T cells peaked at 6 weeks, rather than at 10 weeks, followed by a contraction phase. The peak for the specific IL-2, TNF-α and IL-17 responses was not observed for CD8+ T cells, because the responses were so low.

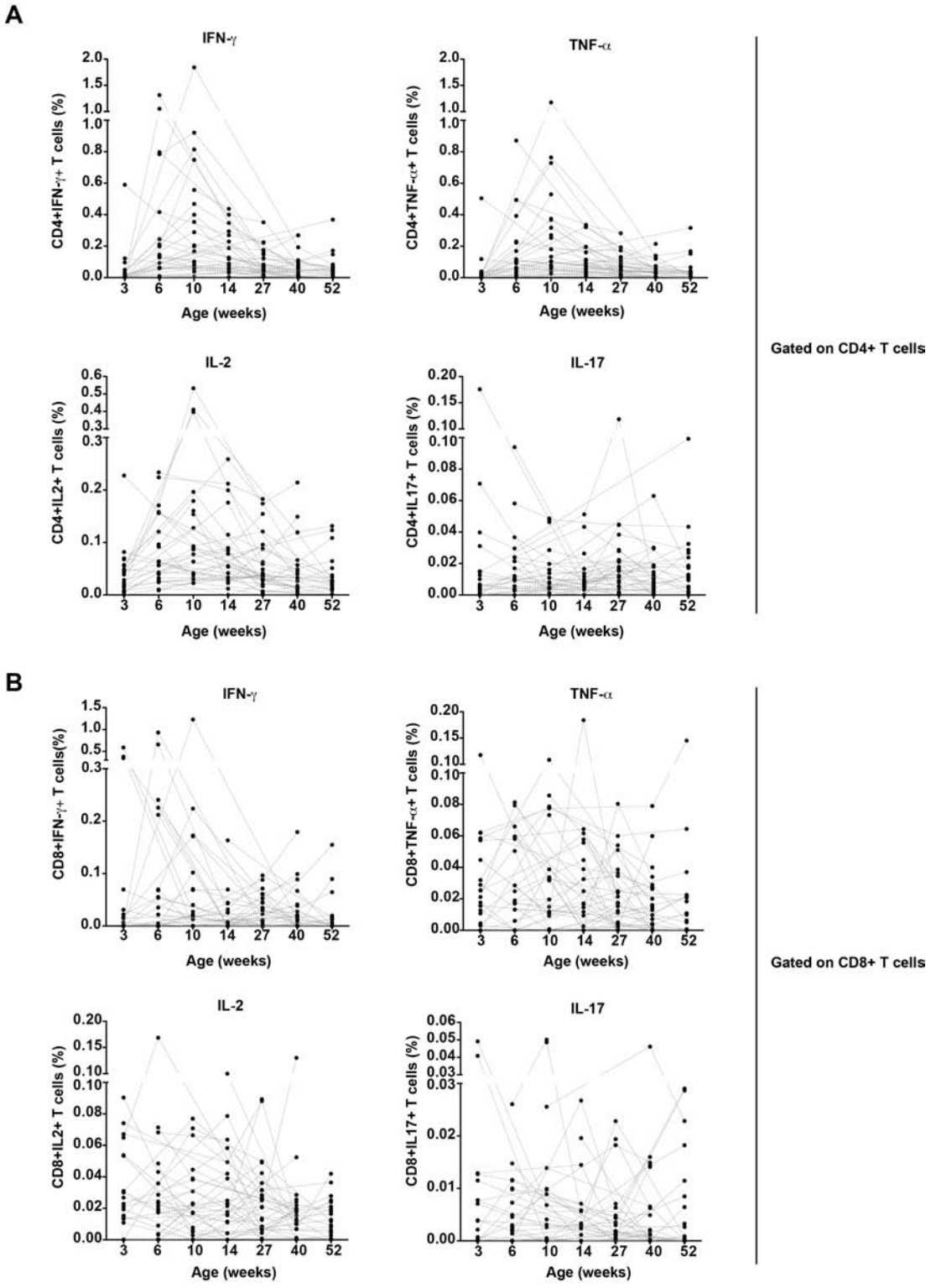

Figure 3.3. Kinetics of cytokine expression of BCG specific T cells over the first year of life. Changes in the frequencies of (A) CD4+ T cells and (B) CD8+ T cells, which produced IFN-γ, IL-2, IL-17 and TNF-α after 12 hrs stimulation of whole blood with BCG (n=42). Each line represents a different infant. The cytokine expression in the unstimulated samples was subtracted from the BCG stimulated samples.

To confirm above findings reflecting longitudinal analysis of individual participants, we also analysed grouped findings at each time point, in a cross-sectional analysis. Different numbers of infants were analysed at each time point, given that blood samples could not be collected from infants at all time points. The number of infants included in the cross sectional analysis is shown in Table 3.1.

Table 3.1 Number of infants included in the cross-sectional analysis

Cross-sectional analysis

Age (weeks)	No of infants
3	23
6	19
10	19
14	19
27	27
40	24
52	21

The cross sectional results mirrored the kinetics of the longitudinal data. There was a significant increase in the frequencies of specific CD4+ T cells, which express IFN-γ, IL-2 and TNF-α at 10 weeks (Figure 3.4A). There was no difference in frequencies of cytokine producing CD4+ T cells at 6 and 10 weeks, when assessed statistically.

As mentioned above, the frequencies of specific CD8+ T cells were very low compared with those of specific CD4+ T cells (Figure 3.4B). There was no significant increase in the frequencies of cytokine positive CD8+ T cells between 3 and 6 weeks, nor between 3 and 10 weeks.

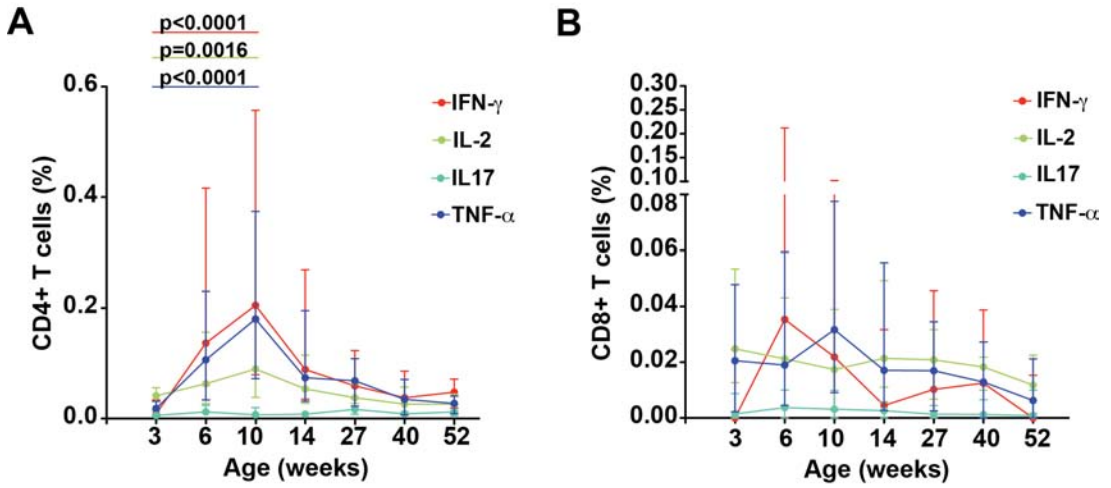

Figure 3.4 Cross-sectional analyses of BCG specific T cell responses over the first year of life. Frequencies of (A) CD4+ and (B) CD8+ T cells, which produced IFN-γ, IL-2, IL-17 and TNF-α after 12 hrs stimulation of whole blood with BCG. The cytokine expression in the unstimulated samples was subtracted from the BCG stimulated samples (n≥19/time point). The horizontal lines at each time point indicate the median and whiskers of the interquartile range. The differences were assessed using a Mann Whitney test. A p value of less than 0.05 is considered as significant.

3.4.3 Combinations of cytokine expression among BCG-specific T cells

To further characterise the BCG induced immune response, cytokine co-expression by CD4+ and CD8+ T cells was investigated. Stimulation of whole blood with BCG for 12 hrs induced 3 dominant populations of cytokine producing CD4+ T cells. The 3 subsets were either triple positive for IFN-γ, IL-2 and TNF-α, or double positive for IFN-γ and TNF-α, or single positive for IFN-γ (Figure 3.5A). The frequencies of specific cytokine positive CD4+ T cells in these 3 subsets were highest at 10 weeks. The specific CD8+ T cell response was dominated by single cytokine positive T cells that produce IFN-γ only (Figure 3.5B).

