Changes in the BCG-Induced T Cell Response Over the First Year of Life.

Thesis Presented for the Degree of

Doctor of Philosophy

by

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Abstract

Bacille Calmette-Guerin (BCG), the only currently available tuberculosis vaccine, is the most widely administered vaccine in the Expanded Programme on Immunisation (EPI). Despite this, many gaps in our knowledge of BCG-induced T cell responses exist. The primary objective of this study was to comprehensively delineate T cell immunity induced by BCG vaccination of newborns and to longitudinally assess BCG-specific T cell responses over the first year of life. Comprehensive analysis of BCG-specific immunity is needed to guide future, novel vaccination strategies against tuberculosis, which may involve a modified BCG prime at birth, and specific boost vaccines within the time frames of the EPI.

Several novel polychromatic flow cytometry reagent panels were designed and optimised to analyse specific components of BCG-specific T cell responses. A novel whole blood proliferation assay that utilises Ki67 as a marker of antigen-specific *in vitro* lymphoproliferation was also optimised.

Cross-sectional examination of BCG-specific T cell cytokine expression and memory phenotypes from 10-14 week old infants showed that BCG vaccination induces distinct T cell subsets that predominantly express Th1 cytokines in multiple combinations. Th1 cytokine expressing cells predominantly expressed an effector memory phenotype, which may reflect persistence of BCG.

Longitudinal changes in T cell turnover kinetics, expression of cytotoxic molecules and differentiation phenotypes by BCG-specific T cells were examined. BCG-specific T cell responses peaked 6 weeks after vaccination and gradually waned over the first year of life. Moreover, these specific T cells progressively differentiated towards more mature phenotypes over time. These T cell functions were also measured directly *ex vivo,* which showed marked alterations in cytotoxic molecule expression and T cell differentiation phenotypes.

The results suggest that low-level antigen exposure, probably through persistence of BCG, or exposure to cross-reactive mycobacteria, results in continuous activation and differentiation of specific T cells over the first year of life. Taken together, these data and the kinetics of the BCG-induced T cell response suggest that boosting of BCG-primed T cells with heterologous tuberculosis vaccines may be ideal after 14 weeks of age. Our findings therefore have important implications for novel vaccination strategies against tuberculosis.

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Contributions to this thesis

The following individuals contributed to work presented in this thesis. Unless specified here, all data were generated by the candidate.

Chapter 2

Sample size calculations for the cross-sectional studies (section 2.7.1), longitudinal study (section 2.7.2) and the longitudinal study phlebotomy schedule (section 2.7.2) were done by Dr Francesca Little.

Chapter 4

Soluble cytokine analysis by multiplex bead assays was done by Dr Sarah Joseph (section 4.3.4).

Chapter 6

Processing, staining and acquisition of Oregon Green proliferation assay samples were done by Lerisa Govender (section 6.2.3). Wendy Mavakla assisted with sample processing for both whole blood and PBMC based proliferation assays.

Publications

The work directly related to this thesis has been published in the following manuscripts and are attached as appendices:

1. **Soares, A.P.**, Scriba, T.J., Joseph, S., Harbacheuski, R., Murray, R.A., Gelderbloem, S.J., Hawkridge, A., Hussey, G.D., Maecker, H., Kaplan, G., Hanekom, W.A. (2008) Bacille Calmette Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. J Immunol 180, 3569-3577.

2. **Soares, A.**, Govender, L., Hughes, J., Mavakla, W., de Kock, M., Stone, L., Barnard, C., Jacobs, G., Nkomba, G., Pienaar, B., Janse van Rensburg, E., Abel, B., Scriba, T.J., Hanekom, W.A. (2010) Novel application of Ki67 to quantify antigen-specific *in vitro* lymphoproliferation. J Immunol Methods 362, 43-50.

Contents

Abbreviations

%	percent
°C	degrees centigrade
Ad35	adenovirus serotype 35
Ag85A	antigen 85 protein A
Ag85B	antigen 85 protein B
AICD	activation induced cell death
APC	allophycocyanin
BCG	bacille Calmette-Guérin
BID	BH3-interacting domain death agonist
BrdU	bromodeoxyuridine
CAD	caspase-activated DNAse
CCL	chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CFP-10	culture filtrate protein-10
CFSE	5,6-carboxyfluorescein diacetate, succinimidyl ester
CME	chloroform-methanol extract
CMV	cytomegalovirus
CO_2	carbon dioxide
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
CXCL	CXC chemokine ligand
Cy5.5-PerCP	cyanine-5.5-peridin-chlorophyll
Cy7	cyanine-7
DC	dendritic cell
DCLP	dichroic long-pass

DNA	deoxyribonucleic acid
DTP-HibV	Diphtheria, tetanus, pertussis, *haemophilus influenza* type B combined vaccine
EL	erythrocyte lysis
ELISA	enzyme linked immunosorbent assay
ELISpot	enzyme linked immunospot
EPI	expanded programme on immunisation
ER	endoplasmic reticulum
ESAT-6	early secretory antigenic target 6
FasL	Fas ligand
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
Fox	forkhead box
FSC	forward scatter
GM-CSF	granulocyte-macrophage colony-stimulating factor
Grm	granzyme
HCW	health care workers
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
ICS	intracellular cytokine staining
IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
iTreg	inducible regulatory T cell
kDa	kilodalton
KO	knockout
L-selectin	leukocyte selectin
L. major	*Leishmania major*
L. monocytogenes	*Listeria monocytogenes*

LCMV	lymphocytic choriomeningitis virus
log	logarithm
LPS	lipopolysaccharide
Lt	lymphotoxin
M. bovis	*Mycobacterium bovis*
M.tb	*Mycobacterium tuberculosis*
MFI	median fluorecent intensity
MHC	major histocompatibility complex
MHC I	MHC class I
MHC II	MHC class II
mL	milliliter
Mø	macrophage
mRNA	messenger RNA
MTCT	maternal to child transmission
MV	measles vaccine
NK	natural killer
nm	nanometer
NO	nitric oxide
nTreg	naturally occuring regulatory T cell
OG	Oregon Green
OPV	oral polio vaccine
PAMPS	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PFC	polychromatic flow cytometry
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate

PMT	photomultiplier tube
PPD	purified protein derivative
PRR	pattern recognition receptor
Qdot	quantum dot
R	receptor
rBCG	recombinant BCG
RNI	reactive nitrogen intermediates
ROI	rective oxygen intermediates
ROR	retinoic-acid-receptor-related orphan receptor
SATVI	South African TB Vaccine Initiative
SEB	Staphylococcal enterotoxin B
SSC	side scatter
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
TB	tuberculosis
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
T_{EMRA}	terminally differentiated memory T cell
TGF	transforming growth factor
Th	T helper lymphocyte
TNF	tumour necrosis factor
TRECS	T-cell receptor excision circles
Treg	regulatory T cell
TST	tuberculin skin test
TT	tetanus toxoid
VV	vaccinia virus
WB-ICC	whole blood – intracellular cytokine
WHO	World Health Organisation

YFV	yellow fever vaccine
γc	gamma chain
μg	microgram
μL	microliter

Chapter 1

Introduction and literature review

1.1. Introduction

Nearly one third of the global population is estimated to be infected with *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB) disease (Dye et al., 1999). Of the 22 countries with the highest estimated TB incidence rates per capita, South Africa is second and in 2007 the Western Cape alone had an incidence of 948 TB cases per 100 000 people (Western Cape Department of Health, 2007; WHO, 2009). TB case numbers are increasing due to the HIV pandemic, the emergence of multi-drug resistant TB strains and the variable efficacy of *M. bovis* bacille Calmette-Guérin (BCG), the only licensed TB vaccine currently available. The need for a more efficacious TB vaccine or improvement of BCG is urgent.

M.tb is commonly transmitted through close contact with an individual with cavitary pulmonary disease, the most common form of TB disease in adults. Unfortunately, BCG is not effective at preventing pulmonary disease in adults and therefore has minimal impact on global TB burden. A more efficacious TB vaccine or novel vaccination strategies to improve the protective efficacy of BCG against pulmonary disease is required to reduce disease transmission. Although TB is primarily a pulmonary disease in adults, disseminated TB and TB meningitis contributes significantly to TB-related mortality and morbidity of children under 5 years of age (Marais et al., 2004). BCG significantly reduces the risk of these forms of disease in infants, has a good safety record in immuno-competant children and high global immunisation coverage (**Figure**

1). Because of this, the current BCG vaccine is likely to remain the cornerstone of novel vaccination strategies against *M.tb* (McShane et al., 2004; Doherty and Andersen 2005; Grode 2005).

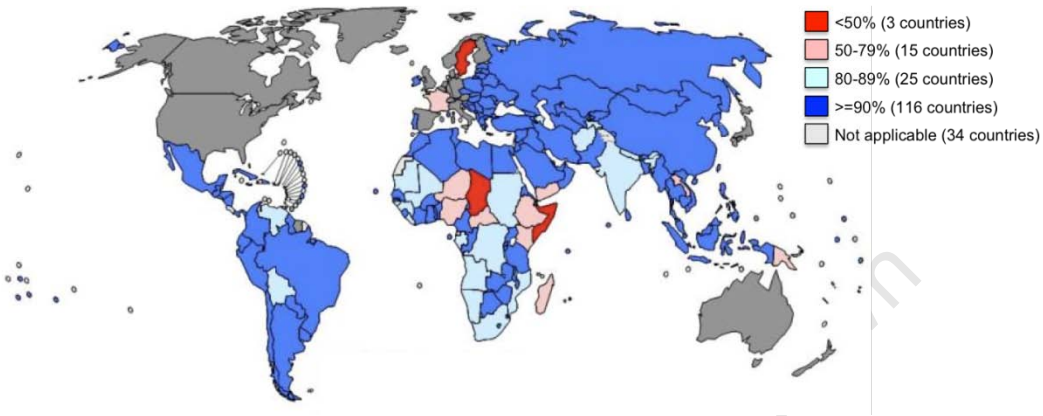

Figure 1. Global immunisation coverage with BCG at birth, 2009. (WHO/UNICEF, 1980-2009)

Boosting the BCG-primed response with a novel vaccine is the most common strategy aimed at enhancing vaccine-mediated protective efficacy (McShane, 2005; Lambert et al., 2009; Kaufmann et al., 2010). Despite this, comprehensive knowledge of T cell subsets, cytokines and cytotoxic molecules that contribute to BCG-specific immunity remains limited (Hanekom, 2005). In addition, longitudinal changes in T cell responses following primary immunisation of infants with BCG remains unknown.

Comprehensive understanding of the human host response to BCG is essential for measuring immunogenicity and longevity of immune responses conferred by novel TB vaccine candidates and for the rational design of new vaccination strategies that aim to improve protection against TB.

1.2. The immune response to *M.tb*

M.tb is acquired through the inhalation of aerosol droplets containing infective bacilli when an individual with cavitary pulmonary TB expels infectious aerosol droplets by coughing. The body's first line of defense against *M.tb* are cells of the innate immune system. Once bacilli are inhaled, lung-resident alveolar macrophages (Mø), the primary cells involved in the initial uptake of *M.tb*, and immature dendritic cells (DC) in the lung, phagocytose the bacteria (**Figure 2**) (Schlesinger 1993; Schlesinger et al., 1996; Henderson et al., 1997). Recognition and phagocytosis of *M.tb* is mediated via pattern recognition receptors (PRR), which recognise pathogen-associated molecular patterns (PAMP) of the bacilli (Janeway and Medzhitov, 1998; Aderem and Ulevitch, 2000). PRRs associated with the recognition and phagocytosis of *M.tb* include complement receptors, scavenger receptors, Toll-like receptors and NOD-like receptors (Schlesinger et al., 1996; Ernst 1998; Ferwerda et al., 2005; Thoma-Uszynski et al., 2001; Krutzik and Modlin 2004). At this point, bacilli may be killed, or may evade host defense mechanisms and begin to multiply (Clemens and Horwitz 1995; Loeuillet et al., 2006). Mycobacteria evade host defense mechanisms by preventing the maturation of phagosomes that contain bacilli, inhibiting the recognition of infected cells by T cells and inhibiting Mø killing mechanisms, such as those mediated by reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Hmama et al., 1998; Ruan et al., 1999; Clemens et al., 2000; Noss et al., 2000; Fratti et al., 2001). Recognition and uptake of bacilli by phagocytic cells trigger the expression of chemokines and pro-inflammatory cytokines necessary for the recruitment of adaptive and innate cells to the site of infection (Friedland et

al., 1993; Schluger and Rom 1997; Kasahara et al., 1998; Underhill et al., 1999)

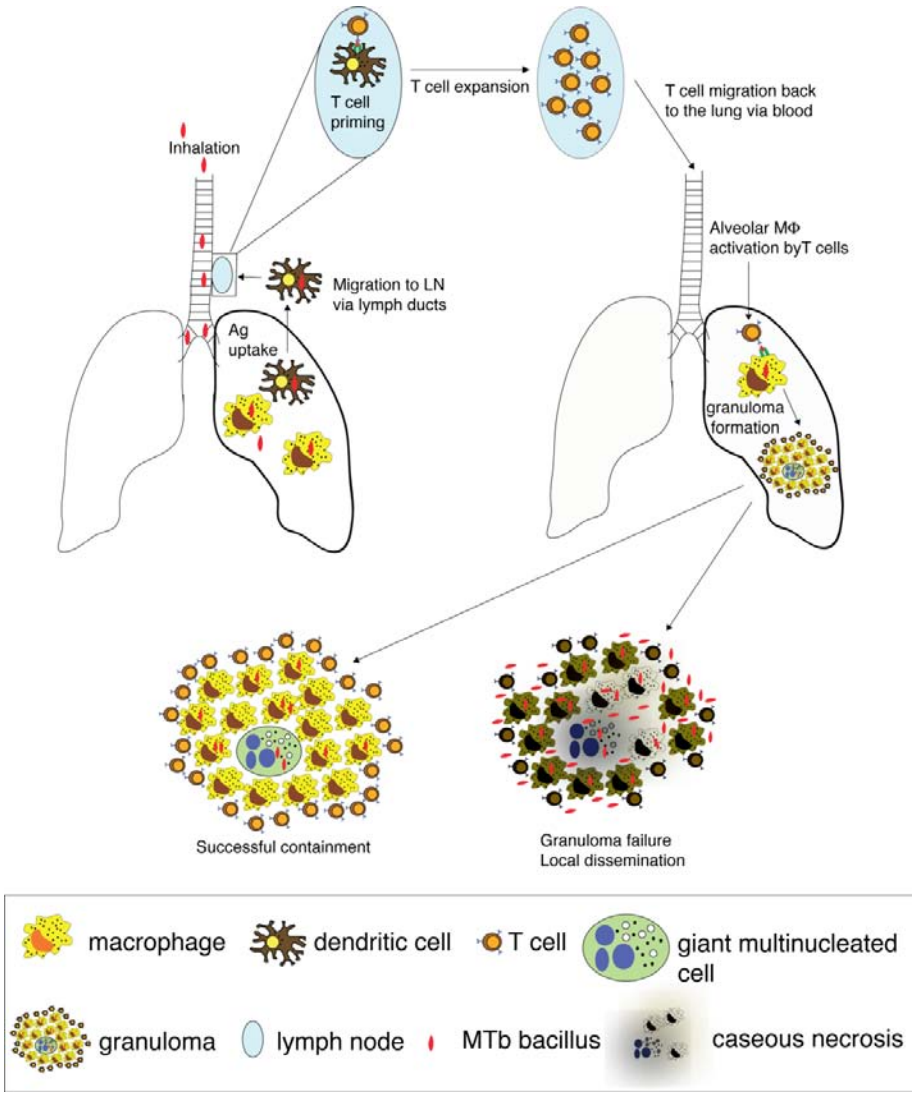

Figure 2. The immune response to M.tb (Hanekom et al., 2007). In the lung M.tb bacilli are phagocytosed by DC and Mø. Activated DC migrate to the lymph nodes (LN) and prime naïve T cells, which differentiate into effector T cells and undergo clonal expansion. Antigen-specific T cells traffic to the lung and activate Mø mycobacterial killing mechanisms. Activated Mø express pro-inflammatory cytokines and chemokines, which leads to the continuous recruitment of T cells and mononuclear cells to the lung, resulting in granuloma formation and containment of the pathogen. Breakdown of protective immunity leads to granuloma failure followed by bacterial dissemination.

DC play a key role in the initiation of adaptive immune responses. DC are professional antigen presenting cells, which form the primary link between innate and adaptive immunity (Mempel et al., 2004). Once activated through pathogen-associated products and danger signals provided by inflammatory cytokines, DC begin to mature and process the pathogen into antigenic peptides, which are loaded onto major histocompatibility complex (MHC) molecules. Simultaneously, DC acquire migratory properties and traffic towards lymph nodes to prime naïve T cells (**Figure 2**) (Schuler and Steinman 1985; Kripke et al., 1990; Pure et al., 1990; Moll et al., 1993; MacPherson et al., 1995; Sozzani et al., 1998). Maturation of DC is also associated with reduced antigen capture activity and enhanced capacity to activate T cells through the up-regulation of MHC, co-stimulatory and adhesion molecules (Viola and Lanzavecchia 1996; Winzler et al., 1997; Rescigno et al., 1997).

In the lymph node fully mature DC prime naïve T cells by: 1) triggering the T-cell receptor (TCR) via pathogen-derived antigenic peptides bound to MHC molecules (signal 1); 2) providing co-stimulatory signals, for example through B7-1 (CD80) and B7-2 (CD86), which interact with CD28 on the T cell (signal 2) and 3) through the expression of pro-inflammatory cytokines (signal 3) (Harding et al., 1992; Curtsinger et al., 1999).