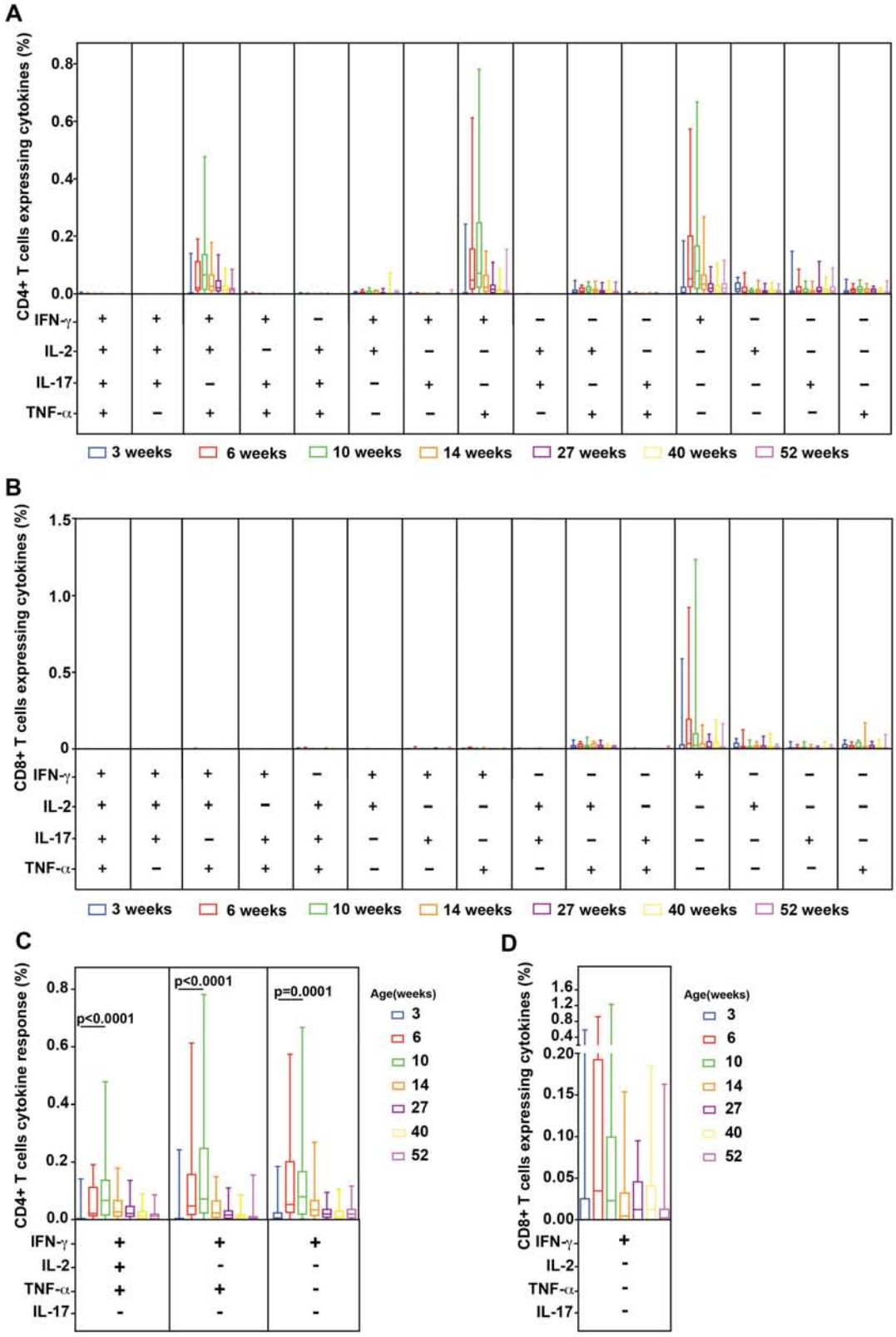

Figure 3.5 Cytokine co-expression by BCG specific T cells. Frequencies of all (A) CD4+, (B) CD8+ T cells and dominant (C) CD4+ and (D) CD8+ which co-expressed cytokines after 12 hrs stimulation of whole blood with BCG. The cytokine expression in the unstimulated samples was subtracted from the BCG stimulated samples. The differences were assessed using a Mann Whitney test. A p value of less of 0.05 is considered as significant.

To investigate the combined expression of cytokines by specific T cells, the proportions of CD4+ and CD8+ T cells producing cytokines were investigated. We observed an increase in the proportion of polyfunctional CD4+ T cells, which co-expressed IFN-γ, IL-2 and TNF-α, over the first year of life (Figure 3.6A). Single cytokine positive cells dominated the total BCG specific CD4+ T cell response. Interestingly, these cells produced either IFN-γ or IL-2 or IL-17 but not TNF-α.

Over the first year of life, a small proportion of specific CD8+ T cells, which predominantly co-expressed TNF-α and IL-2, were detected (Figure 3.6B). Single cytokine positive T cells dominated the specific CD8+ T cell response. At 3, 6 and 10 weeks, the majority of these cells produced IFN-γ. However, we observed an increase in the proportions of specific CD8+ T cells, which produced either TNF-α or IL-2 over the first year of life.

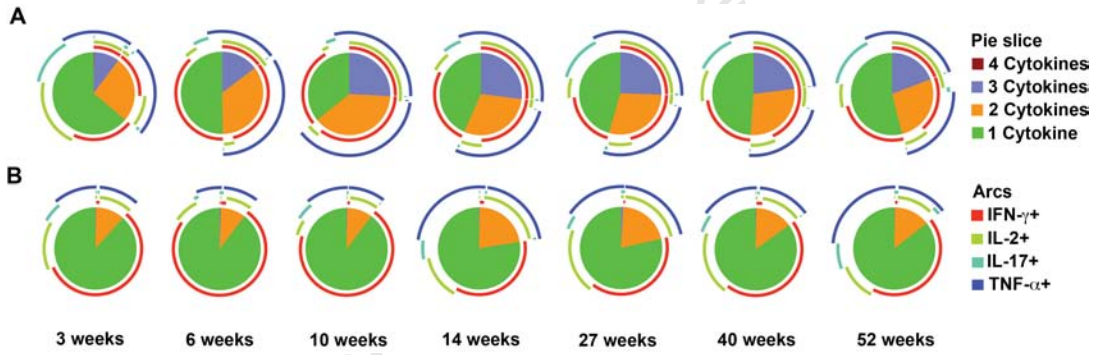

Figure 3.6 Distribution of the BCG specific T cell cytokine responses over the first year of life. Proportions of T cells within the total BCG specific (A) CD4+ and (B) CD8+ T cells that express a given number of cytokines simultaneously after 12 hrs stimulation of whole blood with BCG. Responses are classified and colour-coded according to the number of cytokines that are co-expressed (Pie slice). The outer colour-coded arcs show the contribution of each cytokine to the pie slice.

3.4.4 CD38 expression by total cytokine producing BCG specific CD4+ T cells

To test whether BCG specific CD4+ T cells were highly activated at the peak of the cytokine response, we measured the expression of CD38 in BCG specific CD4+ T cells that produce any of the 4 cytokines IFN-γ, IL-2, IL-17 and TNF-α. These T cells expressed highest levels of CD38 at 3 and 6

weeks. CD38 expression declined by 10 weeks and waned over time (Figure 3.7A, B and C). The data suggests that the BCG specific CD4+ T cells were most highly activated at 3 and 6 weeks.

Changes in CD38 expression over time have to be interpreted with those in non-specific cells. We therefore assessed the kinetics of CD38 expression in the total CD4+ T cell compartment, and showed that this expression gradually declined over the first year of life (Figure 3.7D, E and F). The expression in this population was uniformly higher, than that of the BCG specific CD4+ T cells. The decrease in CD38 expression in BCG-specific CD4+ T cells was more prominent than the decrease in the total population.

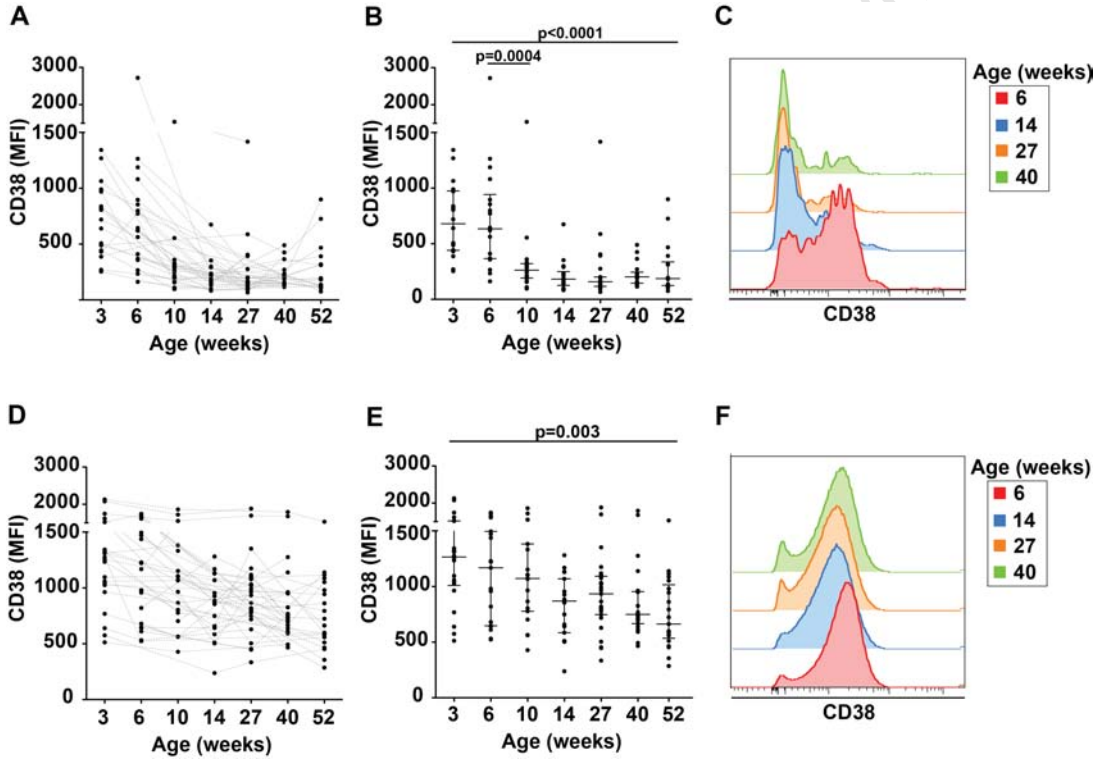

Figure 3.7. Changes in CD38 expression in CD4+ T cells over the first year of life. (A) Longitudinal (n=42) and (B) Cross-sectional analysis of CD38 expression in BCG specific CD4+T cells, identified by expression of any cytokine following incubation of whole blood with BCG for 12 hrs (n≥17/time point). (C) CD38 expression in BCG specific CD4+ T cells of a BCG vaccinated infant. (D) Longitudinal and (E) Cross-sectional analysis of CD38 expression in total (BCG-specific and non-specific) CD4+T cells. (F) CD38 expression in total CD4+ T cells of a BCG vaccinated infant. For longitudinal analysis, each line represents one infant. For cross-sectional analysis, the lines represent the median and the whiskers of interquartile range. The differences were calculated using a Mann Whitney test. A p value of less of 0.05 is considered as significant.

To further characterise the BCG induced immune response in infants, we investigated the CD38 expression according to IFN-γ, IL-2 or TNF-α expression, in BCG-specific CD4+ T cells. In CD4+IFN-γ+ cells there was a decrease in CD38 expression over time (Figure 3.8A). At 3 and 6 weeks IFN-γ+, IL-2+ and TNF-α+ CD4+ T cells are $CD38^{high}$. CD38 expression declined at 10 weeks and waned over time (Figure 3.8B, C, D and E). Interestingly, we found a similar pattern of CD38 expression in BCG specific CD4+ T cells that were triple positive for IFN-γ, IL-2 and TNF-α, or double positive for IFN-γ and TNF-α, or single positive for IFN-γ (data not shown). The BCG specific CD8+ T cell cytokine response was low and we could not investigate the CD38 expression in specific CD8+ T cells.

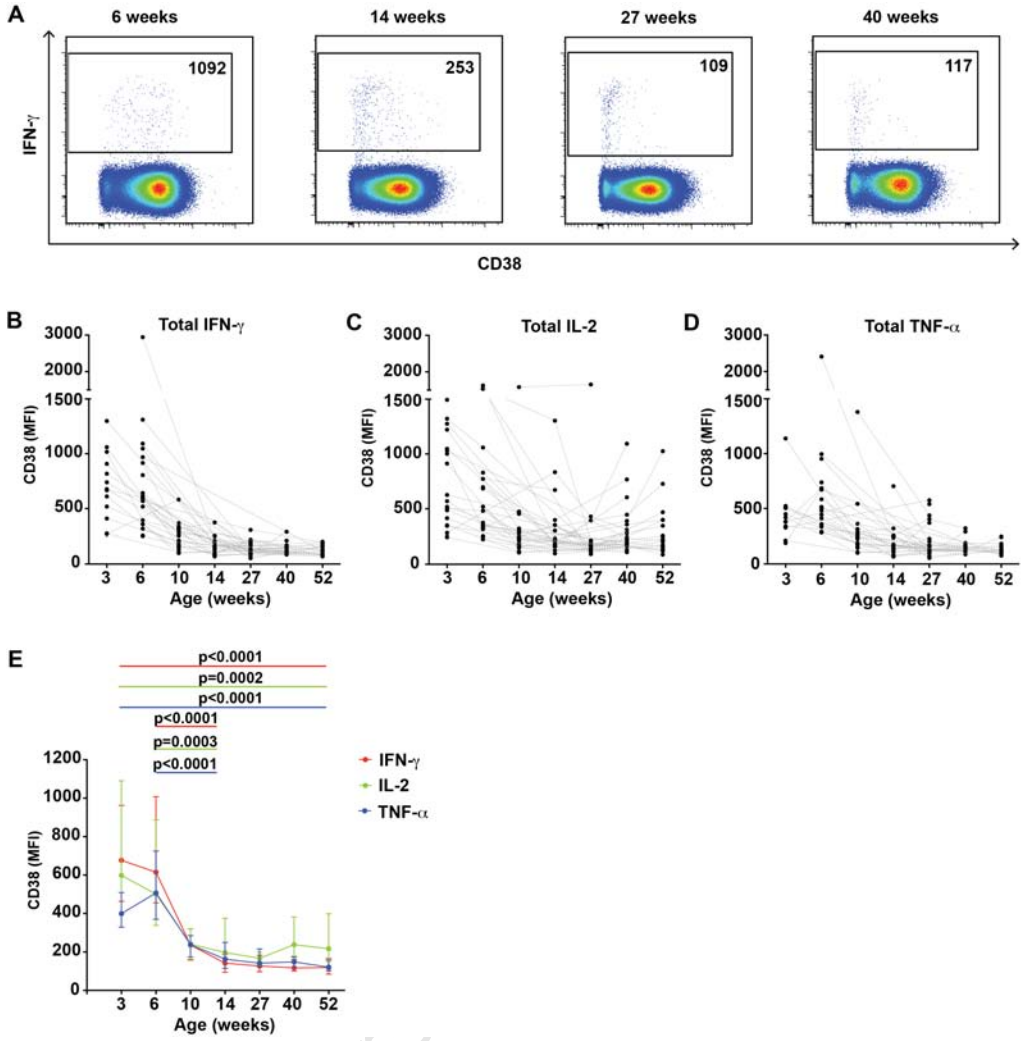

Figure 3.8 CD38 expression in BCG specific CD4+ T cells over the first year of life. (A) Changes in CD38 expression in BCG specific CD4+IFN-γ+ T cells following incubation of whole blood with BCG for 12 hrs. Longitudinal analysis of CD38 expression in BCG specific CD4+ T cells, identified by the production of (B) IFN-γ (n=40), (C) IL-2 (n=42), and (D) TNF-α (n=41). (E) Cross-sectional analysis of CD38 expression in BCG-specific CD4+T cells, identified by IFN-γ or IL-2 or TNF-α (n≥17/time point for each cytokine). For cross-sectional analysis, the lines represent the median and the whiskers of interquartile range. The differences were calculated using a Mann Whitney test. A p value of less of 0.05 is considered as significant.

3.4.5 Bcl-2 expression by total cytokine producing BCG specific CD4+ T cells

To investigate the ability of BCG to induce the formation of long-lived T cells, we used expression of Bcl-2 as a marker of these cells. Bcl-2 expression in BCG specific CD4+ T cells (again identified by expression of IFN-γ, IL-2, IL-17 and/or TNF-α) increased over the first year of life (Figure 3.9A, B and C). This trend was not observed in the total CD4+ T cell compartment, in which a significant decrease in Bcl-2 expression occurred (Figure 3.9 D, E and F). The data suggests that BCG specific CD4+ T cells are more long-lived and more resistant to apoptosis over the first year of life.

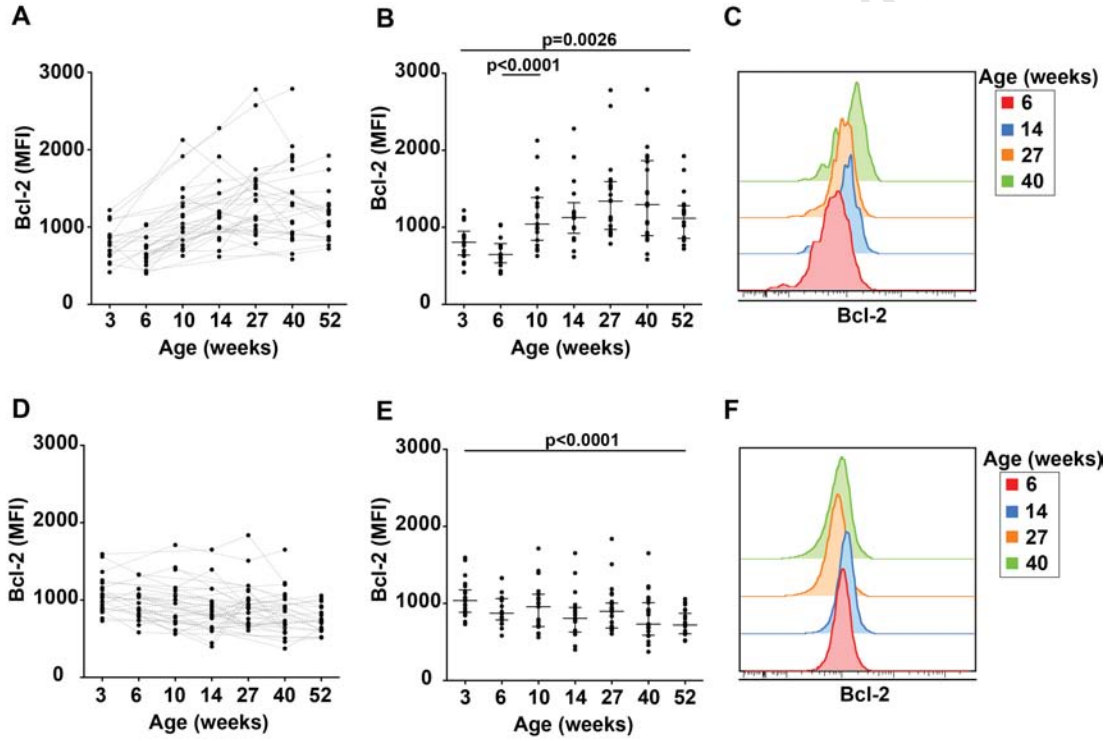

Figure 3.9 Changes in Bcl-2 expression in CD4+ T cells over the first year of life. (A) Longitudinal (n=42) and (B) Cross-sectional analysis of Bcl-2 expression in BCG specific CD4+T cells, identified by expression of any cytokine following incubation of whole blood with BCG for 12 hrs (n≥17/time point). (C) Bcl-2 expression in BCG specific CD4+ T cells of a BCG vaccinated infant (D) Longitudinal and (E) Cross sectional analysis of Bcl-2 expression in total (BCG-specific and non-specific) CD4+T cells. (F) Bcl-2 expression in total CD4+ T cells of a BCG vaccinated infant. For longitudinal analysis, each line represents one infant. For cross sectional analysis, the lines represent the median and the whiskers of interquartile range. The differences were calculated using a Mann Whitney test. A p value of less of 0.05 is considered as significant.

Similarly, we measured the expression of Bcl-2 in BCG specific CD4+ T cells, which produce IFN-γ, IL-2 or TNF-α. In CD4+IFN-γ+ T cells, there is an increase in Bcl-2 expression over time (Figure 3.10A). Longitudinal analysis of Bcl-2 expression in BCG specific CD4+ T cells that produce IFN-γ, IL-2 and TNF-α shows that there is a uniform increase in Bcl-2 over the first year of life (Figure 3.10B, C, D and E). Interestingly, we found a similar pattern of Bcl-2 expression in BCG specific CD4+ T cells that were IFN-γ+IL-2+TNF-α+ or IFN-γ+TNF-α+, or IFN-γ+ only (data not shown). The Bcl-2 expression in BCG specific CD8+ T cells could not be investigated because the cytokine responses in the CD8+ T cells were so low.