Two main subsets of T lymphocytes have been described according to the expression of co-receptors CD4 and CD8, which contribute to signal transduction during antigen recognition (Turka et al., 1991; Julius et al., 1993). Exogenously derived antigens, such as *M.tb* products from the endosome, are presented on MHC class II (MHC II) molecules to T cells expressing the CD4 molecule (CD4+ T cells). Cytosolic antigens are loaded onto MHC class I

(MHC I) molecules for the priming of CD8+ T cells (Bachmann et al., 1996). Once activated, T cells undergo clonal expansion and differentiate into functionally distinct lineages. Cytokines expressed by DC during priming drive the polarisation of naïve helper T (Th) cells into these distinct lineages, which were defined primarily according to cytokine expression and function (Macatonia et al., 1995). Once differentiated, effector T cells leave the lymph nodes to carry out their function at the site of infection, the lung (Mempel et al., 2004). In experimental TB models, specific T cells are detected in the lung approximately 3 weeks after initial aerosol challenge with *M.tb* (Gallegos et al., 2008; Wolf et al., 2008). Wolf et al. suggest that *M.tb* may delay the initiation of adaptive immune responses to evade host immune effector mechanisms. Delaying recruitment of antigen-specific T cells to the site of infection allows for bacterial growth to occur and facilitates establishment of a chronic infection (Wolf et al., 2008).

Persistence of *M.tb* bacilli within Mø, continuous expression of pro-inflammatory cytokines and chemokines leads to the recruitment and accumulation of Mø, T cells and B cells to the site of infection. This localised pro-inflammatory response and aggregation of cells into specific architectural structures results in the formation of a granuloma, the hallmark of *M.tb* infection (**Figure 2**). Recruited *M.tb*-specific T cells participate in granuloma formation by activating Mø anti-microbial mechanisms through the production of cytokines. T cells also lyse *M.tb* infected Mø and extracellular bacilli by releasing cytotoxic molecules (Ottenhoff and Mutis 1990; Bonecini-Almeida et al., 1998; Saunders et al., 2002; Stegelmann et al., 2005). Within the granuloma *M.tb* bacilli may not be completely eliminated but are effectively

contained resulting in latent infection. Of individuals infected with *M.tb* 90% are able to contain the infection throughout their lifetime. Latently infected individuals are immunologically sensitised to *M.tb*-specific antigens but do not display any clinical symptoms of active disease (Brock et al., 2004; Pai et al., 2008). Latent infection may be determined by using *in vitro* tests that measure T cell interferon (IFN)-γ release in response to *M.tb*-specific antigens such as early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 (Brock et al., 2004; Lee et al., 2006; Mahomed et al., 2006; Connell et al., 2008). Sensitisation may also be determined by measuring skin induration caused by delayed type hypersensitivity in response to purified protein derivative (PPD) (Connell et al., 2008). The major disadvantage of the skin test (TST) is that it cannot differentiate between T cell responses primed by non-pathogenic mycobacteria or *M.tb* because PPD contains antigens that are present in both.

Of latently infected individuals, less than 10% will develop active disease during their lifetime. The factors that lead to reactivation of disease are not well defined. However if critical immune regulators required for granuloma maintenance, such as tumour necrosis factor (TNF)-α, or if adaptive immune responses are compromised, the risk of developing active disease increases significantly (Wallace et al., 1997; Keane et al., 2001; Elliott et al., 2004). Although latent infection and active disease are described as a single condition according to clinical definitions, *M.tb* infection is likely to represent a broad spectrum of *in vivo* characteristics based on new information of diverse granuloma morphology, bacterial load and the metabolic state of bacteria within the lung (Barry et al., 2009; Dheda et al., 2010). During active disease,

bacteria multiply and granulomas become caeseous facilitating dissemination of bacilli (**Figure 2**) (Fayyazi et al., 2000). At this point the individual becomes actively infectious and the cycle begins again.

1.3. The role of CD4+ and CD8+ T cells

Recruitment of mycobacteria-specific T cells to the site of infection and activation of Mø is critical for effective containment of *M.tb* within granulomas (Kaufmann, 2001). *M.tb* reside predominantly in vacuoles within alveolar Mø and survive by inhibiting phagosome maturation and acidification (Frehel et al., 1986; Sturgill-Koszycki et al., 1994). Infected Mø are unable to efficiently kill bacilli unless activated by effector molecules expressed by activated T cells. Multiple lines of evidence from murine and human studies suggest that both CD4+ and CD8+ T cells play roles in the adequate containment of *M.tb* as discussed below.

1.3.1. CD4+ T cells

The contribution of CD4+ T cells to the protective immune response during *M.tb* infection has been well described in humans and in experimental animal models of infection (Flynn, 2001; Kaufmann, 2005b). Intravenous challenge of mice deficient in MHC II or CD4+ T cells with *M.tb* leads to significantly increased bacterial loads in the lung, spleen and liver, compared with wild-type controls (Caruso et al., 1999; Saunders et al., 2002). In addition, activation of Mø mycobactericidal mechanisms, cytokine production and granuloma formation is delayed in these mice (Caruso et al., 1999; Saunders et al., 2002).

Studies in healthy, latently infected individuals have demonstrated that antigen-specific CD4+ T cells are recruited to the lung and participate in localised immune responses to mycobacteria-specific antigens (Silver et al., 2003). Silver et al. instilled a crude mycobacterial antigen, *M.tb* PPD, via bronchoscopic challenge into sub-segmental bronchi of healthy infected individuals. Instillation of PPD induced a localised immune response, which was compared to saline challenged bronchi segments within the same individual. The number of CD4+ T cells increased significantly in challenged bronchi segments, and when evaluated *in vitro* by enzyme linked immunospot (ELISpot), these cells expressed IFN-γ in response to mycobacteria-specific antigens. In addition to cytokine expression, CD4+ T cells also have the capacity to express cytotoxic molecules, which inhibit *M.tb* growth *in vitro*. CD4+ T cells that express cytotoxic molecules are detectable in healthy TST-positive individuals and TB patients (Tan et al., 1997; Lalvani et al., 1998a; Canaday et al., 2001; Bastian et al., 2008).

The important role of CD4+ T cells in mycobacteria-specific immunity in humans has further been highlighted in human immunodeficiency virus (HIV)/TB co-infected individuals (Wallace et al., 1997; Elliott et al., 2004). HIV-infected persons have a 10% annual risk of developing active TB disease compared with a 10% lifetime risk in healthy controls (Selwyn et al., 1989; Raviglione et al., 1997; Aaron et al., 2004). In addition, the progressive loss of CD4+ T cells during HIV infection is associated with an increased incidence of active TB (Markowitz et al., 1997), suggesting that CD4+ T cells play a continuous role in effectively containing *M.tb* during latent infection.

These findings highlight the essential role CD4+ T cells in mediating *M.tb* protective immunity through their capacity to express key Th1 cytokines that promote Mø activation, granuloma formation and maintanence.

1.3.2. CD8+ T cells

The key functional characteristics of CD8+ T cells that contribute to effective *M.tb* immunity include their capacity to secrete Th1 cytokines, and capacity to secrete cytotoxic molecules that mediate lysis of infected target cells (Woodworth and Behar, 2006). Although *M.tb* bacilli reside mainly within Mø vacuoles and are thought not to transverse into the cytosol, CD8+ T cell responses against mycobacteria-specific antigens have been reported (Grotzke and Lewinsohn, 2005). Priming of mycobacteria-specific CD8+ T cells occurs through mechanisms of cross-presentation and cross-priming in which exogenously derived antigens are loaded onto MHC I molecules (Kaufmann and Schaible, 2005; Kaufmann, 2005a; Winau et al., 2006). The process of cross-priming requires apoptosis of *M.tb* infected Mø, which release apoptotic blebs containing *M.tb* derived antigens (Winau et al., 2006). Once apoptotic blebs are taken up by bystander DC, antigens transverse into the cytosol and are broken down into peptides by proteasomes (Werner et al., 1996; Wiertz et al., 1996; Pilon et al., 1997). Peptides are translocated into the lumen of the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) and bind to MHC I (Flutter and Gao, 2004). Recently, murine studies have shown that cross-priming and activation of CD8+ T cells by *M.tb* derived antigens occurs *in vivo* (Winau et al., 2006).

Experimental models have shown that CD8+ T cells contribute to control of *M.tb* and participate in long-term containment of the pathogen (Flynn et al., 1992; Silva et al., 1994; Caruso et al., 1999; Lazarevic et al., 2005; Carranza et al., 2006). Lazaveric et al. demonstrated that CD8+ T cells contribute substantially to IFN-γ production over the chronic phase of *M.tb* infection in mice. Interestingly, the expression of CD8 effector molecules changed during the course of infection. Initially, CD8+ T cells expressed cytotoxic molecules but switched to IFN-γ expression as the infection progressed (Lazarevic et al., 2005). The authors' hypothesis was that the change in CD8+ T cell effector function could possibly be attributed to antigenic load during the course of infection.

CD8+ T cells which recognise *M.tb* specific ESAT-6 and antigen 85 protein A (Ag85A) have been described in human studies (Lalvani et al., 1998; Caccamo et al., 2006). Lalvani et al. detected ESAT-6 specific human leukocyte antigen (HLA) class I restricted CD8+ T cells in patients with TB. ESAT-6 specific CD8+ T cells were capable of IFN-γ production and cytolytic activity against infected Mø (Lalvani et al., 1998b). Using MHC pentamers, Caccamo et al. detected Ag85A-specific CD8+ T cells in children with TB (Caccamo et al., 2006). These Ag85A-specific CD8+ T cells were capable of IFN-γ production and perforin expression when stimulated with mycobacteria-specific antigens.

These data demonstrate that CD8+ T cells contribute to the immune control of *M.tb* and inducing this subset through vaccination may contribute significantly to vaccine-mediated protection.

1.3.3. Non-classical T cell subsets

This project focuses on conventional CD4+ and CD8+ T cells, however non-classical T cells such as γδ T cells and CD1 restricted αβ T cells also contribute to antimicrobial immunity.

γδ T cells reside predominantly in epithelial surfaces and are likely to play an important role in the initial interaction between host and pathogen. These T cells are characterised by a unique TCR comprising a γ and δ chain and do not respond to peptide antigens presented by MHC I or II (Xiong and Raulet, 2007). A major subset of human γδ T cells express the Vγ9Vδ2 TCR, which expand in response to viral and bacterial infections (Havlir et al., 1991; Dechanet et al., 1999; Ottones et al., 2000; Poccia et al., 2005). Vγ9Vδ2 T cells recognise non-peptide intermediates in the isoprenoid biosynthesis pathway. Isoprenoids are molecules that play a role in the cellular metabolism of pathogenic bacteria, including *M.tb* (Eberl et al., 2003; Morita et al., 2007).

In humans, exposure to mycobacteria-specific antigens either through *M.tb* infection or BCG vaccination leads to the expansion of Vγ9Vδ2 T cells (Havlir et al., 1991; Li et al., 1996; Hoft et al., 1998; Shen et al., 2002). Mycobacteria-specific Vγ9Vδ2 T cells express IFN-γ and target intracellular and extracellular bacilli through the expression of cytotoxic molecules such as granulysin (Tsukaguchi et al., 1995; Dieli et al., 2001; Worku and Hoft, 2003). Importantly, γδ T cells develop a memory-like response and may therefore contribute to rapid recall responses at the site of infection (Hoft et al., 1998; Shen et al., 2002). In the rhesus monkey, primary immunisation with BCG leads to the expansion of Vγ9Vδ2 T cells and accumulation of these cells in tissue sites, including the lung. Shen et al. demonstrated that Vγ9Vδ2 T cells

mount a rapid recall response after secondary BCG infection and confer protective immunity against lethal *M.tb* challenge (Shen et al., 2002).

Human and experimental data also support a role for CD-1 restricted T cells in the immune response to *M.tb*. CD1 is a MHC-like molecule that associates with β2-microglobulin and is expressed on DC and monocytes (Zeng et al., 1997). Five CD1 genes have been identified in humans that encode a family of distinct CD1 proteins classified into three groups. Group 1 CD1 molecules (CD1a, CD1b and CD1c) present microbial lipid antigens to αβ T cells, while the group 2 CD1 molecule, CD1d, presents these antigens to natural killer (NK) T cells (Jones et al., 2007). CD1e belongs to group 3, is expressed intracellulary and plays a role in CD1b antigen loading (Angenieux et al., 2005; de la Salle et al., 2005). CD1 molecules bind and present lipid and glycolipid antigens derived from *M.tb* cell membrane components including mannosylated lipoarabinomannan, mycolic acids and phosphatidyl inositolmannosides (Sieling et al., 1995; de la Salle et al., 2005; Kaufmann and Schaible, 2005). In guinea pigs, immunisation with mycobacterial lipids induces specific and long-lived CD-1 dependent T cells, which contribute to protective immunity (Hiromatsu et al., 2002; Dascher et al., 2003). Mycobacteria-specific CD1-restricted T cells are detectable in BCG-vaccinated and healthy TST reactive donors (Stenger et al., 1998; Ulrichs et al., 2003). These cells secrete Th1 cytokines and express cytotoxic molecules in response to mycobacterial lipids (Fischer et al., 2004; Stenger et al., 1998; Ulrichs et al., 2003).

1.4. Helper T cell differentiation

As described earlier, Th cells differentiate into specific lineages during clonal expansion of naïve T cells after primary antigenic stimulation by DC. The specific lineages were classified according to cytokine expression. It is critically important that the appropriate T cell lineage is primed and expanded in response to a given pathogen to successfully contain the infection.

The polarisation of naïve T cells towards a specific functional profile is influenced by a number of factors including co-stimulatory signals, antigen dose and the cytokine milieu during priming (Constant et al., 1995; Hosken et al., 1995; Kuchroo et al., 1995; O'Garra and Murphy 1996). Cytokines present during priming are thought to be the most important regulators of T cell polarisation. Cytokines in combination with TCR engagement activate signalling pathways and master transcription factors unique to each lineage which in turn bind and initiate transcription of specific cytokine gene promoters (Jacobson et al., 1995; Kaplan et al., 1996; Lederer et al., 1996; Thierfelder et al., 1996; Szabo et al., 2000; Murphy and Reiner 2002).

The identification of T cell lineages based on expression of signature cytokines and function was first described in 1986 by Mosmann and colleagues. Initially two subsets of antigen-specific murine Th clones were defined, which they designated Th1 and Th2 (Mosmann et al., 1986). Th1 cells are characterised by the production of IFN-γ, but these cells also express interleukin (IL)-2 and lymphotoxin (Lt)-α. Th1 cells are associated with cell-mediated immunity, promote the activation of cytotoxic T cells (CTL), provide protection against intracellular pathogens and mediate delayed type hypersensitivity. IL-12 secreted by activated Mø and DC trigger signal

transducer and activator of transcription (STAT)-4 and the master regulator T box transcription factor, T-bet, to promote Th1 development (**Figure 3**) (Jacobson et al., 1995; Szabo et al., 2000). T-bet further sensitises cells to Th1 polarising cytokines by up-regulating the IL-12 receptor (IL-12R).

Th2 cells support B-cell differentiation, promote IgG class switching and are required for the control of certain parasites, such as helminths (Coffman et al., 1988; Turner et al., 2003). Activation of STAT6 by IL-4 leads to the induction of transcription factors GATA-3 and cMAF, which bind to genes encoding the signature Th2 cytokines, IL-4, IL-5 and IL-13 (**Figure 3**) (Hoet al., 1996; Zheng and Flavell 1997; Kim et al., 1999).

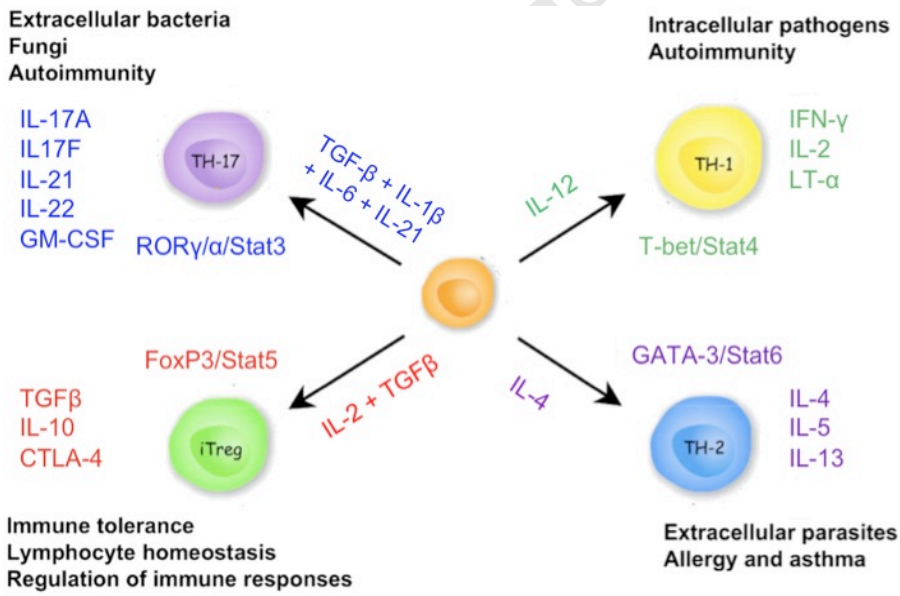

Figure 3. T helper differentiation and regulation. Master transcription factors and cytokines involved in the differentiation of particular Th lineages. Adapted from http://www.citeulike.org/blog/Zephyrus/5402

Since their initial description in the 1980s, additional T cell lineages beyond the Th1/Th2 paradigm have been identified. The novel Th17 lineage was

described based on the expression of IL-17 (Yao et al., 1995). This subset plays a role in the modulation of autoimmune diseases and protection to extracellular bacteria and fungi (Fujino et al., 2003; Park et al., 2005). Th17 signature cytokines include IL-17A, IL-17F, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21 and IL-22. Th17 differentiation is driven by IL-6 and transforming growth factor (TGF)-β in combination with IL-21 and IL-1β (Mangan et al., 2006; Veldhoen et al., 2006). These cytokines activate STAT3, which induces the expression of Th17 master transcription factors retinoic-acid-receptor-related orphan receptor (ROR)-γ and ROR-α (**Figure 3**) (Ivanov et al., 2006; Yang et al., 2008). IL-23, a member of the IL-12 family, is required for Th17 stabilisation and maintenance (Stritesky et al., 2008).

Several CD4+ T cell subsets are involved in regulating effector responses. Regulatory T cells (Treg) limit pathogenic immune responses to self- and foreign-antigens through contact-dependent and independent mechanisms (Takahashi et al., 2000; Nakamura et al., 2001; Chen et al., 2003; Roncarolo et al., 2006; Wan and Flavell 2008). Several Treg subsets have been described according to their origin and mechanisms of suppression. Two major classes of Treg are naturally occurring (nTreg) and inducible (iTreg). forkhead box (Fox)-P3 expressing nTreg originate from the thymus and mediate their suppressive activity through cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) or membrane-bound TGF-β (Takahashi et al., 2000; Nakamura et al., 2001). iTreg develop from conventional T cells and are comprised of IL-10-expressing Tr1 cell, TGFβ-expressing Th3 cells and inducible FoxP3+ T cells (Hara et al., 2001; Zheng et al., 2002). The programming of Treg is predominantly dependent on IL-2 and TGFβ in the

absence of inflammatory cytokines, which up-regulate FoxP3 (**Figure 3**) (Chen et al., 2003).

The cytokines that drive the polarisation of specific Th subsets negatively regulate one another and promote their own lineage. For example, IFN-γ and IL-12 inhibit GATA-3, while GATA-3 increases Th2 cytokine expression and down-regulates IL-12Rβ2 expression, thus inhibiting Th1 development. TGFβ blocks Th1 and Th2 differentiation by inhibiting GATA-3 and T-bet, and promotes Treg differentiation by up-regulating FoxP3 (Gorelik et al., 2000; Gorelik et al., 2002; Chen et al., 2003). However, combined TGFβ and IL-6 signalling suppresses FoxP3 and promotes the differentiation of Th17 (Chen et al., 2003; Bettelli et al., 2006; Zhou et al., 2007).

1.5. Role of cytokines in TB

1.5.1. Th1 and pro-inflammatory cytokines

The importance of Th1 cytokines in primary resistance to *M.tb* is well established. Th1 responses are critical for effective immunity against *M.tb* infections partly through their action on Mø (Flynn et al., 1993; Flynn et al., 1995; Cooper et al., 1997; Ottenhoff et al., 2003; Dorman et al., 2004; van de Vosse et al., 2004). Effective control of *M.tb* bacillary growth is maintained through the induction of nitric oxide (NO) and other RNI within infected Mø. IFN-γ knockout (KO) mice have undetectable levels of RNI mRNA and high bacterial loads. Despite their initial ability to form granulomas, tissue necrosis and dissemination of bacteria to major organs is observed 2 weeks post-*M.tb* challenge (Flynn et al., 1993). Similarly, individuals with genetic mutations that compromise IFN-γ pathways, such as IFN-γR and IL-12R mutations are highly

susceptibility to severe mycobacterial disease (Newport et al., 1996; Altare et al., 1998; Dorman et al., 2004).

Similar susceptibilities are seen in TNF-α deficient mice. Neutralization of TNF-α leads to reduced RNI expression, increased bacillary loads and re-activation of latent TB infection (Botha and Ryffel, 2003). TNF-α, a pro-inflammatory cytokine, synergises with IFN-γ to activate Mø and plays a key role in granuloma formation (Bekker et al., 2001; Roach et al., 2002). The important role of TNF-α mediated immunity in humans has been demonstrated in individuals receiving treatment for inflammatory diseases (Stenger, 2005; Saliu et al., 2006). For example, blocking of TNF-α with cytokine specific inhibitors in patients with Crohns disease or rheumatoid arthritis results in the reactivation of latent *M.tb* infection (Keane et al., 2001; Mohan et al., 2004).

IL-2, although not exclusively associated with Th1 cytokines, is critical for the development of memory CD4+ and CD8+ T cell responses (Dooms, 2006; Williams et al., 2006). T cells primed in the absence of IL-2 fail to induce an effective recall response (Blattman et al., 2003; Dooms et al., 2007). The expression of cytotoxic molecules in CD8+ T cells is also regulated by IL-2 (Janas et al., 2005; Pipkin et al., 2010). In conditions of weak IL-2 signalling, murine CD8+ T cells fail to acquire efficient killing capacity due to a reduction in cytotoxic molecule expression (Pipkin et al., 2010).

1.5.2. Th2 cytokines

In contrast to the important role of Th1 cytokines, a Th2 response may be associated with sub-optimal protection against TB (Bhattacharyya et al.,

1999). For example, Th1 responses to mycobacteria-specific antigens are down-modulated in individuals with helminth infections, which may be related to a Th2 biased immune response induced by parasitic worms (Elias et al., 2001). Elias et al. demonstrated that de-worming of individuals prior to BCG vaccination results in a significant increase in T cell proliferation and IFN-γ expression in response to mycobacteria-specific antigens compared to infected individuals (Elias et al., 2001).

Presence of a Th2 response to mycobacteria-specific antigens during latent infection also appears to be predictive of risk of TB disease (Ordway et al., 2004). Significantly higher frequencies of mycobacteria-specific IL-4 expressing γδ T cells and CD8+ T cells were detected in health care workers (HCW) who develop TB compared to HCW who remain healthy. HCW who developed TB also have increased levels of IL-5 (Ordway et al., 2004).

1.5.3. Treg cytokines

Treg are required for homeostatic control of the immune response but may also inhibit immune responses that eliminate pathogens (Belkaid et al., 2002; Kursar et al., 2007; Scott-Browne et al., 2007). In TB patients, elevated frequencies of Treg are found at the site of infection and in peripheral blood compared with healthy TST positive controls (Ribeiro-Rodrigues et al., 2006; Chen et al., 2007; Garg et al., 2008; Guyot-Revol et al., 2006).

TGFβ and IL-10 levels are elevated at the site of disease in TB patients compared to controls with other lung diseases (Toossi et al., 1995; Bonecini-Almeida et al., 2004). Treg exert their immunosuppressive functions through numerous mechanisms, including the expression of IL-10 and TGFβ (Hara et

al., 2001; Gorelik et al., 2002), which in turn suppress Th1 activity through inhibition of IL-12, IFN-γ and proliferation (Fiorentino et al. 1991; Moore et al., 2001). Therefore, the accumulation of Treg at the site of infection may prevent elimination of mycobacteria and contribute to persistence of *M.tb*.

1.5.4. Other Th cytokines

Other cytokines that contribute to protective immunity against *M.tb* include IL-17 and IL-23. Studies by Khader et al. have shown that protection in mice following ESAT-6 vaccination is dependent on the expression of both these cytokines. In vaccinated mice, memory Th17 cells accelerated the accumulation of protective Th1 cells in the lung through up-regulation of chemokines, CXC chemokine ligand (CXCL)-9, CXCL10, and CXCL11 (Khader et al., 2007). Therefore, the induction of Th17 through vaccination may play a critical role in protective efficacy. However, appropriate regulation of this subset and timing of response is required to prevent pathogenic cellular responses to TB. Cruz and colleagues have shown that repeated vaccination of mice with BCG following *M.tb* infection leads to a significant increase in lung pathology due to excessive inflammation. This increased immunopathologic response was associated with persistent IL-17 responses in the lung (Cruz et al., 2010).

1.6. Cytotoxic T lymphocytes (CTL)

CTL confer protection against intracellular pathogens through direct lysis of infected target cells and the activation of apoptotic pathways. Lysis of infected target cells is mediated by two mechanisms: 1) release of cytotoxic granules containing anti-microbial effector molecules such as perforin, granulysin and

granzymes (Grm) and 2) through engagement of death receptors, such as Fas-Fas ligand (FasL) (Kagi et al., 1994). Studies by Stenger et al. and Canaday et al. suggest that the granule exocytosis pathway more efficiently eliminates *M.tb* infected target cells compared with the death receptor pathway. For example, blocking Fas-FasL interaction decreases T cell cytolytic function by 25%. However, blocking the granule exocytosis pathway reduces cytolytic capacity by >90% (Canaday et al., 2001).

CTL store cytotoxic and cytolytic effector molecules within specialised secretory lysosomes. Upon recognition of target cells, CTL direct granules to the immunological synapse where lysosomes fuse with the plasma membrane. Cytotoxic molecules are then delivered into the target cell and induce both caspase-dependent and independent death pathways.

1.6.1. Granulysin

Granulysin, a member of the saposin-like family of lipid binding proteins, is expressed by numerous cellular subsets including NK cells, NK T cells, CD4+ and CD8+ T cells (Clayberger and Krensky, 2003). This cytolytic protein has broad lytic activity against numerous pathogens including Gram-positive and -negative bacteria, fungi, and parasites (Stenger et al., 1998; Ochoa et al., 2001; Ma et al., 2002). Importantly, early studies by Stenger et al. demonstrated that granulysin can kill extracellular *M.tb* directly and intracellular *M.tb* in combination with perforin (Stenger et al., 1998). In patients with chronic TB infection granulysin and perforin expression at the site of infection, the granuloma, is impaired compared with distal lung parenchyma and uninfected control lungs suggesting that these molecules

contribute to efficient containment of *M.tb* during latent infection (Andersson et al., 2007).

Granulysin disrupts cell membrane integrity directly and can cause mitochondrial damage, which leads to cytochrome c release and the activation of caspase 3 (Kaspar et al., 2001; Okada et al., 2003). In addition, this molecule induces monocytes to express cytokines and chemokines, such as CC chemokine ligand (CCL)-5 and may therefore play a role in cellular recruitment during inflammation (Deng et al., 2005).

1.6.2. Granzymes

Grm are a group of serine proteases. Five human Grm have been described (Grm A, B, H, K, M), which differ on the basis of killing mechanisms, cellular expression and biological function (Sower et al., 1996; Russell and Ley 2002; Lieberman 2003; Chattopadhyay et al., 2009; Harari et al., 2009; Pardo et al., 2009).

Grm induce cell death through multiple mechanisms including direct activation of pro-caspases or targeting molecules that lead to mitochondrial and DNA damage. Grm entry into target cells occurs via reparative endocytosis or receptor mediated endocytosis. However, perforin is required for the efficient activation of Grm mediated death pathways (Froelich et al., 1996; Trapani et al., 2003; Veugelers et al., 2004).

The most well characterised serine proteases are GrmA and B. The molecular pathways of cell death induced by these two Grm are distinct. GrmA induces cell death through caspase independent mechanisms, which involves the activation of nucleases that introduce single-stranded nicks into DNA

(Beresford et al., 1999; Lieberman, 2003). In the absence of a delivery agent such as perforin, GrmA induces the secretion of pro-inflammatory cytokines from fibroblasts, epithelial cells and monocytes (Sower et al., 1996; Beresford et al., 1999; Lieberman and Fan 2003; Metkar et al., 2008).

GrmB activates caspase-dependent cell death pathways and caspase-independent mitochondrial death pathways. GrmB activates caspase-activated DNAse (CAD) by cleaving its associated inhibitor (ICAD) directly or indirectly by activating caspase 3, resulting in DNA fragmentation and apoptosis (Thomas et al., 2000; Sharif-Askari et al., 2001).

GrmB also cleaves BH3-interacting domain death agonist (BID), which disrupts the mitochondrial outer membrane and leads to the activation of a number of pro-apoptotic factors (Yang et al., 1998; Kuwana et al., 2002). Studies using CTL from KO mice have demonstrated that cell death delivery is greatly impaired in the absence of GrmB compared to other granzymes (Heusel et al., 1994). This data suggests that GrmB is one of the main effector molecules in granule exocytosis mediated cell death.

1.6.3. Perforin

Perforin is a 66kDa protein that binds to phospholipid membranes in a calcium dependent manner (Masson and Tschopp, 1985). Early studies demonstrated that perforin is capable of forming pore-like structures on the plasma membrane of target cells (Masson and Tschopp 1985; Podack et al., 1985; Young et al., 1986). In addition, delivery of granzymes in combination with perforin is required for maximum CTL activity (Kagi et al., 1994). Thus it was initially hypothesised that perforin is required for the formation of pores in

target cells to allow delivery of serine proteases. However, more recent studies have shown that Grm are internalized into target cells in a perforin independent manner (Trapani et al., 2003; Veugelers et al., 2004; Shi et al., 2005). In addition, secreted Grm are bound to a serglycin carrier protein, therefore pores formed by perforin on target cells may not be sufficiently large enough to allow entry of these macromolecule complexes (Raja et al., 2002) The revised hypothesis suggests that although perforin is not required for Grm entry into cells, both molecules need to be co-endocytosed for the release of Grm from endosomes into the cytosol or for nuclear translocation (Shi et al., 2005).

As mentioned previously, granulysin in combination with perforin kills intracellular *M.tb* and perforin expression is impaired at the site of infection during chronic TB (Stenger et al., 1998; Andersson et al., 2007).

1.7. Immunological memory

1.7.1. Phases of the T cell response

In order for a vaccine to be effective it must not only prime an appropriate T cell response but also establish a pool of long-lived memory T cells. Memory T cells are antigen experienced T cells, which persist in an antigen and TCR independent manner (Sallusto et al., 2004). These cells are present at higher frequencies and respond rapidly upon pathogen re-encounter, compared with naïve T cells. The heightened and accelerated immune responses conferred by memory T cells mediate rapid clearance of the targeted pathogen before extensive damage is inflicted to the host (Steinbrink et al., 2002).

Memory T cells are established after a number of distinct “phases” following exposure of naïve T cells to antigen, either through infection or vaccination.

After priming in the lymph nodes, antigen specific T cells enter a clonal expansion phase driven by IL-2. Primed T cell "clones" differentiate into antigen-specific Th cells and CTL, leave the lymph nodes and circulate in the periphery or home to infection sites to carry out their effector functions. Once the antigen or pathogen has been cleared, T cells undergo a contraction or death phase in which >90% of the cells undergo apoptosis. The remaining pool of T cells develop into long-lived memory T cells (**Figure 4**) (Kaech et al., 2002; Schluns KS, 2003).

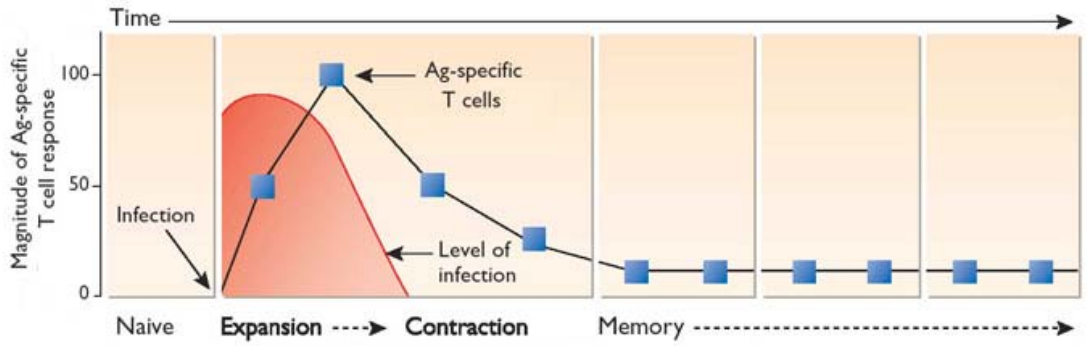

Figure 4. Phases of the T cell response (Badovinac and Harty, 2003)**.** Following acute infection or vaccination, primed naïve T cells enter a clonal expansion phase and differentiate into effector T cells. Once the targeted antigen or pathogen has been cleared, >90% of T cells undergo apoptosis during the contraction phase. The remaining pool of T cells develop into long-live memory T cells.

Survival and transition of effector T cells into memory T cells following contraction is mediated through IL-7R signalling, which up-regulates the expression anti-apoptotic molecules such as Bcl-2 and Bcl-XL (Kaech et al., 2003; Li et al., 2003). Although several memory subsets have been described, a subset referred to as central memory T cells (T_{CM}) are often considered to be "true" long-lived memory T cells as this population persists for longer *in vivo* and confers long-term protection to naive hosts (Wherry et al., 2003;

Zaph et al., 2004a). The delineation of specific memory subsets based on T cell function and phenotype will be discussed in greater detail in the following section. The selective survival of T_{CM} compared with other memory T cell subsets is mediated by transcription factor FoxO3a, which regulates pro-apoptotic and anti-proliferative genes. T_{CM} express significantly higher levels of the transcriptionally inactive forms of FoxO3a compared to T_{EM} (Birkenkamp and Coffer, 2003; Riou et al., 2007). Another characteristic feature of T_{CM} cells is that they undergo homeostatic turnover. This mechanism of self-renewal and survival is primarily mediated through the common gamma chain (γc) cytokines, IL-15 and IL-7 (Zhang et al., 1998; Lantz et al., 2000; Judge et al., 2002; Carrio et al., 2007; Osborne et al., 2007).