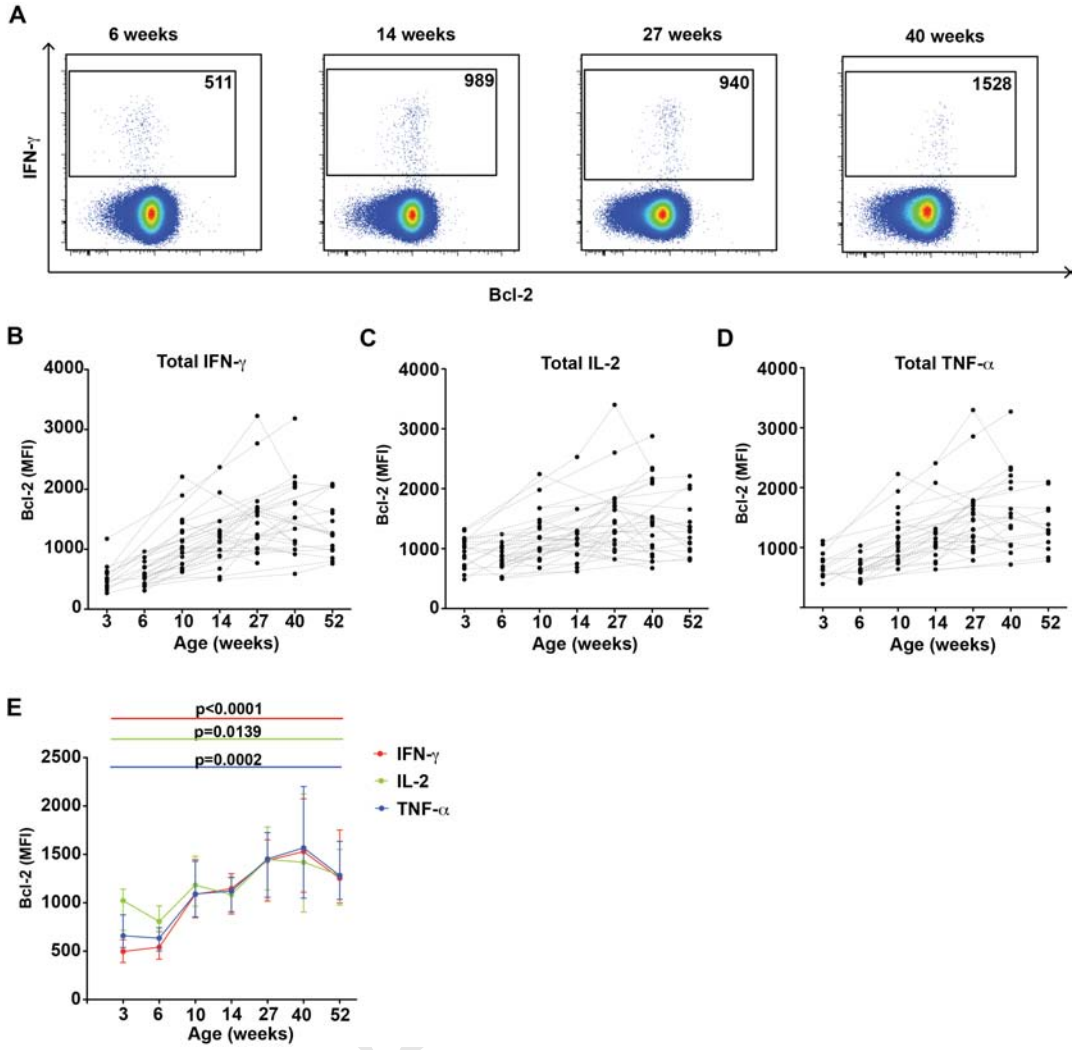

Figure 3.10 Bcl-2 expression in BCG specific CD4+ T cells over the first year of life. (A) Changes in Bcl-2 expression in BCG specific CD4+IFN-γ+ T cells following incubation of whole blood with BCG for 12 hrs. Longitudinal analysis of Bcl-2 expression in BCG specific CD4+ T cells, identified by the production of (B) IFN-γ (n=40), (C) IL-2 (n=42), and (D) TNF-α (n=41). (E) Cross-sectional analysis of Bcl-2 expression in BCG-specific CD4+T cells, identified by IFN-γ or IL-2 or TNF-α (n≥17/time point for each cytokine). For cross sectional analysis, the lines represent the median and the whiskers of interquartile range. The differences were calculated using a Mann Whitney test. A p value of less of 0.05 is considered as significant.

3.4.6 Combined expression of Bcl-2 and CD38 in BCG specific CD4+ T cells

We assessed the co-expression of Bcl-2 and CD38 in BCG specific CD4+ T cells, identified by the expression of IFN-γ, IL-2, IL-17 and TNF-α. We observed a decrease in specific co-expression of Bcl-2 and CD38 over time (Figure 3.11). Over the first year of life, the majority of BCG specific CD4+ T cells became $CD38^{low}$ and $Bcl\text{-}2^{high}$.

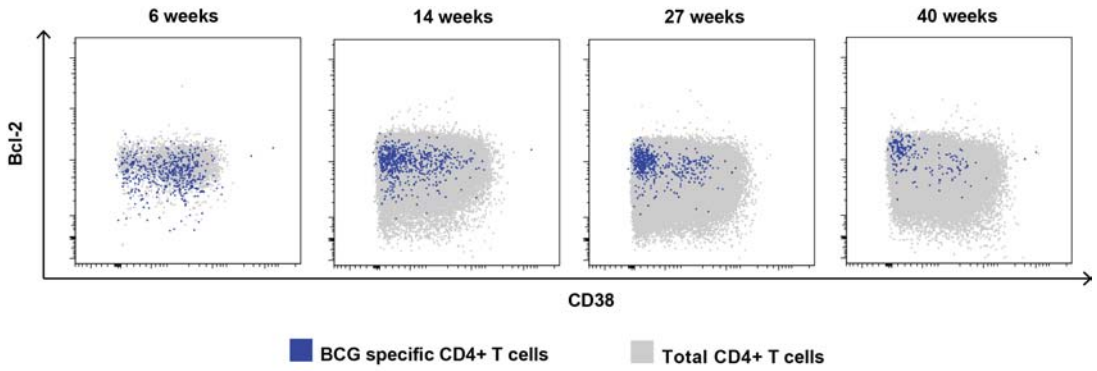

Figure 3.11 Co-expression of Bcl-2 and CD38 in BCG specific CD4+ T cells following 12 hrs incubation of whole blood with BCG.

3.5 Discussion

Multiple novel TB vaccines are currently being tested at the SATVI. Given that infants remain an important target population for novel TB vaccines, there is a need to investigate the longitudinal changes in BCG specific T cell immunity following vaccination of newborns with BCG. Our study revealed that the BCG induced immune responses peak at 10 weeks and that BCG specific T cells become long lived over the first year of life. At the peak effector phase, T cells are more prone to apoptosis (Wherry et al., 2004; Wherry et al., 2007); we therefore propose that immunisation with novel booster TB vaccines at 10 weeks would result in T cell exhaustion and a suboptimal vaccine response. Our data suggests that the BCG induced immunity should be boosted after 14 weeks, following the contraction of the immune response and the formation of long-lived T cells.

In contrast to the peak at 10 weeks found in our study, a study in TST negative adults has reported that the peak of the BCG vaccine response occurs at 4 weeks following vaccination with BCG (McShane et al., 2004). On the other hand, Nabeshima et al. found the peak responses occurred at 8 weeks following BCG vaccination of TST negative health care workers (Nabeshima et al., 2005). It is possible that the immune system of these adults could have been primed by environmental mycobacteria or by non-mycobacterial organisms that contain cross-reactive antigens. It is known that the presence of pre-existing immunity can modulate the immune responses to BCG (Black et al., 2002; Scriba et al., 2011). Alternatively, the discrepancies in results could be reflective of the immaturity of the infant immune system. Innate cells from infants are less capable at priming Th1 responses at an early age, which could account for the delayed peak observed at 10 weeks in infants (Gans et al., 1998; Levy, 2007).

Infant cells became more polyfunctional over the first year of life. We propose that as infant immune system matures over time, they are more able to produce multiple cytokines simultaneously. Polyfunctional T cells have been associated with protection against *Leishmania major* (Darrah et al., 2007). The importance of polyfunctional T cells in the protection against TB remains

unclear. Work by Sutherland et al. showed that the frequencies of polyfunctional CD4+ T cells, which co-express IFN-γ, IL-2 and TNF-α, are significantly higher in TB cases than individuals with latent TB infection (Caccamo et al., 2010; Sutherland et al., 2009), suggesting that polyfunctional T cells may be associated with poor outcome. In contrast, Day et al. has shown that there is an increase in polyfunctional T cells during anti-TB therapy, suggesting that presence of these T cells may be associated with a recovering and more optimal immune response (Day et al., 2011). Future studies investigating the immune correlates of TB protection will help in the better understanding of the role of polyfunctional T cells in the context of TB. Interestingly, it has been shown that TB patients have a higher proportion of TNF-α single positive cells and IFN-γ+TNF-α+ T cells than individuals with latent infection (Day et al., 2011; Harari et al., 2011). This suggests that although these cytokines are important in the protection against TB, these cytokines may contribute to excess inflammation during TB disease.

We found that CD38 expression is higher at 3 weeks in the total CD4+ T cell compartment than in BCG specific CD4+ T cells. It is very unlikely that all CD4+ T cells are highly activated at 3 weeks in BCG vaccinated infants. Besides being expressed by activated T cells, CD38 is also expressed early on by immature T cells (Shubinsky and Schlesinger, 1997; Tenca et al., 2003). Approximately, 90% of lymphocytes in newborns are CD38 positive. CD38 expression is downregulated on mature T cells but is re-expressed upon activation. This could account for the high levels of CD38 expression at observed 3 weeks and the gradual decrease in CD38 expression over the first year of life in the total CD4+ T cell compartment. Overall, our data suggests that CD38, on its own, may not be an appropriate activation marker to be used in infants. We will assess whether BCG specific CD4+ T cells are able to co-express CD38 and other markers of activation such as HLA-DR in future experiments at SATVI.

The capacity of BCG to induce long-lived T cells in humans has not been studied to date. Recently, it has been hypothesised that BCG is able to induce the formation of effector memory T cells but not long-lived central memory T cells (Orme, 2010). The author proposed that T cell exhaustion due to the

constant exposure to environmental mycobacteria leads to the depletion of effector memory T cells. Given that central memory T cells are not present, the pool of effector T cells is not replenished leaving BCG vaccinated individuals susceptible to develop TB disease. In support of this hypothesis, it has been shown that BCG establishes a chronic infection in mice and a very low proportion of antigen specific cells express a central memory phenotype (Henao-Tamayo et al., 2010). Our data are not in accord with this hypothesis since we found that Bcl-2 expression in BCG specific CD4+ T cells increases significantly over the first year of life, indicating that BCG vaccination of newborns establishes a pool of long-lived T cells.

Overall, our study provides a comprehensive characterisation of the BCG specific T cell responses over the first year of life. Our data significantly contributes to our knowledge on the kinetics of the T cell responses following vaccination with BCG and is important in guiding future TB vaccination strategies.

Chapter 4. Immune recognition of latency and reactivation associated antigens following vaccination of newborns with BCG.

4.1 Introduction

The most efficient intervention to control the TB pandemic remains vaccination. BCG does not prevent the establishment of latent Mtb infection in the majority of vaccinated individuals, or the reactivation of latent Mtb infection to cause TB disease. Current novel TB vaccines contain immunodominant antigens of Mtb, which are expressed during early Mtb infection and during TB disease (Abel et al., 2010; Scriba et al., 2010). However, we now appreciate that different sets of antigens are expressed during latency and during reactivation of latent Mtb, suggesting that novel TB vaccines to target latency and reactivation should include these antigens.