The generation of long-lived memory T cells is primarily observed following acute infection or after antigen clearance and may not develop in scenarios of prolonged antigen persistence. In humans, T cells displaying qualities of long-lived memory cells are detectable in peripheral blood many years after primary immunisation with vaccine antigens that are efficiently cleared, such as tetanus toxoid (TT) and the attenuated strain of the yellow fever virus (Cellerai et al., 2007; Miller et al., 2008). However, specific T cells generated under conditions of antigen persistence fail to acquire a characteristic long-lived phenotype (Appay et al., 2002b). For example, HIV-specific and cytomegalovirus (CMV)-specific T cells are largely skewed towards a highly differentiated effector phenotype and do not express markers associated with long-lived potential such as BCL-2 and the IL-7R (Champagne et al., 2001; Appay et al., 2002b; van Leeuwen et al., 2005; Golden-Mason et al., 2006; Lv

et al., 2010). The same observation has been made in experimental models of chronic infection. Following chronic lymphocytic choriomeningitis virus (LCMV) infection, murine CD8+ T cells isolated after viraemic control express low levels of IL-7R, IL-15R and show reduced homeostatic proliferation and survival capacity (Wherry et al., 2004). In combination with data from acute viral infection models, the authors suggest that T cells require a rest period after antigen-stimulation for efficient transition into long-lived antigen-independent T cells.

1.7.2. Phenotypic markers for the identification of memory T cell subsets

Memory cells are categorised into a number of subsets based on anatomical location, phenotype and function (Sallusto et al., 1999; Wherry et al., 2003). A number of markers expressed on the cell membrane including chemokine receptors and antigens involved in the regulation of T cell activation and survival have been useful for discrimination of specific T cell memory subsets (Hamann et al., 1997; Kaech et al., 2003; Gourley et al., 2004; Cellerai et al., 2007; Miller et al., 2008). The most commonly used markers in humans include CC chemokine receptor (CCR)-7, leukocyte selectin (L-selectin, CD62L), CD45RA, CD27 and CD28 (Michie et al., 1992; Sallusto et al., 1999; Tomiyama et al., 2004; Fritsch et al., 2005).

CCR7 is a chemokine receptor for CCL-19 and CCL-21, which are constitutively expressed by stromal cells in secondary lymphoid organs (Gunn et al., 1998; Cyster 1999). CD62L is a cell adhesion molecule that mediates rolling of leukocytes through high endothelial venules (Gallatin et al., 1983).

Thus, CCR7 and CD62L play an essential role in facilitating the homing and entry of naïve and memory T cells into lymph nodes.

CD45 is an important regulatory component of TCR signal transduction and therefore essential for effective T cell activation (Mustelin et al., 1989; Koretzky et al., 1990). Combined analysis of the CD45 isoforms, CD45RA and CD45RO, distinguishes naïve from antigen-experienced T cells (Ralph et al., 1987). Loss of CD45RA and acquisition of CD45RO expression is observed during the transition of naïve T cells into memory T cells. However, a subset of antigen-experienced T cells has been shown to express CD45RA (Johannisson and Festin 1995; Caccamo et al., 2006).

The co-stimulatory molecules, CD27 and CD28, enhance cell cycling and promote T cell survival by maintaining the expression of the IL-7R and by up-regulating the expression of anti-apoptotic molecules (Hendriks et al., 2000; Okkenhaug et al., 2001; Carr et al., 2006). In addition, the expression of CD27 and CD28 provides information about the function and progressive differentiation of T cells driven by antigen encounter. T cells with an early differentiation phenotype express both molecules and have high proliferative potential. T cells progress through an intermediate differentiation stage associated with the loss of CD27 on CD4+ T cells and CD28 on CD8+ T cells. Highly differentiated T cells lack the expression of both CD27 and CD28 and express high levels of cytolytic molecules (Appay et al., 2002a; Tomiyama et al., 2004). The accumulation of highly differentiated T cells is typically observed during chronic viral infections (Marchant et al., 2003; Miles et al., 2008).

1.7.3. Memory T cell subsets and pathways of differentiation

Two main subsets of memory T cells were first described in human blood based on CCR7 and CD62L expression (Sallusto et al., 1999). T_{CM} cells are identified by the expression of lymph node homing receptors, CCR7 and/or CD62L, but also express CD27 and CD28 (Wherry et al., 2003). T_{CM} cells reside mainly in draining lymph nodes and once presented with antigen proliferate rapidly and differentiate into effector T cells with cytokine producing properties (Wherry et al., 2003). Because proliferative capacity is a key feature for this subset and cytokine expression is relatively delayed compared to T_{EM}, longer term T cell assays such as the cultured ELISPOT assay and proliferation assays are required to accurately measure this population (Combadiere et al., 2004; Reece et al., 2004; Hanekom et al., 2008).

T_{EM} cells are identified by the loss of CCR7 expression and down-regulation of CD62L and/or CD27 (Wherry et al., 2003; Schiott et al., 2004). T_{EM} patrol sites where pathogens may first be encountered and have an increased capacity to home to inflamed tissue through the expression of chemokine receptors such as CCR2 and CCR5 (Sallusto et al., 2004; Hansen et al., 2009). These cells have limited proliferative potential but are highly functional and release cytokines and cytotoxic molecules rapidly once presented with antigen.

Terminally differentiated memory T cells lack CCR7 and CD62L expression but express CD45RA and are proposed to be derived from effector T cells or T_{EM}. This subset also expresses CD57, a marker associated with a state of replicative senescence (Brenchley et al., 2003). This subset is thought to be the most differentiated antigen-experienced subpopulation based on function

and short telomere lengths (Hamann et al., 1999; Fritsch et al., 2005). Terminally differentiated T cells may express effector molecules but have lost the ability to proliferate and are likely to undergo apoptosis after pathogen clearance.

A number of models describing the differentiation pathways of T_{EM} and T_{CM} have been proposed. Based on observations of acute infection of mice with LCMV or the intracellular bacterium, *Listeria monocytogenes* (*L. monocytogenes*), a linear model of differentiation was originally proposed by Wherry and colleagues (Wherry et al., 2003). Wherry et al. demonstrated that the differentiation of memory CD8+ T cells is a continuous linear process from effector T cell to T_{EM} to $T_{CM.}$ Following expansion, effector T cells either underwent apoptosis following pathogen clearance or became $T_{EM,}$ which gave rise to T_{CM} (**Figure 5A**).

This study also showed that the conversion from T_{EM} to T_{CM} was imprinted during priming of naive T cells and that T_{CM} convert into effectors and T_{EM} upon secondary infection. A similar linear model of differentiation for CD4+ T cells was also described (Swain 1994; Hu et al., 2001). Swain et al. demonstrated that after adoptive transfer, Th1 and Th2 effector T cells generated *in vitro* displayed a long-lived memory phenotype when recovered from their adoptive hosts.

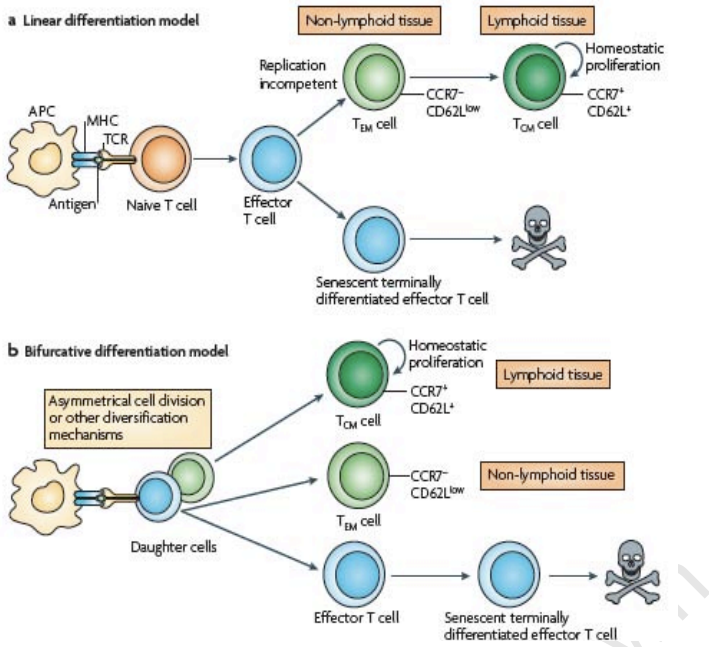

Figure 5. Models of memory T cell differentiation (Ahmed et al., 2009)**. A.** In the linear differentiation model, effector T cells are generated following stimulation of naive T cells with antigen. Effector T cells either become terminally differentiated T cells, which die by apoptosis soon after antigen clearance, or differentiate into T_{EM} which differentiate into long-lived T_{CM}. **B.** The branched differentiation model suggests that following priming, asymmetric T cell division gives rise to two daughter cell subsets with different differentiation fates: one subset gives rise to long-lived T_{CM} cells and the other T_{EM} and effector T cells.

Non-linear or bifurcated differentiation pathways have also been described for both CD4+ and CD8+ memory T cell development. Recent studies by Chang et al. suggest that asymmetric division of naïve T cells, pre-determined during priming, gives rise to daughter cells, which either display properties of short-lived effector T cells (T_{EM}) or are long-lived memory precursors (T_{CM}) (**Figure 5B**) (Chang et al., 2007).

Whatever the differentiation pathway, it is well established that cells fated toward an effector lineage express IFN-γR, T-bet and GrmB and memory precursors express lower levels of GrmB, T-bet and higher IL-7Rα and CD62L

expression. Adoptive transfer experiments demonstrated that both lineages confer protection to naïve hosts upon challenge, however memory precursors confer protection over a longer period. A branched differentiation model in which memory CD4+ T cells do not have to progress through a fully differentiated effector phase has also been proposed (Song et al., 2005). Based on the analysis of chemokine receptors, Song et al. described the development of functional effector and memory CD4+ T cells at multiple points along the classical pathway from naïve to memory T cell differentiation. Although the differentiation and linear relationship between distinct memory T cell subsets remains uncertain, it is clear that the induction of long-lived memory T cells may be key for establishing vaccine-mediated protection.

1.8. BCG and novel TB vaccine strategies

BCG, an attenuated strain of *M. bovis,* remains the only licensed TB vaccine currently available. BCG vaccination is effective at preventing severe forms of childhood TB, such as TB meningitis and miliary TB, but has variable efficacy against pulmonary disease (Colditz et al., 1995; van Rie et al., 1999; Trunz et al., 2006). The reasons for this variable efficacy have not been well defined but a number of hypotheses have been proposed. These include host genetic differences, differences between BCG vaccine strains and interference by environmental mycobacteria or other pathogens (Elias et al., 2001; Black et al., 2002; Castillo-Rodal et al., 2006). Because BCG is not effective at preventing pulmonary disease in adults it has minimal effect on global TB burden. Therefore, a number of novel TB vaccines are currently being developed in an attempt to improve protective efficacy and reduce TB case

numbers. These include pre-exposure vaccines, which aim to replace BCG, or boost BCG primed responses. Since BCG prevents severe forms of TB and over 80% of infants worldwide have been immunised with this vaccine, it is likely that BCG will be retained until a new, safe TB vaccine with improved benefits is available. Consequently, one of the approaches for the design of novel vaccination strategies is to include BCG in a heterologous prime-boost regimen or to replace the current BCG with an improved BCG (Lambert et al., 2009; Kaufmann et al., 2010).

A rational strategy for the improvement of BCG has been to introduce or over-express key mycobacteria-specific antigens or to insert non-mycobacterial genes (Eddine A.N 2005; Grode 2005; Hoft et al., 2008). For example, recombinant BCG (rBCG)-30 expresses the 30-kDa major secretory protein of *M.tb*, Ag85B. rBCGΔureC:Hly+ is an example of a rBCG containing non-mycobacterial genes. rBCGΔureC:Hly+ secretes listeriolysin, a pore forming toxin produced by *L. monocytogenes* and maintains an acidic phagosomal pH for optimal listeriolysin activity. Listeriolysin expression facilitates escape of BCG from the phagolysosome into the cytoplasm and thus facilitates efficient priming of CD8+ T cells (Horwitz and Harth 2003; Grode 2005; Sun et al., 2009).

A second strategy is to boost BCG-primed responses with a heterologous vaccine (McShane et al., 2004; McShane 2005; Abel et al., 2010). Several studies in animals and humans have demonstrated that boosting BCG-specific responses with a non-replicating viral vector vaccine can be achieved safely and rapidly (Beveridge et al., 2007; Hawkridge et al., 2008; Abel et al., 2010). Examples of such vaccines include MVA85A, a non-replicating

recombinant vaccinia vector expressing Ag85A and AERAS-402, a non-replicating Adeno virus serotype-35 (Ad35) vector expressing Ag85A, Ag85B, and TB10.4 (Kaufmann et al., 2010). Recently, a novel subunit vaccine that combines Ag85B, ESAT-6 and Rv2660c was shown to efficiently boost BCG-primed T cell responses in mice (Aagaard et al., 2011). This booster vaccine significantly reduced lung bacterial loads during late-stage *M.tb* infection compared with mice that received BCG only (Aagaard et al., 2011). In addition to vaccine candidates outlined above, a number of other vaccine candidates, including attenuated mycobacteria such as M. vaccae, are currently undergoing clinical trials (Lambert et al., 2009; Kaufmann et al., 2010). Most of these vaccines aim to boost BCG-primed responses however the kinetics of BCG-specific T cell responses following primary immunisation of infants remains unknown. Previous work in chronic viral infection models suggest that primary immune responses should be boosted after the peak effector phase and following the transition of effector T cell into a stable memory population (Wherry et al., 2003; Wherry et al., 2004; Wrammert et al., 2009). Boosting during the effector phase of the T cell response may lead to T cell exhaustion and death. Therefore, a major limitation in the optimal implementation of novel TB vaccines is the lack of kinetic data on BCG-specific responses, which hampers the design of appropriate prime-boost immunisation regimens.

1.9. T cell responses following BCG vaccination of infants

Almost 9 decades after BCG was first administered, BCG-specific T cell effector responses remain poorly characterised and the memory phenotypes and kinetics of specific T cells following vaccination of infants are unknown.

Infants are more susceptible to infectious diseases, which is predominantly attributed to limitations of both innate and adaptive effector mechanisms of the neonatal immune system (Harris et al., 1992; Goriely et al., 2001). For example, neonatal DC remain phenotypically immature and express lower quantities of IL-12 in response to stimulation with lipopolysaccharide (LPS), CD40 ligation or polyinosinic–polycytidylic acid compared with adult DC (Goriely et al., 2001). Thus, this lack of maturity translates to diminished capacity to prime T cells and polarisation of Th1 responses (Harris et al., 1992; Goriely et al., 2001; Langrish et al., 2002). Despite this, adult like IFN-γ responses following BCG vaccination of infants have been reported (Vekemans et al., 2001; Ota et al., 2002). Studies have shown that vaccination of infants induces specific CD4+ and CD8+ T cells capable of IFN-γ recall responses to mycobacteria-specific antigens (Marchant et al., 1999; Smith et al., 1999; Vekemans et al., 2001; Hussey et al., 2002; Ota 2002; Davids et al., 2006; Murray et al., 2006). In the studies conducted by Vekemans *et al* and Davids *et al*, analysis of peripheral blood mononuclear cells (PBMC) by four colour flow cytometry revealed that mycobacteria-specific CD4+ T cells are a source of IFN-γ (Vekemans et al., 2001; Davids et al., 2006). BCG vaccination also up-regulates the expression of cytotoxic molecules. Specific CD8+ T cells from 10-14 week old BCG vaccinated infants' up-regulate Grm, perforin and lyse *M.tb* infected target cells (Hussey et al., 2002; Murray et al., 2006). BCG vaccination of neonates has also been shown to induce the production of IL-5, IL-13 and IL-10, which are detectable by enzyme-linked immunosorbent assay (ELISA) at low levels in plasma following stimulation of whole blood with BCG or mycobacteria-specific

antigens (Marchant et al., 1999; Vekemans et al., 2001; Ota 2002).

Despite the studies described above, comprehensive knowledge of BCG-specific T cell responses and kinetics following vaccination of infants remains limited. This can be attributed to a number of factors: Firstly, since soluble cytokine production was pre-dominantly assessed in plasma or supernatant following incubation of whole blood, intracellular cytokine expression and the phenotypic profiles of antigen-specific T cells could not be discerned. Secondly, T cell profiling was restricted to four colour flow cytometry which limits the ability to gauge the potential complexity of T cell mediated immune responses. Thirdly, IFN-γ expression was selected as the primary measure of immunogenicity; however, measuring a single component of the immune response may underestimate the magnitude and complexity of BCG-induced immunity. Lastly, our understanding of BCG-specific T cell immunity in infants is largely based on cross-sectional studies. To model the kinetics of T cell responses following vaccination detailed longitudinal studies are required.

1.10. Objectives

The primary objective of this thesis is to comprehensively delineate T cell immunity induced by BCG vaccination of newborns and to longitudinally asses BCG-specific T cell responses over the first year of life. This entails developing and optimising several novel polychromatic flow cytometry (PFC) reagent panels to allow for detailed dissection of various components of the immune response including T cell cytokine and cytotoxic molecule expression, T cell memory subsets and differentiation phenotypes. A novel whole blood proliferation assay will also be optimised to be implemented in future studies to monitor antigen-specific T cell proliferation and cytokine expression following vaccination of infants. Optimised reagent panels will first be used for cross-sectional analysis of a 10-14 week old infant cohort to determine which functional and phenotypic markers are expressed on BCG-specific T cells. Longitudinal changes in T cell turnover kinetics, expression of cytotoxic molecules and differentiation phenotypes will then be examined directly *ex vivo* and on BCG-specific T cells.

Chapter 2

Materials and methods

2.1. Recruitment of study participants

2.1.1. Adult participants

Pilot studies for the initial development and optimisation of PFC reagent panels were completed using adult whole blood. Healthy TST positive and negative donors were recruited at the Institute of Infectious Disease and Molecular Medicine (IIDMM) at the University of Cape Town (UCT). Blood was collected by qualified phlebotomists within the guidelines of written consent. This protocol was approved by the UCT research ethics committee (REC REF: 038/2003).

2.1.2. Infant participants - Cross-sectional studies

The aim of the cross-sectional studies was to determine which functional and phenotypic markers are expressed on specific T cells after BCG vaccination of infants. Healthy, 10-14 week old infants routinely vaccinated at birth with intradermal BCG (Danish strain 1331; Statens Serum Institut), were recruited at South African TB Vaccine Initiative (SATVI) clinical sites in Worcester and surrounding areas. Peripheral venous blood was collected by qualified nurses within the guidelines of written consent and HIV pre- and post-test counselling was provided. This protocol was approved by the UCT research ethics committee (REC REF: 016/2001) and written informed consent was provided by a legal guardian.

2.1.3. Cross-sectional studies exclusion criteria

1. Infants who had not received BCG within 48 hours of birth, as is routine.
2. Infants <10 weeks or >14 weeks of age
3. Infants who were HIV positive
4. Infants with acute or chronic disease at the time a blood draw was scheduled
5. Infants on TB prophylaxis or treatment

2.1.4. Infant participants- Longitudinal study

Participants were recruited in the Worcester district. Mothers were verbally informed about this study by SATVI study nurses after delivery at Eben Donges Hospital and the Worcester Community Health Centre maternity units. Only mothers who had undergone screening for HIV infection by the state-run programme to prevent maternal to child transmission (MTCT) of HIV and who were HIV negative were approached. Mothers that expressed interest in participating in the study were visited at home where informed consent was obtained from her or another appropriate legal guardian, and a follow-up phlebotomy schedule was assigned.

At specified scheduled time points, infants visited the Maria Pieterse clinic site in Worcester. All had been routinely vaccinated at birth with intradermal BCG (Danish strain 1331; Statens Serum Institut). At each visit the infant's medical history was taken and a general examination was performed prior to phlebotomy procedures. Peripheral venous blood was collected within the guidelines of the protocol. Exclusion criteria for this longitudinal study are

listed below. This protocol was approved by the UCT research ethics committee (REC REF: 323/2006) and by the Western Cape Department of Health (19/18/RP01/2008).

2.1.5. Longitudinal study exclusion criteria

1. Infants who had not received BCG within 48 hours of birth, as is routine.
2. Infants with a birth weight of <2.5kg, as these infants immune systems may be relatively "immature" and the maximum amount of blood we could safely draw at each time point may be insufficient to allow completion of all assays.
3. Infants with chronic disease within the first three weeks of life, e.g., congenital heart disease and chronic lung disease. (Chronic disease that developed after three weeks of age, e.g., asthma, was not an exclusion criterion.)
4. Acute disease in the first month of life, such as newborn respiratory disease and sepsis.
5. Infants whose mothers were HIV positive, as HIV exposure or infection of infants may affect the BCG-induced immune response.
6. Infants whose mothers had not been screened for HIV infection by the state-run program to prevent MTCT of HIV.
7. Clinically significant anaemia. This was detected by the examination of the infant during each visit. If the study personnel suspected anaemia, a finger prick haemoglobin examination was done. A Hb<9.5 g/dL led to exclusion of the infant from that blood draw and the infant was referred for evaluation and treatment of anaemia.

8. Acute disease at the time a blood draw was scheduled.
9. Missed scheduled appointments on more than two occasions.
10. TB prophylaxis or treatment at any point during the one-year follow-up period.
11. *M.tb* infected infants. *M.tb* infection was determined at each time point by measuring T cell proliferation following a 6 day whole blood culture with ESAT6/CFP10 fusion protein.

2.2. Longitudinal study design

To determine the kinetics of BCG-specific T cell responses following vaccination, infants were followed up from 3 weeks of age to 52 weeks of age. Blood specimens were drawn from each infant at only three to four of the following seven time-points (ages): 3, 6, 10, 14, 27, 40 or 52 weeks. This schedule was chosen to coincide with the standard Expanded Programme on Immunisation (EPI) schedule (**Table 1**).

Table 1. EPI vaccine schedule in South Africa during the study follow-up period (2008 – 2009).

At birth	**6 weeks**	**10 weeks**	**14 weeks**	**6 months**	**9 months**	**>12 months**
BCG						
OPV	OPV	OPV	OPV			OPV (18 months)
		DTP-HibV	DTP-HibV	DTP-HibV		DTP-HibV (18 months)
					MV	MV (18 months)

OPV - Oral polio vaccine

DTP-HibV - Diphtheria, tetanus, pertussis, *haemophilus influenza* type B combined vaccine

MV - Measles vaccine

Infants were allocated to a limited number of time points as it was unlikely that parents would allow infant phlebotomy seven times during the year. Also, the volume of blood that can be safely drawn from infants cannot exceed 3mL/kg over an 8 week period. The randomised allocation of infant participants to specific phlebotomy schedules is discussed in section 2.7.2 of this chapter.

2.3. Assays

2.3.1. Whole blood intracellular cytokine (WB-ICC) assay

Co-stimulants anti-CD28 and anti-CD49d (both from BD Biosciences) at a final concentration of 1µg/ml each, were included with antigens, as pilot experiments had shown enhancement of the specific cytokine response of T cells. Whole blood was incubated with the following antigens (final concentrations): 1.8 x 10^6cfu/mL Danish BCG (Danish strain 1331; Statens Serum Institut) or 20ug/mL *M.tb* PPD (Statens Serum Institut; Batch RT48). Whole blood incubated with co-stimulants only served as the negative control. Positive controls included: 5ug/mL phytohaemagglutinin (PHA; Sigma-Aldrich) or 10µg/ml Staphylococcal enterotoxin B (SEB; Sigma-Aldrich). Prepared antigens were stored overnight at 4°C and transported on ice packs the following morning to the clinic sites.

At the clinic, whole blood was collected into heparinised syringes by veni-puncture. Immediately following phlebotomy, 1mL of whole blood was added drop-wise directly into the Sarstedt tubes containing prepared antigens, after these had been brought to room temperature. Whole blood was added to antigens within 18 hours of antigen preparation. Tubes were vortexed and

transferred into a portable incubator set to 37°C for transport to the Cape Town SATVI lab site. The portable incubator, adapted to plug into a vehicles lighter outlet, maintains whole blood samples at an optimal temperature of 37°C during transport. At the SATVI lab site, samples were transferred into a programmable waterbath set to 37°C.

After 7 hours of incubation from the initial bleed time, 100 µL of plasma was removed from each sample and immediately stored at -80°C. These samples were collected for later analysis of soluble cytokines by multi-plex bead array. After collection of plasma, Brefeldin A (Sigma-Aldrich) was added at a final concentration of 10µg/mL, to trap cytokines intracellularly. Tubes were vortexed and transferred immediately into a 37°C waterbath and stimulated for an additional 5 hours. After a total stimulation time of 12 hours, the waterbath temperature was reduced to 21°C to down-regulate stimulation. Cells were harvested the following morning within 10 hours of temperature reduction.

To harvest cells, whole blood was incubated with 2mM EDTA (Sigma-Aldrich) for 15 minutes at room temperature. To lyse red blood cells and fix white blood cells, 1mL whole blood was transferred into 15 ml Sterillin tubes containing 9mL, BD FACS lysing solution (BD Biosciences) and incubated at room temperature for 10 minutes. Cells were centrifuged at 300 x g for 10 minutes at room temperature. Supernatants were decanted and the remaining cells were resuspended in a cryo-preservation solution consisting of 500µL 1% human AB serum (Western Cape Blood Transfusion Service) in RPMI 1640 (Lonza BioWhittaker) and 500µL 20% DMSO (Sigma-Aldrich) in fetal calf serum (Gibco). Resuspended cells were transferred into cryo-vials

(Corning) and placed in Mr. Frosty's (Merck) containing iso-propanol (Sigma-Aldrich) which allows for step-wise freezing at a rate of -1°C/min. Mr Frosty's remained at -80°C for 24 hours prior to transfer of cells into liquid nitrogen.

2.3.2. Cytotoxicity assay

Whole blood (125µL diluted 1:5 in warm RPMI 1640) was incubated with 1 x 10^5cfu/mL BCG, 1ug/mL PHA (positive control) or medium only (NIL, negative control) in 24-well culture plates at 37°C in 5% CO_2. On day 3, 250uL plasma was removed from each sample and immediately stored at -80°C for later analysis of soluble cytokines by multi-plex bead array. After collection of plasma, whole blood was incubated with 2mM EDTA for 15 minutes at room temperature to harvest cells. To lyse red blood cells and fix white blood cells, 1mL of whole blood was transferred into 15 mL Sterilin tubes containing BD FACS lysing solution and incubated at room temperature for 10 minutes. Cells were pelleted at 300 x g for 10 minutes at room temperature and cryo-preserved as described for the WB-ICC assay.

2.4. Antibodies

The development and optimisation of each PFC reagent panel designed to address the aims of this thesis is outlined in chapter 3. Conjugated antibodies selected for the analysis of T cell functional and phenotypic profiles are tabulated in chapter 3 or listed under the materials and methods section of each appropriate chapter.

2.5. Staining Protocols

A “one step” and “two step” staining method were optimised to assess the functional and phenotypic profiles of T cells, respectively.

2.5.1. One step staining method

Cryo-preserved cells were rapidly thawed in a waterbath set to 37°C. Thawed cells were transferred into Falcon tubes containing 1mL of 1 x PBS (BioWhittaker) and pelleted at 300 x g for 10 minutes at 4°C. Cells were resuspended in 1mL 1 x BD Perm/Wash solution (BD Biosciences), vortexed and incubated for 10 minutes at room temperature to permeabilise cells. Permeabilised cells were pelleted and supernatants decanted prior to staining. The remaining volume after decanting was approximately 60µL. Cells were incubated with fluorescent-conjugated antibodies for 1 hour in the dark at 4°C. Stained cells were washed in 1mL BD Perm/Wash buffer, resuspended in 100µL BD Perm/Wash buffer and acquired immediately on a LSR II flow cytometer (BD Biosciences).

2.5.2. Two step staining method using BD Perm/Wash buffer

To assess memory T cell phenotypes a two step staining method was used. This method allows for staining of all surface markers first, followed by staining of intracellular cytokines.

Cryo-preserved cells were rapidly thawed in a waterbath set to 37°C. Thawed cells were transferred into falcon tubes, containing 1mL 1 x PBS and pelleted at 300 x g for 10 minutes at 4°C. Cells were resuspended in 1mL BD Perm/Wash solution and incubated at room temperature for 10 minutes to

permeabilise cells. Cells were pelleted, decanted and stained for all surface markers for 1 hour in the dark at 4°C. Stained cells were washed twice in 1mL BD Perm/Wash buffer and then stained for 1 hour at 4°C with antibodies against intracellular cytokines. Stained cells were washed in 1mL BD Perm/Wash, pelleted and resuspended in 100µL of BD Perm/Wash buffer. Cells were acquired immediately on a LSR II flow cytometer.

2.6. Data acquisition

All flow cytometric data was acquired on a BD LSR II equipped to detect 13 individual fluorescent signals excited by 405nm violet, 488nm blue and 633 red lasers. Mirror and filter configurations are tabulated in **Appendix A**. Compensation corrections and data analysis was completed using FACSDiva (BD Biosciences) or FlowJo (Tree Star Inc, Ashland, OR). Histograms and bivariant dotplots were selected to display data with 0 and negative log scaling (bi-exponential scaling) (Tung et al., 2004).

2.7. Statistical considerations

Statistical analysis and graphical representation was done using Prism (GraphPad, San Diego, CA, USA). Statistical significance (p values) was calculated using non-parametric statistical tests, as most data were not normally distributed. A p value of less than 0.05 was considered significant. Correlations amongst variables were tested by the Spearman test.

2.7.1. Sample size calculations for the cross-sectional studies

Sample size calculations were based on data from pilot experiments that recorded the peripheral blood frequency of BCG-specific CD4+ T cells (IFN-γ-expressing CD4+ T cells after 12 hours stimulation of whole blood), measured at 10-14 weeks of age, in children vaccinated with BCG at birth. The mean frequency was 0.30% with a standard deviation of 0.27%. When the sample size in 29, a two-sided 95.0% confidence interval for the single mean will extend 0.100 from the observed mean, assuming that the standard deviation is known to be 0.270 and the confidence interval is based on the large sample z statistic.

2.7.2. Sample size calculations for the longitudinal study

Sample size calculations were based on pilot data from the cross-sectional studies that recorded the peripheral blood frequency of BCG-specific CD4+ T cells (IFN-γ-expressing CD4+ T cells after 12 hours stimulation of whole blood), measured at 10 weeks of age, in 33 children vaccinated with BCG at birth. The mean frequency was 0.54% with a standard deviation of 0.29%. Assuming that the standard deviation of the frequency measurements at 1 month and at 1 year will both be 0.29%, and assuming a within-subject correlation between 1-month and 1-year frequencies of 0.75 (i.e. 56% of the variability in the 1-year frequencies can be accounted for by their linear association with the 1-month frequencies), the standard deviation for the difference in the mean frequencies at 1 month and 1 year is 0.205%. To detect a relative change of 20% in CD4+ T cell frequencies from 1 month to 1 year, translates into a difference of 0.108% between the mean frequencies,

based on a 1 month frequency of 0.54%. Using a paired t-test to detect a difference of 0.108%, with 80% power and at a two-sided 5% level of significance, when the standard deviation of the differences is 0.205%, a sample size of 32 subjects per time point is required. To reduce the frequency of bleeds, infants were assigned to 2 of the first 4 bleed time points and 2 of the last 3 time points. Since infants were only allocated to half the number of visits, the sample size was doubled to 64 and an additional two recruited to obtain round numbers. The total sample size was therefore 66 infants. The sample size could be increased to 90 infants, to compensate for loss to follow-up, acute disease, or unanticipated sample loss, etc, which may have resulted in obtaining fewer than the proposed 33 infant for each time point.

There were 6 possible schedules of 2 out of the first 4 visits and 3 possible schedules of 2 out of the later 3 visits. Half the number of infants were allocated to consecutive visits than to other schedules among the earlier visits. The allocation of infant participants to specific phlebotomy schedules is listed in **Appendix B**. Details of participant specific follow-up time points (time given in weeks), including volumes of blood drawn at specific time points are tabulated in **Appendix C**.

Chapter 3

Optimisation of polychromatic flow cytometry reagent panels for the characterisation of BCG-specific T cell subsets and functional profiles

3.1. Introduction

Characterising the human host response induced by BCG vaccination is challenging due to the highly complex nature of the T cell compartment. Previous studies have relied on assays such as the IFN-γ ELISPOT, ELISA or intracellular cytokine staining (ICS) combined with four colour flow cytometry to measure mycobacteria-specific immunity (Vekemans et al., 2001; Ota et al., 2002; Vekemans et al., 2004; Davids et al., 2006; Murray et al., 2006). However, these assays do not accurately reflect the complexity of vaccine-specific responses, which encompass multiple cellular subsets with numerous effector functions (De Rosa et al., 2004; Cellerai et al., 2007; Miller et al., 2008). Further, measuring the production of a single effector molecule, such as IFN-γ, and collecting limited data on the immunophenotype of antigen-specific cells, limits the rate at which we acquire knowledge on vaccine induced responses.

The field of PFC has progressed rapidly due to the development of flow cytometers equipped with multiple lasers and advanced detection systems and the availability of numerous novel fluorescent dyes (Roederer et al., 1996; Perfetto et al., 2004; Chattopadhyay et al., 2006a). PFC, which is based on conventional flow cytometric principles, allows measurement of more than six

parameters or characteristics on a single cell level. PFC is the only technological platform currently available that allows complex analysis of multiple T cell subsets and functions.

At the initiation of this project, comprehensive knowledge of BCG-specific immunity in infants was very limited, and PFC reagent panels specifically designed to measure BCG-induced T cell function and phenotypes had not been developed. This chapter focuses on the steps undertaken to develop and optimise novel reagent panels designed to address the objectives of this study.

3.2. Aims

To address the objectives of this study, four reagent panels, each aimed at assessing specific components of T cell mediated immune responses relevant to mycobacterial immunity, were optimised. These included panels measuring: i) T cell cytokine expression profiles ii) memory phenotypes of T cell subsets iii) expression of cytotoxic molecules and T cell differentiation stage and iv) T cell proliferative capacity. Reagent panels were designed and optimised by completing the following sub-aims:

1. Identify relevant cellular markers for measurement of BCG-specific T cell immunity.
2. Optimise reagent panels by:
    a. Titrating antibodies.
    b. Optimising staining reagents and protocols.
    c. Assess false-positives and any potential antibody interactions within reagent panels by fluorescence minus one (FMO).

3.3. Materials and methods

3.3.1. Study participants and phlebotomy

Optimisation of reagent panels was performed on whole blood from healthy adults. Study participants were recruited as described in chapter 2 (section 2.1.1) and blood was collected by qualified phlebotomists within the guidelines of written consent.

3.3.2. Immuno-fluorescence staining

All samples used for optimisation experiments were stained using the "one step staining method" described in chapter 2 (section 2.5.1.), unless stated otherwise.

3.2.3. Titration of antibodies

Each commercially obtained antibody conjugate was individually titrated by serial dilution to determine staining performance and optimal antibody titre. Conditions such as temperature, light exposure and different staining buffers may influence staining and antibody performance; therefore titrations were completed under the same conditions as experimental staining. Twice the volume recommended by the manufacturer was selected as the starting titer to ensure antigen saturation. At least eight and up to twelve two-fold serial dilutions were completed per antibody. Data were analysed using histograms and dotplots to determine background staining (noise), separation and saturation titers and signal-to-noise ratios. A separation titer is defined as the dilution at which optimal resolution between unstained (noise) and stained (signal) cells is observed. A saturation titer is defined as the highest dilution at

which 100% staining of the targeted antigen is achieved. A 'signal-to-noise' ratio of the median fluorescent intensity (MFI) of the positive population divided by that of the negative population was calculated for each dilution. When appropriate, biological negative controls were included in titration experiments to determine the level of non-specific antibody binding or noise and to guide analysis of titration data. The MFI and the frequency of the positive population were plotted against antibody volume (µL). The optimal antibody volume typically lies on the plateau or peak area of both plots, i.e. a dilution that results in the maximum frequency of positively stained cells and in the largest separation (highest signal-to-noise ratio) between stained and unstained cells. Final dilutions for each antibody were selected by assessing all of the experimental outcomes listed above.