It is generally accepted that Mtb switches to an alternative metabolic state in order to adapt and survive the challenging conditions encountered during latency. These conditions are likely to include oxygen and nutrients deprivation in the host cell (Leyten et al., 2006; Tufariello et al., 2003). Expression profiling of Mtb grown under hypoxic and low nutrient conditions in vitro, simulating latency, have shown that sets of genes termed the dormancy regulon (DosR) and starvation regulon are upregulated (Betts et al., 2002; Schnappinger et al., 2003; Voskuil et al., 2003). In addition, 100 genes were found to be upregulated during reactivation of Mtb in vitro (Sherrid et al., 2010). Resuscitation promoting factors (Rpfs) have also been shown to be involved during Mtb reactivation in vitro (Kana et al., 2008; Mukamolova et al., 2002). Rpfs are important for Mtb virulence, and are required for the resuscitation of Mtb from dormancy.

Numerous human studies have investigated the immune responses to latency associated and reactivation associated antigens. These studies have compared responses to these antigens between TB patients and individuals with latent infection (Black et al., 2002; Commandeur et al., 2011; Demissie et al., 2006; Govender et al., 2010; Leyten et al., 2006; Schuck et al., 2009). As a general rule, latently infected individuals appear to recognise more latency and reactivation associated antigens, and have higher IFN-γ responses to

these antigens, compared with TB diseased patients. In contrast, there appears to be higher recognition of immunodominant antigens in TB diseased patients, compared with latently infected individuals. These findings suggest that latency and reactivation associated antigens may be preferentially expressed during latency and reactivation in humans, and may therefore be targeted by the immune system for protection during these stages. Whether BCG primes an immune response to latency and reactivation associated antigens in infants is unknown. This is the focus of this study.

There is at least 97% amino acid homology in the DosR regulon of BCG Pasteur and Mtb H37Rv (Lin et al., 2007). Recently, expression of starvation antigen Rv2660 by BCG has been shown in vitro (Aagaard et al., 2011; Brosch et al., 2007). Moreover, using PCR, Mukamolova et al. showed that the BCG genome contains genes that encode for Rfps (Mukamolova et al., 1998). These data highlights the ability of BCG to express latency and reactivation associated antigens, raising the possibility that BCG vaccination induces an immune response to these antigens.

In this chapter, we assessed the immune recognition of multiple latency and reactivation associated antigens by infants following newborn BCG vaccination. Given that latency and reactivation associated antigens are essential for the survival and reactivation of Mtb, these antigens may be potential candidates for novel TB vaccines. We propose that these antigens, if recognised following BCG vaccination, can be used to boost the BCG induced immune response, so that Mtb can be targeted for prevention of latency, or reactivation of latent Mtb.

4.2 Aims

The objective of this study is to determine the immune recognition of latency and reactivation associated antigens following BCG vaccination of infants. The following aims were addressed:

- To determine the proportion of infants that recognise latency and reactivation associated antigens
- To determine the most widely recognised latency and reactivation associated antigens in BCG vaccinated infants
- To determine quantitative differences in IFN-γ production by PBMC after 5 day stimulation with latency and reactivation associated antigens

4.3 Materials and methods

4.3.1 Study participants

Participants were randomly selected from those who took part in a completed study of BCG-induced correlates of risk of TB disease (Kagina et al., 2010). For the latter study, infants were vaccinated with Japanese BCG (Tokyo 172; Japan BCG laboratory) at birth. At 10 weeks of age, blood was collected and PBMC isolated and cryopreserved. For the project reported here, infants who did not develop TB disease during 2 years of follow up were randomly selected. The study protocol was approved by the University of Cape Town Research Ethics Committee.

4.3.2 Latency-associated antigens

The antigens used were first classified into different groups based on available literature, or on the information provided by Genolist (http://genolist.pasteur.fr/Tuberculist) and Tuberculist (http://tuberculist.epfl.ch) (Behr et al., 1999; Betts et al., 2002; Mukamolova et al., 2002; Schnappinger et al., 2003; Sherrid et al., 2010; Talaat et al., 2004; Voskuil et al., 2003). The complete list of antigens is provided in Table 4.1.

For the first round of screening, we selected 9 antigens, 7 of which have been shown to be widely recognised in infants from the UK and Malawi (Hazel Dockrell, personal communication, blue in table), and 2 of which have been shown in Cape Town to be differentially recognised between individuals with latent TB infection and active TB disease (red in table) (Govender et al., 2010). The remaining 89 latency-associated antigens were then assessed in a second round of antigen screening (black in table). The immune recognition of latency and reactivation associated antigens was assessed at 2 different concentrations since some BCG vaccinated infants have been shown to respond to either low (1 µg/ml) or high (10 µg/ml) concentrations of starvation antigens Rv2659 and Rv2660 in Cape Town (Jane Hughes and Willem Hanekom, personal communication).

Table 4.1 List of latency and reactivation associated antigens, which were assessed in BCG, vaccinated infants. DosR antigens, which have previously been shown to be the most widely recognised in infants from the UK and Malawi, are highlighted in blue (see text). Starvation antigens, which have been shown in previous projects to be differentially recognised between latently infected and TB diseased individuals in Cape Town, are highlighted in red.

Antigen	Classification	Antigen	Classification
Rv0079	Dos Regulon	Rv1996	Dos Regulon
Rv0080	Dos Regulon	Rv1997c	Dos Regulon
Rv0081	Dos Regulon	Rv1997n	Dos Regulon
Rv0140	Reactivation	Rv1998	Dos Regulon
Rv0246	Reactivation	Rv2003c	Dos Regulon
Rv0251c	Reactivation	Rv2004c	Dos Regulon
Rv0288	Classical	Rv2005c	Dos Regulon
Rv0331	Reactivation	Rv2006	Dos Regulon
Rv0384c	Reactivation	Rv2007c	Dos Regulon
Rv0569	Dos Regulon	Rv2028c	Dos Regulon
Rv0570c	Dos Regulon	Rv2029c	Dos Regulon
Rv0570n	Dos Regulon	Rv2030c	Dos Regulon
Rv0571c	Dos Regulon	Rv2032	Dos Regulon
Rv0572c	Dos Regulon	Rv2034	Starvation
Rv0573c	Dos Regulon	Rv2090	Reactivation
Rv0574	Dos Regulon	Rv2389c	Rescuscitation processing
Rv0753c	Reactivation	Rv2450c	Rescuscitation processing
Rv0867c	Rescuscitation processing	Rv2465c	Reactivation
Rv1009	Rescuscitation processing	Rv2466c	Reactivation
Rv1073	Reactivation	Rv2558	Starvation
Rv1115	Reactivation	Rv2623	Dos Regulon
Rv1130	Reactivation	Rv2624c	Dos Regulon
Rv1131	Reactivation	Rv2625c	Dos Regulon
Rv1471	Reactivation	Rv2626c	Dos Regulon
Rv1506c	RD6 related	Rv2627c	Dos Regulon
Rv1507c	RD6 related	Rv2628	Dos Regulon
Rv1508c	RD6 related	Rv2629	Dos Regulon
Rv1509	RD6 related	Rv2630	Dos Regulon
Rv1510	RD6 related	Rv2631	Dos Regulon
Rv1511	RD6 related	Rv2658c	Starvation
Rv1512	RD6 related	Rv2659c	Starvation
Rv1513	RD6 related	Rv2660c	Starvation
Rv1514	RD6 related	Rv2661c	Starvation
Rv1515	RD6 related	Rv2662	Starvation
Rv1516	RD6 related	Rv2745c	Starvation
Rv1717	Reactivation	Rv3054c	Reactivation
Rv1733c	Dos Regulon	Rv3126c	Dos Regulon
Rv1734c	Dos Regulon	Rv3127	Dos Regulon
Rv1735c	Dos Regulon	Rv3128c	Dos Regulon
Rv1736c	Dos Regulon	Rv3129	Dos Regulon
Rv1736n	Dos Regulon	Rv3130c	Dos Regulon
Rv1737c	Dos Regulon	Rv3131	Dos Regulon
Rv1738	Dos Regulon	Rv3132c	Dos Regulon
Rv1812c	Dos Regulon	Rv3133c	Dos Regulon
Rv1813c	Dos Regulon	Rv3134c	Dos Regulon
Rv1874	Reactivation	Rv3223c	Reactivation
Rv1875	Reactivation	Rv3307	Reactivation
Rv1884c	Rescuscitation processing	Rv3463	Reactivation
Rv1955	Starvation	Rv3862c	Reactivation

Recombinant antigens were provided as lyophilized powder by our collaborators in Leiden, The Netherlands. These antigens were synthesised by expression in *E.coli*, followed by purification with chromatography. Contaminants, including endotoxins were further removed by washing with 60% isopropanol (Franken et al., 2000). The purity of the recombinant proteins was verified by a negative Western blot using anti-*E.coli* antibodies and by an endotoxin level of less than 50 IU/mg. The antigens were reconstituted in 0.05% v/v DMSO, followed by dilution with PBS to a concentration of 100 mg/ml or 1 mg/ml.

4.3.3 Thawing and restimulation of PBMC

Cryopreserved PBMC were thawed quickly in a water bath at 37°C. Cells were transferred to a 15 ml conical tube (Corning) containing 12.5% heat inactivated human AB serum in RPMI (complete medium) and 2.5 µg/ml DNAase (Sigma-Aldrich). The PBMC were pelleted and resuspended in complete medium and rested overnight at 37°C, 5% CO2 in a humidified incubator.

The following day, the cells were resuspended using a sterile Pasteur pipette. The number of viable PBMC was obtained using a Coulter Counter (Beckman Coulter) and the cell suspension was adjusted to 2 x 10^6 cells/ml using complete medium, containing 2mM L-Glutamine (Bio Whittaker). Two hundred thousand cells were placed in each well of a 96 well round-bottomed plate (NUNC). PBMC were left unstimulated (negative control) or stimulated with latency and reactivation associated antigens at 1 and at 10 µg/ml. PHA was used as a positive control at 2 µg/ml. PBMC were stimulated for 5 days at 37°C and 5% CO_2 in a humidified incubator. The supernatant was collected after 5 days of stimulation and stored in sealed 96 well plate (NUNC) at -20°C.

4.3.4 IFN-γ ELISA assay

The production of soluble IFN-γ was used as an indication of antigen recognition. To detect soluble IFN-γ, the Ready-SET-GO! ELISA kit (eBioscience) was used. Briefly, ELISA plates were first coated with capture

antibody (NIB42), blocked with assay buffer and fixed with 15% v/v sucrose solution. Supernatant from 5 day stimulated PBMC samples was thawed and added to the pre-coated ELISA plate, followed by incubation for 2 hrs at room temperature. After washing, a biotin-conjugated detection antibody (4S.B3) was added followed by the enzyme streptavidin-HRP. TMB was used as substrate and the reaction was stopped using 2M H_2SO_4. Absorbance for each plate was read at 450 nm and 650 nm wavelengths using the VerseMax ELISA plate reader. The data was acquired using the Softmax pro software v4.5.1 (Molecular Devices Corporation).