3.3.4. Effects of fixation on phenotypic marker resolution

Because all whole blood samples used in this study were fixed prior to staining with antibodies, the effects of fixation on the resolution of surface markers was evaluated. For freshly stained samples, 1mL of adult whole blood was added directly to 5mL erythrocyte lysis (EL) buffer that does not contain a fixative (RNeasy kit, Southern Cross Biotechnology), vortexed and incubated on ice for 20 minutes. For fixed samples, 1mL of whole blood was added directly to 9mL FACS lysing solution, vortexed and incubated at room temperature for 15 minutes.

After lysis, cells were pelleted and stained with a combination of the following antibodies: CD3-Pacific Blue, CD4-Alexa Fluor 700, CD8-PerCP-Cy5.5, CD45RA-PE-Cy7, CCR7-APC and CD27-PE.

All antibodies were obtained from BD Biosciences (BD Biosciences) except for CCR7-APC (R&D Systems).

3.3.5. Optimisation of staining protocols for phenotypic surface marker analysis

Three different staining protocols (outlined below) were evaluated in parallel. Each protocol was tested on whole blood incubated with BCG for a total of 12 hours as described previously in chapter 2 (section 2.3.1.).

Samples were stained with: CD3-Pacific Blue, CD8-PerCP-Cy5.5, CD45RA - PE-Cy7, CD27-PE, CCR7-APC, IFN-γ-Alexa Fluor 700 and IL-2-FITC. All antibodies were obtained from BD Biosciences.

i. One step staining method

Refer to the "one step staining method" in chapter 2 (section 2.5.1.)

ii. Two step staining method using BD Pharmingen stain buffer and BD Perm/Wash buffer

Cryo-preserved cells were rapidly thawed in a waterbath set to 37°C. Thawed cells were transferred into Falcon tubes containing 1mL of 1 x PBS and pelleted at 300 x g for 10 minutes at 4°C. Cells were resuspended in 1ml *BD Pharmingen stain buffer* (BD Biosciences) vortexed, pelleted and the supernatants decanted prior to staining. Cells were stained with antibodies against all surface markers for 1 hour in the dark at 4°C. Stained cells were washed in 1mL BD staining buffer and resuspended in BD Perm/Wash for 10 minutes at room temperature. Pelleted cells were stained for all intracellular

cytokines in the dark for 1 hour at 4°C. Cells were washed, pelleted and resuspended in 100µL of BD Perm/Wash buffer prior to acquisition.

iii. Two step staining method using BD Perm/Wash buffer

Refer to the "two step staining method" in chapter 2 (section 2.5.2.)

3.3.6. FMO

In FMO experiments one reagent is omitted at a time to assess potential false positives or other issues arising from antibody interactions in large panels. A whole blood sample stained with all antibodies from a single panel was compared with a series of samples stained with the same panel excluding each individual antibody (Perfetto et al., 2004). FMO controls were completed for all reagent panels under the same experimental conditions planned for the final analysis of infant samples, e.g., FMO controls for the cytotoxicity panel were tested on whole blood stimulated with BCG for 3 days. Gating strategies were based on data obtained from samples stained with the full reagent panel, before being applied to FMO control data.

3.3.7. Determining which activation markers allow detection of antigen-specific T cells in a 3 day assay

The expression of CD69, HLA-DR and Ki67 were evaluated as potential markers of antigen-specific T cells following a 3 day culture of whole blood with BCG. Markers of activation were screened on samples collected from six healthy BCG-vaccinated infants (age range, 3 - 40 weeks). Markers were evaluated on whole blood cultured with BCG for 3 days as described

previously in chapter 2 (section 2.3.2). Samples were stained with: CD3-Pacific Blue, GrmB-Alexa Fluor 700, CD69-PE-Cy7, HLA-DR-PerCP and Ki67-PE. All antibodies were obtained from BD Biosciences.

3.4. Results

3.4.1. Selecting cellular markers for the analysis of BCG-specific T cell responses

Development of each reagent panel began with selecting markers relevant to mycobacteria-specific immunity and to the objectives of this study. Specific marker combinations were selected to: 1) identify T cell lineages; 2) indentify T cell memory and differentiation phenotypes and 3) T cell functional capacity (proliferative capacity, expression of cytokines and cytotoxic molecules). Markers included for analysis within each final reagent panel are listed in section 3.4.8 at the end of this chapter. Several markers were screened initially but excluded from final reagent panels due to poor discrimination between positive and negative populations, reduced specificity or protocol limitations. These included: CD62L, CD154, CD137, CD69, HLA-DR and CD127. The rationale for excluding each marker is given in the discussion section of this chapter or in other relevant chapters.

3.4.2. Assigning fluorochrome conjugates to markers

After selecting antigens of interest, fluorescent conjugates were assigned to each marker. Allocation of fluorochrome-conjugates was guided by several selection criteria including: instrument configuration (**Appendix A**), commercial availability of antibodies, antigen expression levels, fluorochrome staining index and the amount of spectral overlap contributed by each fluorochrome (Baumgarth and Roederer, 2000). Antigens which were expressed on a small population of cells (rare), or which had low expression levels, were assigned to fluorochromes with a high staining index, i.e., PE, PE

tandem fluorochromes and APC (Maecker and Trotter, 2008). Dimmer fluorochromes, such as the violet dyes AmCyan and Pacific Blue were assigned to highly expressed markers such as the lineage markers CD4 and CD3. These markers have distinct negative and positive populations (bimodal distribution) that are usually well resolved with dimmer dyes. Fluorochromes were also selected based on emission wavelengths and potential spectral overlap between detectors. Fluorochrome emission wavelengths often overlap and "spillover" into other channels. A high percentage of spillover may decrease sensitivity in some channels as a result of data spread (Maecker et al., 2004). Fluorochrome-conjugates assigned to specific markers for each final reagent panel are listed at the end of this chapter in section 3.4.8.

3.4.3. Optimal antibody titers

All antibodies were titrated to ensure optimal reagent performance. The titration of Ki67-PE for the cytotoxicity reagent panel is shown in **Figure 1A-C** as a representative example. Titration data were analysed by dotplots (**Figure 1A**) and histograms (**Figure 1B**) to determine the frequencies and MFI of stained and unstained cells. **Figure 1C** shows the titration curve of antibody volume in relation to the MFI and percentage of Ki67 expressing CD3+ T cells. A plateau was established between 0.2µL to 0.4µL in which no significant increase in the percentage of Ki67+CD3+ T cells was observed. Resolution between stained and unstained cells diminished significantly at titers below 0.025µL (**Figure 1A**). Although the highest signal-to-noise ratio was observed at 0.4µl, 0.2µl was chosen as the optimal volume for this specific conjugate as it corresponded to the saturation titer (**Figure 1A and**

C), allowed good discrimination between stained and unstained cells (**Figure 1B**), and non-specific binding/noise was minimal (NIL, **Figure 1A**). Optimal titers for all antibody-conjugates in each final reagent panel are listed in section 3.4.8 of this chapter.

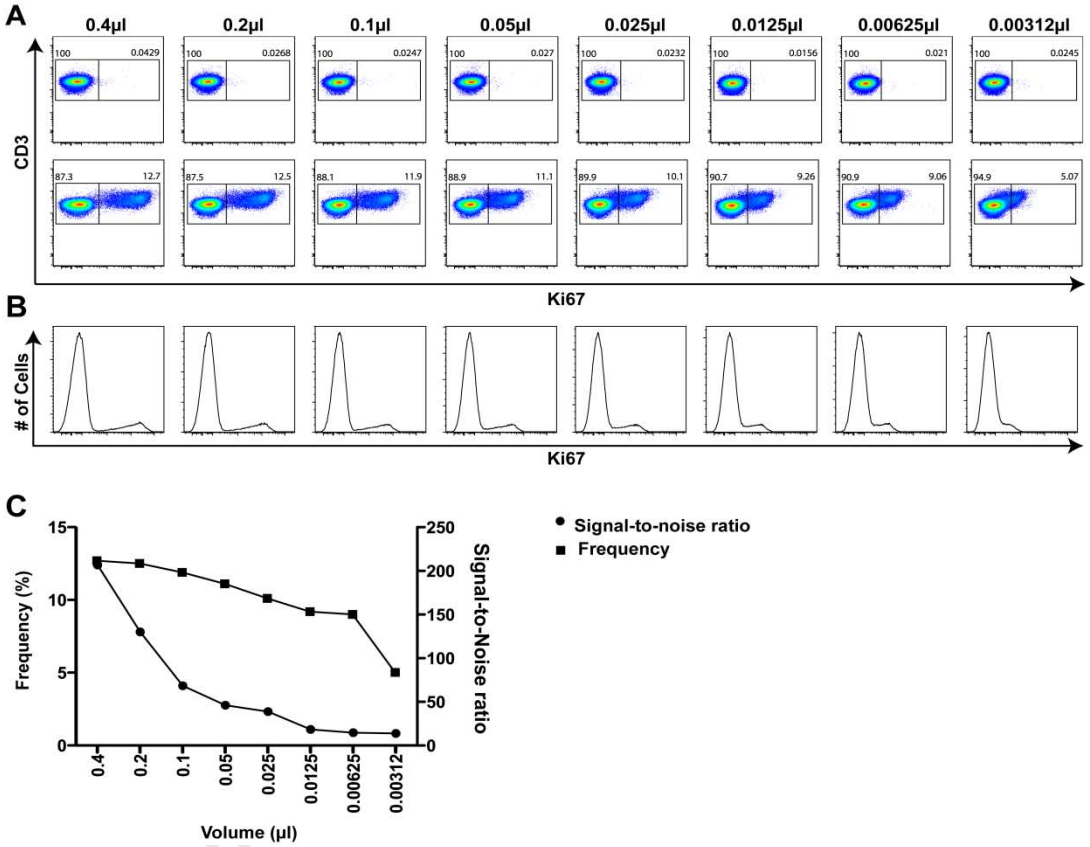

Figure 1. Titration of Ki67 PE. The frequency of Ki67+CD3+ and Ki67-CD3+ T cells in whole blood after 3-day culture with medium only (NIL) or BCG displayed as (**A**) pseudocolour dotplots and (**B**) histograms. The values above each dotplot indicate final antibody volume. (**C**) Frequencies of Ki67+CD3+ T cells after background (NIL) subtraction and signal-to-noise ratios in relation to antibody volume

3.4.4. Effects of fixation on phenotypic marker staining

To determine the effects of paraformaldehyde fixation on phenotypic marker staining and antibody performance, fresh whole blood was stained with

antibodies and compared to whole blood stained post-fixation with FACS lysing solution.

Reliable resolution between negative and positive staining populations was maintained for CD45RA, CCR7 and CD27 after fixation and results were comparable to freshly stained samples (**Figure 2A and B**).

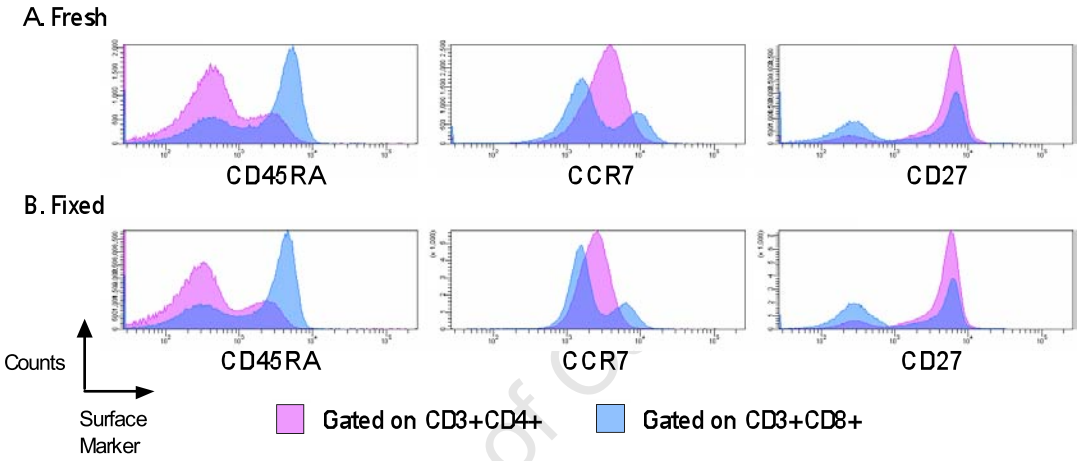

Figure 2. The effect of fixation with FACS lysing solution on CD45RA, CCR7 and CD27 staining. Histogram overlays of CD4+ (purple shading) and CD8+ T cells (blue shading) illustrating the resolution of memory markers in (**A**) fresh and (**B**) fixed whole blood.

Conjugates which exhibited reduced staining intensity due to fixation were excluded from panels, when suitable fixation-resistant antibodies could not be found. An example of such a conjugate was the CD127-PE antibody from BD Bioscience (**Figure 3A and B**).

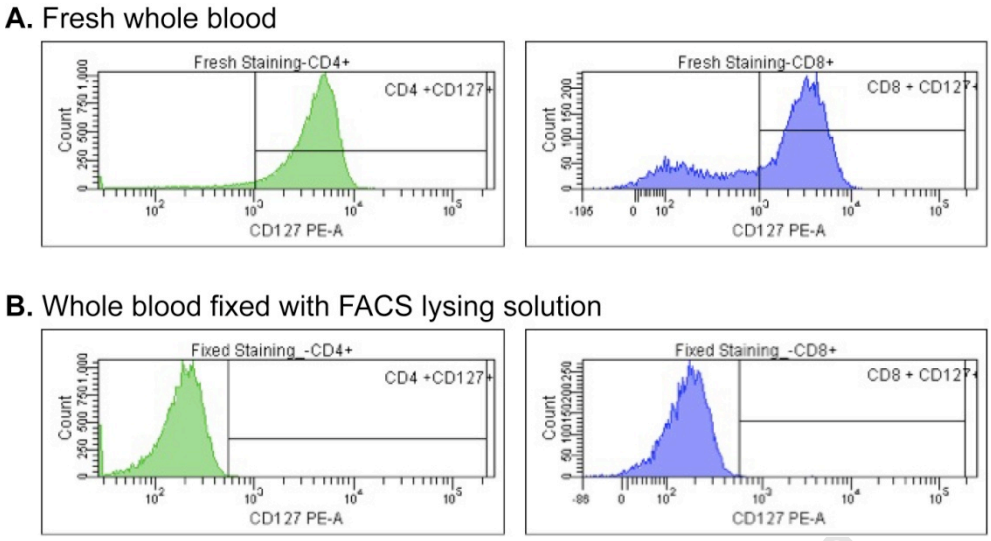

Figure 3. The effect of fixation with FACS lysing solution on CD127 staining. Histograms showing CD127 expression on CD4+ (green shading) and CD8+ T cells (blue shading) after staining (**A**) fresh and (**B**) fixed whole blood.

3.4.5. Optimising of staining protocols for the analysis of phenotypic surface markers

Three staining protocols were tested to determine the effect of permeabilisation and staining techniques on the resolution of surface markers and on antibody performance. Experiments were run in parallel and the levels of CCR7, CD45RA and CD27 expression compared. The discrimination of CCR7-negative and positive populations was more clear when using a two-step staining method with BD Perm/Wash solution for both CD3+CD8- and CD3+CD8+ T cells (**Figure 4A and B**). Discrimination between negative and positive CCR7 populations was lost on CD3+CD8- T cells with the other two staining protocols and a difference in CD45RA staining was observed using the 'two step with staining buffer' method (**Figure 4A and B**). Resolution was

maintained for CD3, CD4, CD8, CD27 and CD28 (data not shown) for all other staining/permeabilisation protocols tested. The two step staining method using BD Perm/Wash buffer was selected as the optimal protocol for the analysis of phenotypic surface markers (phenotypic profile panel).

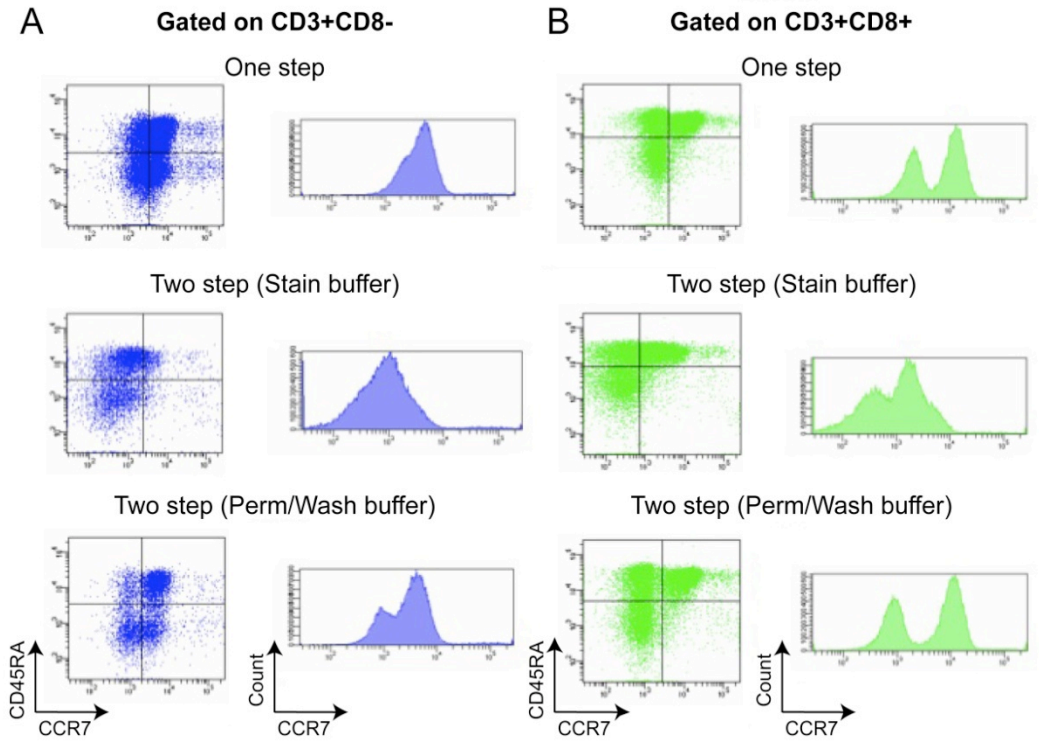

Figure 4. Optimisation of staining protocols for the analysis of phenotypic surface markers. CCR7 and CD45RA staining in (**A**) CD3+CD8- and (**B**) CD3+CD8+ T cells shown as dotplots and histograms using 3 different staining protocols.

3.4.6. FMO

FMO controls were used to assess potential false positives related to fluorochrome interactions, spectral overlap or tandem-dye degradation (Perfetto et al., 2004). This allows detection of fluorescence in secondary

channels because the primary fluorochrome is omitted from the panel. For this study FMO controls were run for all fluorochrome-conjugates and not only tandem dyes or channels requiring high sensitivity. FMO controls for the cytotoxicity reagent panel are shown in **Figure 5**. This panel is shown as a representative example as it was the most complex panel optimised within the study. Detailed results from the cytotoxicity panels FMO controls are listed in **Table 1**. Secondary fluorescence due to spillover remained low in all channels for the cytotoxicity reagent panel (values highlighted in grey, **Table 1**) and all other panels designed for this study (data not shown). The highest secondary fluorescence was detected in the PerCP-Cy5.5 channel (**Table 1**). Since this fluorochrome was assigned to a highly expressed marker, CD28 (**Figure 5)**, the amount of background fluorescence detected in this channel was negligible compared to the positively stained population. Importantly, the frequency of each positive population remained consistent between each FMO control and were comparable to control samples stained with the full 9-colour reagent panel (**Table 1**).

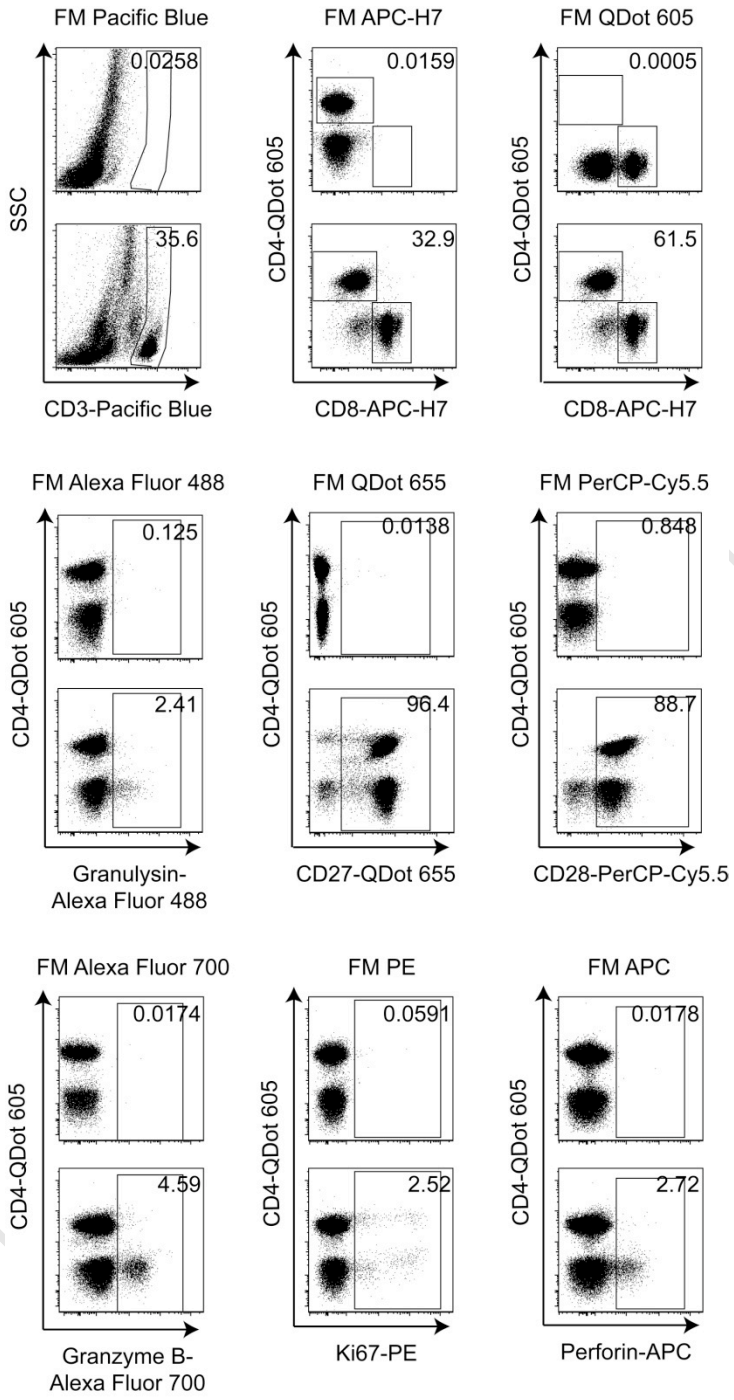

Figure 5. Cytotoxicity panel FMO controls. The label above each dotplot indicates the fluorescent conjugated antibody excluded from the full reagent panel. All dotplots are gated on CD3+ T cells except the first dotplot (FM Pacific Blue), which is gated on lymphocytes. Below each FMO control dotplot is a dotplot showing BCG stimulated whole blood stained with the full reagent panel. FM; fluorescence minus

Table 1. FMO results for the cytotoxicity panel.

FM	Pacific Blue	QDot 605	APC-H7	QDot 655	PerCP-Cy5.5	Alexa Fluor 488	Alexa Fluor 700	PE	APC
Pacific Blue	0.0258	51.6	27.4	81.4	74.9	6.93	5.65	2.25	7.08
QDot 605	37.7	0.00521	32.9	96.5	89.3	2.34	4.72	2.45	2.63
APC-H7	35.8	61.5	0.0159	96.3	89	2.32	4.9	2.45	2.71
QDot 655	34.9	61.6	32.8	0.0138	88.8	2.5	4.7	2.67	2.35
PerCP-Cy5.5	36.4	61.5	32.6	96.3	0.848	2.37	4.78	2.53	2.33
Alexa Fluor 488	35.3	61.5	32.4	96.4	88	0.125	4.7	2.51	2.31
Alexa Fluor 700	33.1	62.5	31.8	96.6	89.7	2.29	0.0174	2.35	2.71
PE	35.5	61.4	32.7	96.4	87.3	2.39	4.74	0.0591	2.56
APC	35.1	61.2	32.8	96.5	89.6	2.8	4.73	2.63	0.0178
Full reagent panel									
BCG stimulated WB	35.6	61.5	32.9	96.4	88.7	2.41	4.59	2.52	2.72

The values shaded in grey indicate the amout of backgrond fluorescence within a specific channel when the antibody detected in that channel is omitted. Values are reported as a frequency of CD3+ T cells except for 'FM Pacific Blue' which shows the percentage of spillover as a frequency of the lymphocyte population. Frequencies obtained from BCG stimulated whole blood samples stained with the full 9-colour reagent panel are are shown as positive controls.

3.4.7. Determining which activation markers allow detection of antigen-specific T cells in a 3 day assay

One of the aims of this study was to characterise BCG-specific cytotoxic molecule expression during the first year of life. Detection of de novo expression of cytotoxic molecules within antigen-specific CTL by flow cytometry is challenging due to the high levels of pre-formed granules containing these molecules (Appay et al., 2002c). To resolve this, the expression of activation antigens CD69, HLA-DR and Ki67 were evaluated as potential markers of antigen-specific T cells after stimulation of whole blood with BCG. Representative dotplots in **Figure 6A** show the expression of CD69, HLA-DR and Ki67 by CD3+ T cells under different stimulatory conditions. Background HLA-DR expression on unstimulated cells (NIL; median, 1.92%) was consistently higher compared with CD69 (median, 0.051%) and Ki67 (median, 0.69%). After stimulation of whole blood with BCG, the expression of all markers increased significantly above background levels (NIL) (**Figure 6A and B**); however after background (NIL) subtraction the frequency of Ki67+CD3+ T cells was higher in four of the six donors compared to CD69+CD3+ and HLA-DR+CD3+ T cells (data not shown). Therefore, Ki67 consistently identified a greater frequency of antigen-specific T cells following background (NIL) subtraction. Ki67 expression also identified a greater percentage of BCG-specific GrmB+ T cells in four of the six donors (**Figure 7A and B**). These data show that Ki67 is more sensitive than CD69 or HLA-DR at identifying BCG-specific T cells in a 3 day culture.

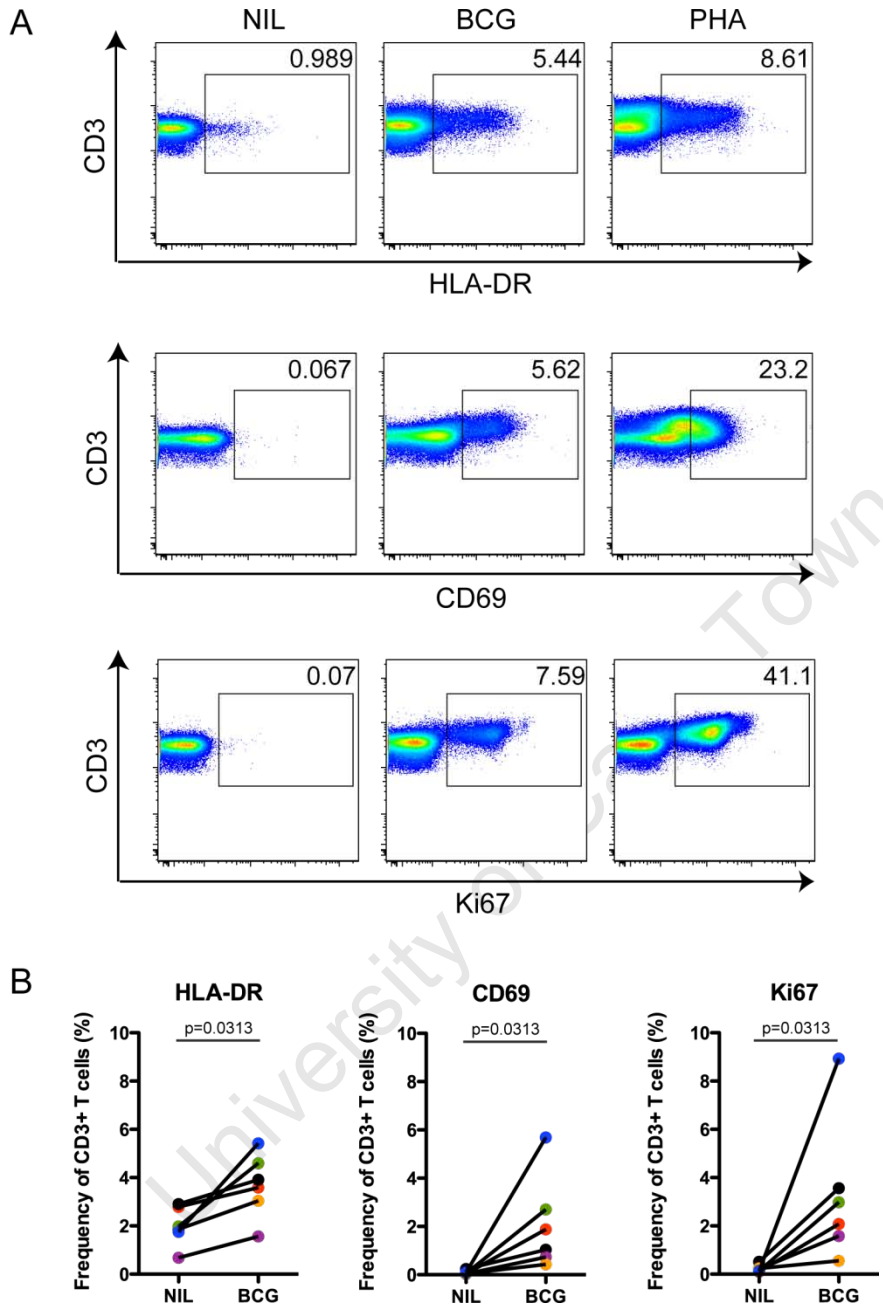

Figure 6. Activation markers HLA-DR, CD69 and Ki67 are up-regulated after incubation of whole blood with BCG. (**A**) Representative example showing the frequencies of HLA-DR, CD69 and Ki67 expressing CD3+ T cells after incubation of whole blood with medium only (NIL), BCG or PHA over 3 days. (**B**) Expression of activation markers in whole blood from 6 donors after 3-day culture with medium only (NIL) or BCG. Each colour represents an individual donor. Differences were calculated using the Wilcoxon matched pairs test.

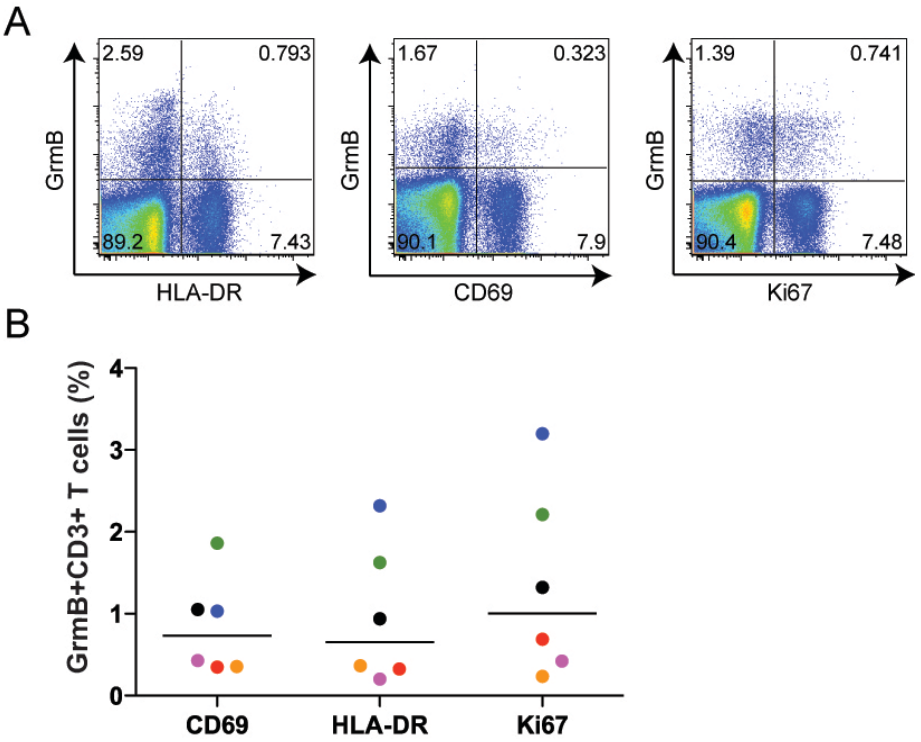

Figure 7. CD69, HLA-DR and Ki67 identify a subset of BCG-specific GrmB+ T cells. (**A**) Representative example showing the frequencies of GrmB+ T cells co-expressing HLA-DR, CD69 and Ki67 after incubation of whole blood with medium only (NIL), BCG or PHA over 3 days. Dotplots are gated on CD3+ lymphocytes. (**B**) Frequencies of GrmB+ T cells detected by CD69 or HLA-DR or Ki67 expression in 6 donors. Each colour represents an individual donor. The horizontal line indicates the median.

3.4.8. Final PFC reagent panels

When this project was initiated, the availability of fluorochrome-conjugates was still limited. To assess all markers of interest, two reagent panels were developed to analyse T cell cytokines profiles and an additional two to analyse memory T cell phenotypes. **Table 2A-F** lists markers included for analysis within each final reagent panel and optimal antibody volumes for each fluorochrome conjugate.

Table 2. Markers and fluorochrome conjugates included in final reagent panels and optimal antibody titers.

Antibody	Fluorochrome	Clone	Optimal volume per reaction (µL)
A. Cytokine Profile Panel 1			
CD3	Amcyan	SK7	5
CD4	Alexa Fluor 700	RPA-T4	1
CD8	PerCP-Cy5.5	SK1	10
TNF-α	PE-Cy7	MAb11	5
IL-2	Alexa 610 PE	5344.111	5
IFN-γ	FITC	25723.11	5
IL-10	APC	JES3-19F1	1
B. Cytokine Profile Panel 2			
CD3	Amcyan	SK7	5
CD4	Alexa Fluor 700	RPA-T4	1
CD8	PerCP-Cy5.5	SK1	10
TNF-α	PE-Cy7	MAb11	5
IL-2	Alexa 610 PE	5344.111	5
IL-4	FITC	MP4-25D2	2
IFN-γ	PE	25723.11	10
IL-10	APC	JES3-19F1	1

Table 2 (continued). Markers and fluorochrome conjugates included in final reagent panels and optimal antibody titers.