4.3.5 Data analysis and interpretation

IFN-γ standards (4-500 pg/ml) were included in each plate and absorbance readings of each standard dilution were used to design a standard curve of absorbance against IFN-γ concentrations using Softmax pro software v4.5.1. A positive response in an antigen containing well was defined as being above the mean plus 3 standard deviations of all the negative controls, of all the infants, examined together, and above the limit of detection of the assay. We considered the limit of detection of the assay, as being twice the lowest IFN-γ standard.

Results were discarded from the analysis if they did not have a positive PHA response. An antigen was considered as recognised if a positive IFN-γ response was induced at either 1 or 10 μg/ml. For the analysis of IFN-γ responses, the background, as defined by the IFN-γ level in the unstimulated control, was subtracted from that of the antigen stimulated sample.

4.4 Results

4.4.1 Recognition of selected DosR and starvation antigens by BCG vaccinated infants

To determine whether BCG primed an immune response to latency-associated antigens, we first investigated the immune recognition of 7 DosR and 2 starvation antigens in BCG vaccinated infants. We found that the 9 latency-associated antigens were recognised in a minority of BCG vaccinated infants (Figure 4.1A), with DosR antigen Rv1733 being the most frequently recognised. A wide range of quantitative IFN-γ production was shown in those who did have responses above the lower limit of detection (Figure 4.1B). The lower limit of detection of the assay was 8 pg/ml.

The results indicate that most infants vaccinated with BCG, do not recognise these 9 latency associated antigens.

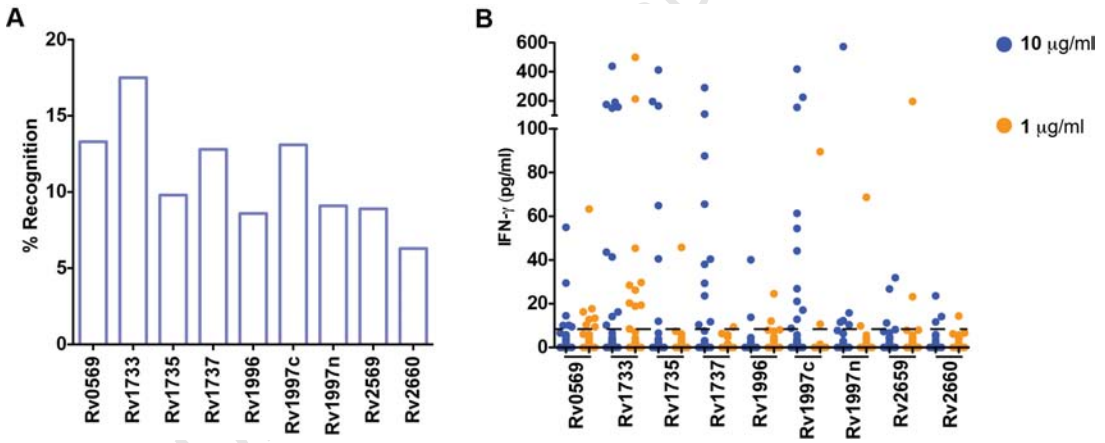

Figure 4.1. Immune recognition of DosR and starvation antigens by BCG vaccinated infants. (A) Proportion of infants who recognised latency associated antigens (n≥80/antigen) and (B) IFN-γ production following 5 day incubation of infant PBMC with 10 and 1 μg/ ml of latency-associated antigens. The dotted line shows the lower limit of detection of the assay.

4.4.2 Recognition of a wider range of latency and reactivation associated antigens, following vaccination of newborns with BCG

Screening of the other 89 latency and reactivation associated antigens in BCG vaccinated infants revealed that the immune recognition of these antigens ranged from 0 to 38% (Figure 4.2). Among the 89 antigens screened, DosR antigens Rv1733c, Rv2004 and Rv3128, as well as reactivation antigen Rv1874, were the most widely recognised, in 30-38% of infants. Our findings validated the main finding from the first set of antigens screened (section 4.4.1): individual latency associated antigens were recognised by a minority of infants.

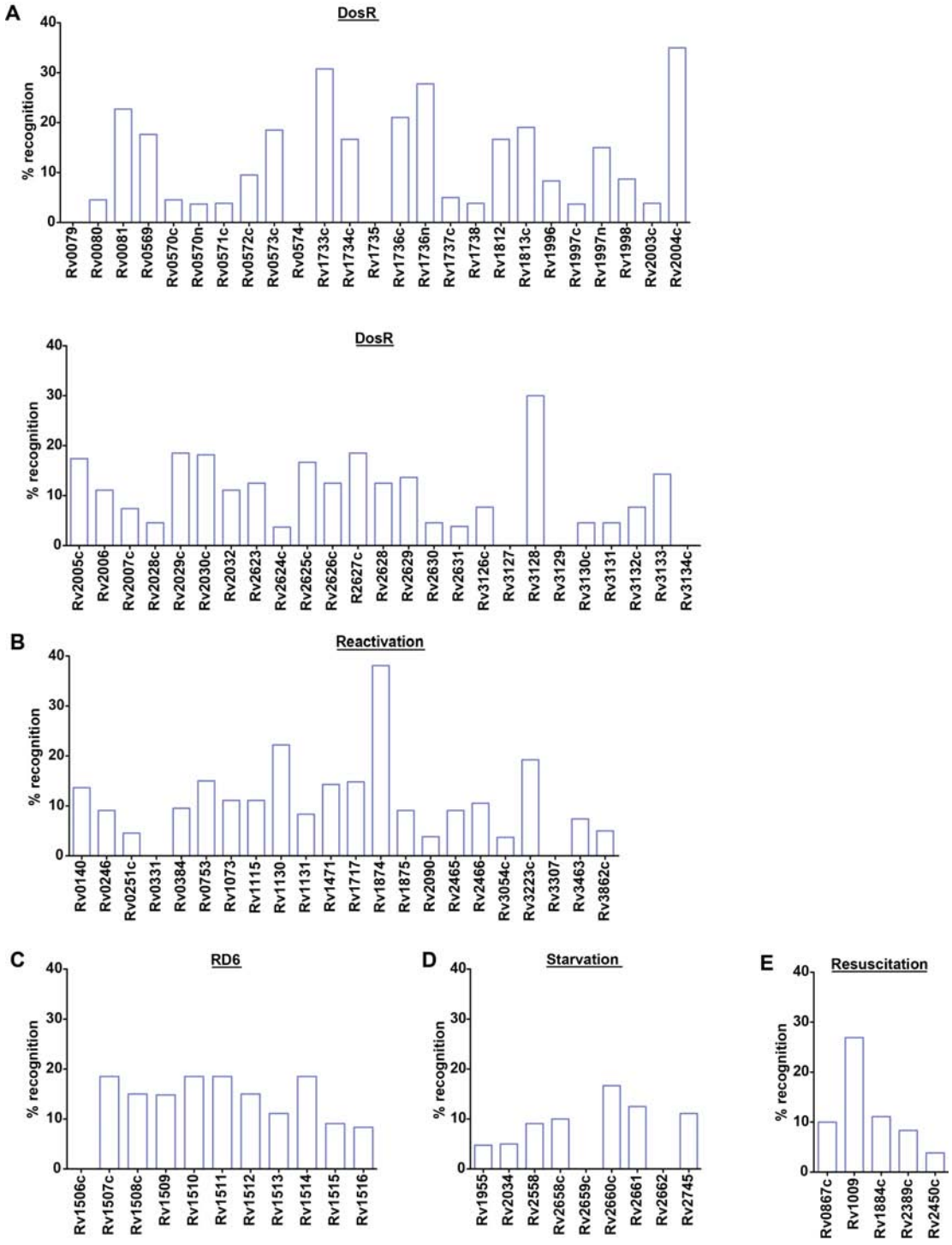

Figure 4.2 Proportion of BCG vaccinated infants who recognised latency and reactivation associated antigens after 5 day stimulation of infant PBMC (n≥17/antigen) with (A) DosR antigens (two figures), (B) Reactivation antigens, (C) Starvation antigens, (D) RD6 related antigens and (E) Resuscitation promoting factors. Bars are not visible in the graphs if none of the infants recognised that antigen.

4.4.3 Recognition of latency and reactivation associated antigens at 1 or 10 µg/ml

We stratified the immune recognition of latency and reactivation associated antigens according to the concentration of antigens used to stimulate infant PBMC. We observed a general trend for more latency and reactivation associated antigens to be recognised at 10 µg/ml, compared with 1 µg/ml (Figure 4.3). As an example, DosR antigen Rv0081 is recognised by 22.7% of BCG vaccinated infants at 10 µg/ml compared with 4.6% of infants who recognise the same antigen at 1 µg/ml. Importantly, 25 antigens were recognised either at 1 µg/ml or at 10 µg/ml, but not at both concentrations, suggesting that inclusion of both concentrations is critical for assessing responses.

As seen during the first analysis, a wide range of IFN-γ production was detected in those who had responses above the limit of detection (Figure 4.4). Stimulation of infant PBMC with 10 µg/ml antigen generally induced higher production of IFN-γ, compared with PBMC stimulated with 1 µg/ml antigen, but not invariably.

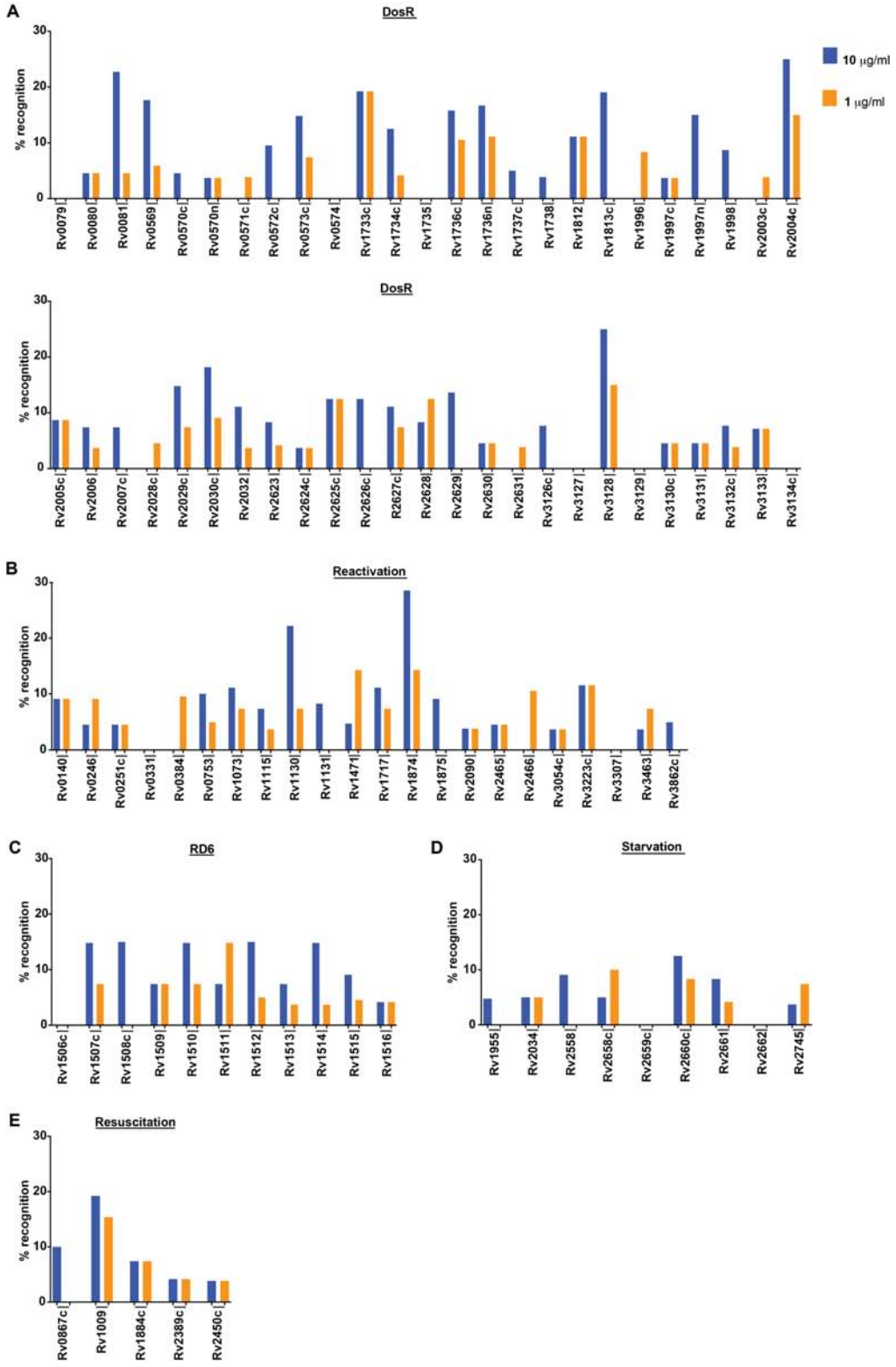

Figure 4.3 Proportion of BCG vaccinated infants who recognised latency and reactivation associated antigens at 10 or 1 µg/ml antigens after 5 day stimulation of infant PBMC (n≥17/antigen) with (A) DosR antigens (two figures), (B) Reactivation antigens, (C) Starvation antigens, (D) RD6 related antigens and (E) Resuscitation promoting factors. Bars are not visible in the graphs if none of the infants recognised that antigen at 10 or 1 µg/ml.

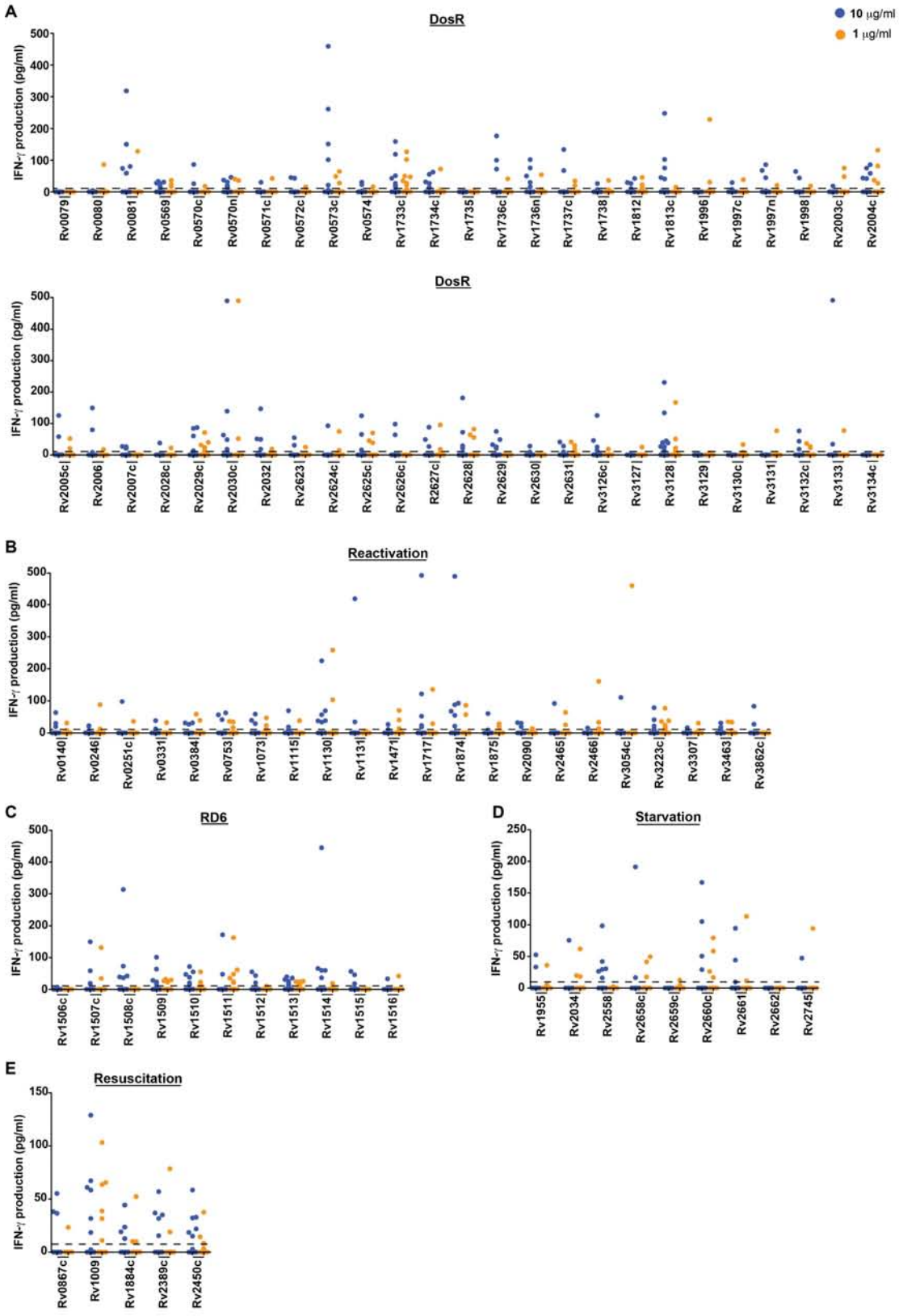

Figure 4.4 IFN-γ responses following 5 day stimulation of infant PBMC (n≥17/antigen) with 10 or 1 μg/ml (A) DosR antigens, (B) Reactivation antigens, (C) Starvation antigens, (D) RD6 related antigens, and (E) Resuscitation promoting factors. The dotted line shows the lower limit of detection of the assay.

4.4.4 Recognition of any or multiple latency and reactivation associated antigens in infants, following vaccination with BCG

We assessed the proportion of BCG vaccinated infants that recognised any, or multiple, latency and reactivation associated antigens. Forty two percent did not recognise any of these antigens, whereas 58% of BCG vaccinated infants recognised at least one latency or reactivation associated antigens (Figure 4.5A).

The findings were stratified according to the antigen concentration that was used to stimulate infant PBMC. Our analysis revealed that 50% and 60% of BCG vaccinated infants do not recognise any latency or reactivation associated antigens at 10 and 1 µg/ml, respectively (Figure 4.5B).

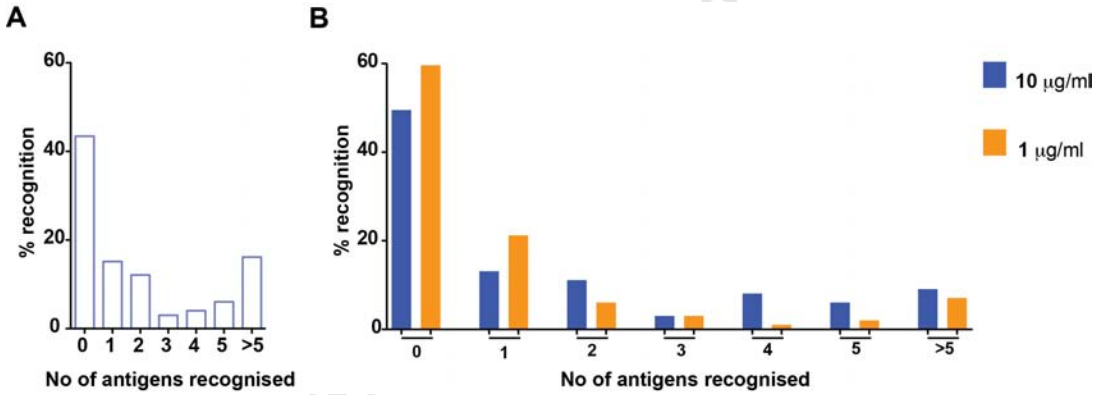

Figure 4.5 Co-recognition of multiple latency and reactivation associated antigens in BCG vaccinated infants. (A) Recognition of any latency or reactivation associated antigens after 5 days stimulation of infant PBMC with these antigens, and (B) Recognition of latency and reactivation associated antigens stratified according either 10 or 1 µg/ml. No bars of are visible if infants did not recognise any given number of antigens.

4.5 Discussion

Success of new TB vaccines is likely to depend, in part, on the choice of antigens to be included in the vaccine. Since Mtb appears to express different antigens during early infection, latency, reactivation and active disease, the ideal vaccine should target Mtb at all life stages. In this study, we showed that all latency and reactivation associated antigens tested are recognised by a minority of infants following BCG vaccination, even though a majority of infants recognise at least 1 latency or reactivation antigen. We have therefore not identified a single antigen that could ideally be included for universal boost vaccination, following BCG. Regardless, combinations of antigens such as Rv1733c, Rv2004, Rv3128 and Rv1874 in a boost vaccine should induce a secondary immune response in approximately 58% of BCG vaccinated infants. The concern is that a secondary immune response to these most commonly recognised antigens might not be induced in 46% of BCG vaccinated infants.

Only two studies have assessed the immune recognition of latency associated antigens following BCG vaccination, but these were conducted in adult populations (Geluk et al., 2007; Lin et al., 2007). Our findings agree with findings from these studies, which also showed an absence of immune recognition of DosR antigen, as measured by IFN-γ production following incubation of whole blood with DosR antigens for 6 days. Our results expand on these studies by showing that, in addition to DosR antigens, antigens expressed during starvation and during Mtb reactivation, are also relatively poorly recognised, on a population basis, following vaccination of newborns with BCG. The poor immune recognition of these antigens could reflect the fact that BCG does not express these antigens following vaccination, even though the genes encoding for latency and reactivation associated antigens are contained within the BCG genome (Lin et al., 2007; Mukamolova et al., 1998).

We found that an antigen in the new TB vaccine H56, that is, Rv2660, was recognised by 5.5% of BCG vaccinated infants only. Despite the lack of recognition in BCG vaccinated infants, pre-clinical testing of the H56 vaccine

(which contains ESAT6, Ag85B and Rv2660 and the adjuvant CAF01) in mice showed that the vaccine successfully boosted the BCG induced immune responses (Aagaard et al., 2011). H56 boosted mice had Rv2660 specific responses. BCG is able to express Rv2660 in vitro (Aagaard et al., 2011; Brosch et al., 2007). While Rv2660 was non immunogenic and did not confer protection on its own, mice immunised with the H56 vaccine, following BCG vaccination, had significantly reduced bacterial burdens, compared with mice who received BCG followed by an ESAT6-Ag85B fusion protein-containing vaccine H1 (The H1 vaccine also contains the same adjuvant CAF01). The outcomes were assessed 24 weeks post Mtb challenge. This supports the hypothesis that despite the lack of immune recognition of latency and reactivation associated antigens in our study, these antigens might still have induced a specific immune response, but that this was not detectable by our assay system. This implies that latency and reactivation associated antigens could possibly still be used in new TB vaccines to boost the BCG induced immunity; this will have to be tested in clinical studies. Alternatively, using T cell lines to assess the immune responses to latency and reactivation associated antigens could be more sensitive.

A surprising finding in our study is the immune recognition (0-18% recognition) of RD6 related antigens in some BCG vaccinated infants, given that Japanese BCG lack RD6 regions (Behr et al., 1999). This raises the possibility of false positive results, or that some BCG vaccinated infants in our cohort could have been exposed to Mtb, resulting in immune recognition of RD6 related antigens. A limitation of our study is that infants were not assessed for Mtb infection at 10 weeks of age, when blood was collected. However, the rate of Mtb infection among our infants was estimated to be 2-4% annually, implying that the possibility of a 10-week old infant being Mtb infected is very low (Middelkoop et al., 2008). Further, all infants who were from households where anyone had TB disease or any cough were excluded.

A limitation of our study was the lack of a mycobacterial control such as PPD or an immunodominant antigen such as Ag85b, although it is unlikely that the lack of immune responses to latency and reactivation associated antigens is due to assay determinants. We found that the viability of infant cells after

thawing was excellent, ranging from 85% to 96%, and that the majority of infants responded to positive control PHA.

Interestingly, while the majority of infants did not recognise any particular antigens, we found that 58% of the BCG vaccinated infants do recognised at least 1 latency or reactivation associated antigen. The data indicates that infant responses to these antigens are heterogeneous, on a population level. In this regard, Finan et al. showed that there is up to 10 log-fold differences in IFN-γ, IL-13 and IL-15 cytokine expression after 6 days stimulation of infant whole blood with BCG within a population of Gambian infants (Finan et al., 2008). This highlights the variability of immune responses to BCG on a population basis.

Our study raises an important question regarding whether the inability of BCG to prevent establishment of latent Mtb infection, or reactivation of Mtb, could be related to the lack of immune responses to latency and reactivation associated antigens following vaccination. Moreover, whether infants who recognise latency and reactivation associated antigens are protected against TB disease compared with those that did not recognise any of these antigens remains unknown. This question will be addressed in future studies at SATVI. The immune responses to the most widely recognised antigens from this study will be assessed in a cohort of infants who were followed up for 2 years, during which some infants developed TB disease whereas other infants remained healthy.

Overall, to the best of our knowledge, this is the first time that such an extensive panel of latency and reactivation associated antigens were screened in BCG vaccinated infants. Despite the lack of immune recognition of these antigens following BCG vaccination, we propose that latency and reactivation associated antigens should be included in novel TB vaccines to complement, rather than boost the BCG induced immunity. Ultimately, novel TB vaccination strategies that target Mtb immunodominant, latency and reactivation associated antigens may have a significant impact on the TB pandemic.

Chapter 5. General discussion and conclusion.

Despite the discovery of Mtb by Robert Koch in the late 19th century, we are still in search of more effective vaccination strategies against TB. Intensive research has paved the way for development of 14 new TB vaccines currently in clinical trials. BCG remains central to novel vaccine strategies, in which infants are vaccinated with BCG at birth, followed by a booster vaccine at later ages. Infants are important targets of novel TB vaccines; the studies described in this thesis focused on infants and addressed two key questions pertaining to TB vaccinology.

The first question was when to optimally boost BCG induced immunity in infants. In chapter 3, we described longitudinal changes in the BCG specific T cell immunity, over the first year of life. Our data revealed that the peak of the vaccine response is at 10 weeks; therefore boosting at 10 weeks (or before) is not ideal and may result in suboptimal vaccine responses. We propose that BCG induced immunity should be boosted after 14 weeks (or thereafter), following the contraction of the immune response and the formation of long-lived T cells.

The grand challenge is what the optimal age, after 14 weeks, would be for administering a novel vaccine. There are two possibilities. In the first scenario, a new TB vaccine could be introduced within the EPI schedule. While this would have multiple benefits, including reduced number of clinic visits and therefore increased vaccine coverage, recent evidence suggests that co-administration of the novel TB vaccine MVA85A with other EPI vaccines may result in reduced immunogenicity of MVA85A (Ota et al., 2011). Therefore, potential co-administration of new TB vaccines with other EPI vaccines would require interference studies.

In the second scenario, a new vaccine administration time point would be allocated, specifically for the administration of the novel TB vaccine. However, implementation of a new vaccination time point has huge implications for public health care structures, including significant demand on resources, which may be limited in settings where the vaccine is needed most. Ultimately, research-driven pilot implementation of the vaccine, within the EPI

schedule or at a new vaccination time point, is needed to assist informed decision-making about the most suitable vaccine schedule.

Despite important progress being made in the field of TB vaccines, the aim of most current TB vaccines is to prevent onset of TB disease; this may not lead to the complete eradication of Mtb (Kaufmann, 2010). There is a need for a new TB vaccine that is effective against all life stages of the bacilli. Therefore, we addressed the question whether BCG induces immunity to latency and reactivation associated antigens, which could then be boosted during follow-up vaccination. In chapter 4, we assessed the immune recognition of multiple latency and reactivation associated antigens following BCG vaccination of newborns. Our data showed that latency and reactivation associated antigens are recognised by a minority of BCG vaccinated infants. This supports the idea that the inability of BCG to induce sterilising immunity may be attributed to a lack of immune responses against latency and reactivation associated antigens (Dey et al., 2011; Vekemans et al., 2004).

Therefore, novel TB vaccination strategies are needed to induce immune responses against latency and reactivation associated antigens. We propose that this can be achieved through the design of a recombinant BCG, which expresses latency and reactivation associated antigens. In this case, a novel TB vaccine containing latency and reactivation associated antigens can also be used to boost the immunity primed by the recombinant BCG. The boost could be a subunit or a viral vectored vaccine, and could contain latency and reactivation associated antigens, as well as, antigens expressed during early infection and TB disease, as BCG does induce responses to most of the latter immunodominant antigens.

Detailed studies are still required to guide the choice of latency and reactivation associated antigens for future inclusion in new TB vaccines. Importantly, safety of these antigens in new TB vaccines should be determined, as resuscitation-promoting factors (Rpfs) have been shown to be important in Mtb virulence, for example.

Collectively, the studies presented in this thesis contribute to our knowledge of the BCG induced immunity in infants. The data provide a framework for

guiding novel TB vaccination strategies and design of novel TB vaccines against TB.

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Appendix I

BCG (*Mycobacterium bovis* bacillus Calmette Guerin Danish 1331)

Supplier: Statens Serum Institut

Cat No: 4203166

Bcl-2 PE

Supplier: BD Biosciences

Cat No: 340576

Brefeldin-A

Supplier: Sigma Aldrich

Cat No: B-7651

CD3 PacBlue

Supplier: BD Biosciences

Cat No: 558117

CD4 Qdot605

Supplier: Invitrogen

Cat No: Q10008

CD8 Horizon V500

Supplier: BD Biosciences

Cat No: 560775

CD38 PE-Cy7

Supplier: BD Biosciences

Cat No: 335825

DMSO

Supplier: E Merck

Cat No: BB103234L

FCS

Supplier: Adcock Ingram

Cat No: 14-501AIH

DNase

Supplier: Sigma Aldrich

Cat No: D4513

ELISA Ready-SET-GO!

Supplier: eBioscience

Cat no: 88-7316

FACS Lysing Solution

Supplier: Scientific Group

Cat No: B-D 349292

Heat inactivated AB serum

Supplier: Sigma Aldrich

Cat No: H4522

IFN-γ Alexa Fluor 700

Supplier: BD Biosciences

Cat No: 557995

IL-2 PerCp-Cy5.5

Supplier: BD Biosciences

Cat No: 560708

IL-17 Alexa Fluor 647

Supplier: eBiosciences

Cat No: 51717871

L-Glutamine

Supplier: Biowhittaker

Cat No: 17-605E

Perm/Wash Buffer

Supplier: Scientific Group

Cat No: 554723

PHA

Supplier: Bioweb

Cat No: HA16

PBS

Supplier: BioWhittaker

Cat No: 17-517Q

PPD

Supplier: Statens Serum Institut

Cat No: 2390

RPMI

Supplier: Adcock Ingram

Cat No: 12-702F

SEB

Supplier: Sigma Aldrich

Cat No: S4881

Sucrose

Supplier: Sigma Aldrich

Cat No: S7903

Sulphuric acid

Supplier: Sigma Aldrich

Cat No: 320501

TMB

Supplier: Sigma Aldrich

Cat No: T0440-1L

TNF-α FITC

Supplier: BD Biosciences

Cat No: 340511

Appendix II

Complete medium

12.5% Human AB serum in RPMI

Cryo-preservation solution

1 part RPMI + 1 part (20% v/v DMSO in FCS)