Antibody	Fluorochrome	Clone	Optimal volume per reaction (µL)
C. Phenotypic Profile Panel 1			
CD3	Pacific Blue	UCHT1	1
CD4	PerCP-Cy5.5	SK3	10
IL-2	FITC	5344.111	5
IFN-γ	Alexa Fluor 700	B27	1
CD45RA	PE-Cy7	L48	5
CCR7	APC	150503	10
CD27	PE	M-T271	5
D. Phenotypic Profile Panel 2			
CD3	Pacific Blue	UCHT1	1
CD8	PerCP-Cy5.5	SK1	10
IL-2	FITC	5344.111	5
IFN-γ	Alexa Fluor 700	B27	1
CD45RA	PE-Cy7	L48	5
CCR7	APC	150503	10
CD27	PE	M-T271	5

Table 2 (continued). Markers and fluorochrome conjugates included in final reagent panels and optimal antibody titers.

Antibody	Fluorochrome	Clone	Optimal volume per test (µL)
E. Cytotoxicity Panel			
CD3	Pacific Blue	UCHT1	1
CD4	Qdot 605	S3.5	0.5
CD8	APC-H7	SK1	0.6
CD28	PerCP-Cy5.5	L293	7
CD27	Qdot 655	CLB27/1	0.1
Perforin	APC	δG9	5
Granulysin	Alexa Fluor 488	RB1	5
GrmB	Alexa Fluor 700	GB11	1
Ki67	PE	B56	0.1
F. Lymphoproliferation Panel			
CD3	QDot605	UCHT1	1
CD8	PerCP-Cy5.5	SK1	5
Ki67	PE	B56	0.2
IFN-γ	Alexa Fluor 700	B27	1
TNF-α	PE-Cy7	MAb11	3
IL-2	APC	MQ1-17H12	0.5
BrdU	FITC	B44	5

3.5. Discussion

The goal of the experiments outlined in this chapter was to develop and optimise PFC reagent panels to comprehensively characterise BCG-induced T cell immunity in infants. Panel development began with the selection of T cell effector molecules and phenotypic markers relevant to the aims of this study. Markers were selected to allow for: the delineation of T cell subsets (CD3, CD4 and CD8), identification of memory T cell populations or T cell differentiation stage (CCR7, CD45RA, CD27, CD28), antigen specificity or proliferative potential (Ki67) and functional capacity (numerous cytokines and cytotoxic molecules). A number of markers and fluorochrome combinations were screened during the development and optimisation of reagent panels and many were excluded from final reagent panels due to protocol limitations or poor performance. For example, CD62L is useful for the identification of long-lived memory T cell subsets (Sallusto et al., 1999; Cellerai et al., 2007; Miller et al., 2008). However, this marker exhibited diminished resolution between positive and negative populations after activation of T cells to such an extent that discrimination between the two populations was challenging. Protocol limitations led to the exclusion of markers such as CD127, which could not be detected on fixed samples and CD154, a marker of antigen-specificity, which requires antibody addition during cell culture for optimal performance (Chattopadhyay et al., 2006b).

After selecting antigens of interest, fluorescent conjugates were assigned to each marker based on antigen density, fluorochrome brightness and spectral overlap (Baumgarth and Roederer, 2000).

Titration of antibodies is critical for interpretable and reliable data and to ensure optimal staining performance. Excessive antibody concentrations increase non-specific binding, leading to a reduction in signal-to-noise ratios and reduce the dynamic range of detection. Conversely, too low a concentration results in diminished resolution between negative and positive populations and may not sufficiently stain the highest antigen expression level. Since batch-to-batch variations are common among tandem conjugates and Qdots, these dyes were re-titrated when different lots were received (Wu et al., 2007; Hulspas et al., 2009). Tandem dyes are a group of dyes in which two fluorescent molecules are covalently linked; one fluorescent molecule (donor) is excited and transfers energy to the second fluorescent molecule (acceptor), which emits light (Baumgarth and Roederer, 2000). Tandem dye variability is related to changes in acceptor to donor ratios during the manufacturing process (Baumgarth and Roederer, 2000; Hulspas et al., 2009). QDots are nanometer sized crystalline clusters synthesised from a variety of semiconductor materials. QDot preparation methods may alter particle size, shape or introduce small surface defects resulting in the synthesis of particles with different optical properties (Wu et al., 2007). Therefore, whenever possible, tandem dye conjugates and QDots from the same lot were used for the analysis of infant samples.

FMO controls were used to assess potential staining artefacts and the amount of background fluorescence expected in each channel (Maecker et al., 2004; Perfetto et al., 2004; Tung et al., 2004). When tandem dyes are included in reagent panels, artefacts are of particular concern as these conjugates are prone to disassociation when exposed to light, elevated temperatures and

fixatives containing paraformaldehyde (Hulspas et al., 2009). FMO controls are also useful for determining the extent of data spread between channels (Maecker et al., 2004). Although fluorochromes were carefully selected to limit this, data spread was observed in some reagent panels and gating strategies were adjusted accordingly to accommodate for this error.

Staining protocols and reagents used for ICS may alter the epitope structure of certain proteins and influence the performance of antibodies (Lee et al., 1999; Berhanu et al., 2003; Kivisakk et al., 2003). All samples processed within this study were fixed prior to cryo-preservation with an erythrocyte lysing buffer containing paraformaldehyde. Fixing cells stabilises cellular membranes and prevents linkage between intracellular and extracellular proteins (Pollice et al., 1992). However, paraformaldehyde alters three-dimensional protein conformations resulting in modified epitope structures and therefore the ability of an antibody to recognise its target antigen (Pollice et al., 1992; Macey and McCathy, 1993; McCarthy et al., 1994). This is a potential explanation for the lack of CD127 staining when using the BD Biosciences antibody on fixed whole blood samples. In this study, the anti-CCR7 antibody was particularly sensitive to variations in staining conditions compared to other surface markers. The reason for this variability remains unclear as the components of commercially obtained reagents are not disclosed by manufacturers.

The development of PFC has finally provided immunologists with an appropriate tool that allows dissection of complex immune responses. PFC has several advantages over conventional four colour flow cytometry in that it offers increased accuracy in identifying T cell populations and the ability to

obtain detailed information from a single sample. This reduces assay variability and maximises data accumulated from small sample volumes, such as the paediatric samples used in this study. The disadvantages of this technology are predominantly due to higher reagent costs and the length of time required to optimise large reagent panels and analyse complex data sets. This chapter outlines the optimisation processes required for the development of PFC panels specifically designed to measure multiple aspects of T cell mediated immunity after BCG vaccination of infants. Although thorough evaluation of panels is required to obtain accurate and reliable data, the advantages of using this technology far outweigh the amount of time and effort required for optimisation.

Chapter 4

BCG vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles

4.1. Introduction

Our knowledge of immunity induced by BCG vaccination is incomplete, particularly after human newborn vaccination. However, infants will be targets of novel and more efficacious TB vaccines in the future (Hoft, 2008) and a better understanding of the immune response induced by newborn BCG vaccination is likely to facilitate development of improved vaccines.

Experimental evidence suggests that both CD4+ and CD8+ T cells are important for protection against *M.tb* (Flory et al., 1992; Flynn et al., 1992; Caruso et al., 1999; Lazarevic et al., 2005). In humans, the role of CD4+ T cells has been highlighted by an increased risk of disease after infection with *M.tb* when CD4+ T cell numbers decline in HIV infected persons (Elliott et al., 2004). CD4+ and CD8+ T cells contribute to anti-mycobacterial immunity through the production of the Th1 cytokine, IFN-γ. Numerous studies have demonstrated that BCG vaccination of newborns induces CD4+ and CD8+ IFN-γ expressing T cells (Vekemans et al., 2001; Black GF, 2002; Davids et al., 2006; Murray et al., 2006). IFN-γ expression has been the primary focus of these studies as production of this cytokine is typically measured to monitor *M. tb*-specific immune responses (Meier et al., 2005; Dogra, 2006).

Although IFN-γ plays a crucial role for the effective control of this pathogen (Ottenhoff et al., 2003; Dorman et al., 2004), T cells are capable of expressing other cytokines critical for the adequate containment of *M. tb* (Flynn, 2004).

The pro-inflammatory cytokine, TNF-α, synergizes with IFN-γ to activate macrophage anti-mycobacterial activity and plays a key role in granuloma formation and maintenance. (Bekker et al., 2001; Roach et al., 2002). The role of TNF-α has been underscored by high rates of reactivation of latent TB following treatment of rheumatoid arthritis patients with specific inhibitors of this cytokine (Stenger, 2005; Saliu et al., 2006). IL-2 drives the optimal development of memory T cell populations and is required for T cell proliferative capacity (Dooms et al., 2007). Previous studies have also shown that BCG vaccination of infants induces TNF-α, which is detectable in plasma by ELISA (Davids et al., 2006). However, the expression of all Th1 cytokines, on a single cell basis, has not been delineated.

Although BCG vaccination of infants has been shown to induce low levels of Th2 cytokines, detection was in plasma (Marchant et al., 1999; Hussey et al., 2002; Ota, 2002) and cell-associated expression has not reported. In contrast to Th1 cytokine responses, Th2 cytokines are thought to be associated with suboptimal anti-mycobacterial T cell mediated immunity. Individuals who develop active TB have increased levels of soluble IL-5 detectable in plasma and increased frequencies of IL-4-expressing γδ and CD8+ T cells (Ordway et al., 2005).

IL-10 expression was also assessed since this cytokine is likely to be an important regulator of effector T cell responses against *M. tb* (Boussiotis et al., 2000) and IL-10 expression is induced by newborn BCG vaccination (Marchant et al., 1999; Vekemans et al., 2001; Ota et al., 2002).

The memory phenotype of T cells induced by BCG vaccination of newborns has not been described. Memory subsets may be distinguished according to

their functional capacity, such as cytokine production or proliferative potential, and according to expression of specific surface markers (Sallusto et al., 1999; Kaech, 2003; Sallusto et al., 2004; Fritsch et al., 2005; Song et al., 2005). Commonly used surface markers include chemokine and co-stimulatory receptors, receptors involved in differentiation and markers of activation (Appay et al., 2002a; Cellerai et al., 2007; Miller et al., 2008). The original classification of human CD4+ and CD8+ T cells into T_{EM} and T_{CM} subpopulations was defined according to CD45RA and CCR7 expression (Sallusto et al., 1999). T_{CM} cells express CCR7, but not CD45RA, and represent a long-lived population, which expand rapidly in lymph nodes upon subsequent antigen encounter (Sallusto et al., 2004; Huster, 2006). Expression of IL-2, which is critical for the development and survival of memory T cells (Dooms et al., 2004), is pre-dominantly associated with T_{CM} (Sallusto et al., 1999). In contrast, T_{EM} lack both CCR7 and CD45RA expression, have limited proliferative capacity but immediate effector functions such as IFN-γ production and cytolytic capacity (Sallusto et al., 1999; Sallusto et al., 2004). A third subset, terminally differentiated memory cells (T_{EMRA}), express CD45RA, lack CCR7 expression and are the most differentiated subpopulation. T_{EMRA} express less or no IL-2, and less IFN-γ compared with $T_{EM,}$ and are more likely to undergo apoptosis after encountering antigen (Geginat et al., 2003; Fritsch et al., 2005). Naïve, or T cells that have not encountered antigen, characteristically express both CD45RA and CCR7 (Sallusto et al., 1999; Geginat et al., 2003; Fritsch et al., 2005) A combination of markers other than CCR7 and CD45RA may also differentiate memory subsets of antigen-experienced cells. Fritsch *et al*. proposed the phenotypic

classification of CD4+ T cell populations based on expression of CCR7 and the TNFR family member CD27 (Fritsch et al., 2005). T_{CM} were defined as CD27+ and CCR7+, T_{EM} as CD27+ and CCR7–, and T_{EMRA} as CD27– and CCR7–.

In this chapter the cytokine and memory phenotype of BCG-specific T cells were measured in a cross-sectional analysis of a 10-14 week old infant cohort.

4.2. Aims

Although several investigators have characterised BCG-specific T cell responses in infants, these studies used assays that could measure a limited number of cytokines or phenotypic markers at a time and thus underestimated the complexity of the response. The primary objective of this study was to determine which functional and phenotypic markers are expressed on BCG-specific T cells after BCG vaccination of infants. A short-term ICS assay was used to cross-sectionally examine BCG-specific T cell mediated immunity in blood from 10-14 week old infants, routinely vaccinated with BCG at birth.

To achieve this, two cross-sectional analysis were designed to address the following aims:

1. Comprehensively assess BCG-specific CD4+ and CD8+ Th1 and Th2 intracellular cytokine expression (cross-sectional study 1).
2. Assess the levels of secreted Th1 and Th2 cytokines in plasma after incubation of whole blood with BCG (cross-sectional study 1).
3. To determine the memory phenotypes of BCG-specific CD4+ and CD8+ T cells after BCG vaccination (cross-sectional study 2).

4.3. Materials and methods

4.3.1. Study participants and phlebotomy

Infants were recruited as described in chapter 2 (section 2.1.2). Twenty-nine infants were recruited into the first cross-sectional study to assess BCG-specific T cell cytokine expression and 29 into a second study characterising T cell memory phenotypes. Blood was collected by qualified phlebotomists within the guidelines of written consent.

4.3.2. Antibodies

The following monoclonal antibodies were used characterise BCG-specific T cell responses:

i. Intracellular cytokine panel

CD3-Amcyan, CD4-Alexa Fluor 700, CD8 PerCPCy5.5, IFN-γ-FITC or IFN-γ-PE, IL-4-FITC, IL-2-Alexa Fluor 610 PE, TNF-α-PE-Cy7 and IL-10-APC.

ii. Memory phenotype panel

CD3-Pacific Blue, CD4-PerCPCy5.5 or CD8-PerCPCy5.5, IFN-γ-Alexa Fluor 700, IL-2-FITC, CD27-PE, CD45RA-PE-Cy7 and CCR7-APC.

All antibodies were obtained from BD Biosciences except for CD3-Amcyan and IL-2-Alexa Fluor 610 PE, which were custom conjugated by H. T. Maecker (BD Biosciences, San Jose, CA) and CCR7-APC was obtained from R&D Systems.

4.3.3. Whole blood intracellular cytokine (WB-ICC) assay

Whole blood was stimulated with specific antigens for a total of 12 hours as described previously in chapter 2 (section 2.3.1.).

4.3.4. Plasma cytokine detection (multi-plex bead assay)

To assess soluble Th1 and Th2 cytokines in plasma, plasma was collected from whole blood cultured with BCG for 7 hours, and cryopreserved. Levels of IFN-γ, TNF-α, IL-12p70, IL-2, IL-4, IL-5, IL-10 and IL-13 were measured in thawed plasma with multiplex beads following the manufacturer's instructions (Bio-Rad Laboratories), and read on a luminometer (Luminex). The range of detection for all cytokines was 1.95–32,000pg/mL. An optimal plasma dilution of 1:4 was determined in pilot experiments. Background cytokine levels measured in plasma harvested from whole blood incubated with co-stimulants only were subtracted from BCG-stimulated blood.

4.3.5. Immuno-fluorescence staining and flow cytometric analysis

i. Intracellular cytokine panel

Stimulated whole blood was stained with fluorochrome-conjugated antibodies using a one step staining method as described in chapter 2 (section 2.5.1).

Cytokine profiles of T cells were analysed using FACSDiva based on the following gating strategy: T cells were first defined based on expression of CD3. CD3+ T cell lineages were differentiated according to CD4 and CD8 expression. Expression of all cytokines were assessed in CD4+ and CD8+ T cell populations. To determine co-expression of Th1 cytokines, IFN-γ expressing cells were selected on a IFN-γ vs. SSC dotplot. Expression of IL-2

and TNF-α were assessed within IFN-γ positive (+) and IFN-γ negative (-) populations.

Cut-offs to determine positive cytokine expression in CD4+ and CD8+ T cells from blood incubated with BCG were set using cells incubated with co-stimulants only (NIL, negative control). Negative control (background) values for cytokine expression were not subtracted from BCG-induced responses, as the median backgrounds was low for all CD4+ T cells (0.001%; range, 0.000%-0.01%) and for CD8+ T cells (0.000%; range, 0.000%-0.01%). The sensitivity of the LSR II used in this study was 0.01%. Therefore, cytokine responses above 0.01% were considered reliable. At least 266 610 (median = 508 509) CD4+ cells and 86 421 (median = 188 498) CD8+ cells were acquired for each condition. Angled cut-off lines were necessary for some fluorochromes because of increased variance (data spread) after instrument compensation. SEB was an excellent positive control for induction of all cytokines, except IL-4. HICK-2 cells (BD Biosciences), processed as per manufacturer's protocol were used as a positive control for IL-4.

ii. Memory phenotype panel

Stimulated whole blood was stained with fluorescent-conjugated antibodies using a two step staining method as described chapter 2 (section 2.5.2).

Phenotypic profiles of BCG specific T cells were analysed using FACSDiva according to the following gating strategy: T cell populations were first identified in a SSC vs. CD3 dotplot (**Figure 1A**). T cell lineages were differentiated according to expression of CD4 or CD8 (**Figure 1A**). CD4+ and CD8+ T cells expressing IFN-γ and/or IL-2 were defined as antigen-specific T

cells. IFN-γ+, IFN-γ+IL-2+ and IL-2+ T cell subsets were each analysed for the expression of CD45RA. (**Figure 1B**) CD45RA+ and CD45RA- populations were then delineated according to CCR7 and CD27 expression (**Figure 1**). T cell phenotyping was carried out on samples which had at least 50 cytokine positive events.

Cut-offs to determine positive cytokine expression in CD4+ and CD8+ T cells from blood incubated with BCG were set using cells incubated with co-stimulants only (NIL, negative control) as previously described. Negative control (background) values were not subtracted from BCG-induced cytokine responses as the endpoint of this analysis was the phenotype of cytokine expressing T cells.

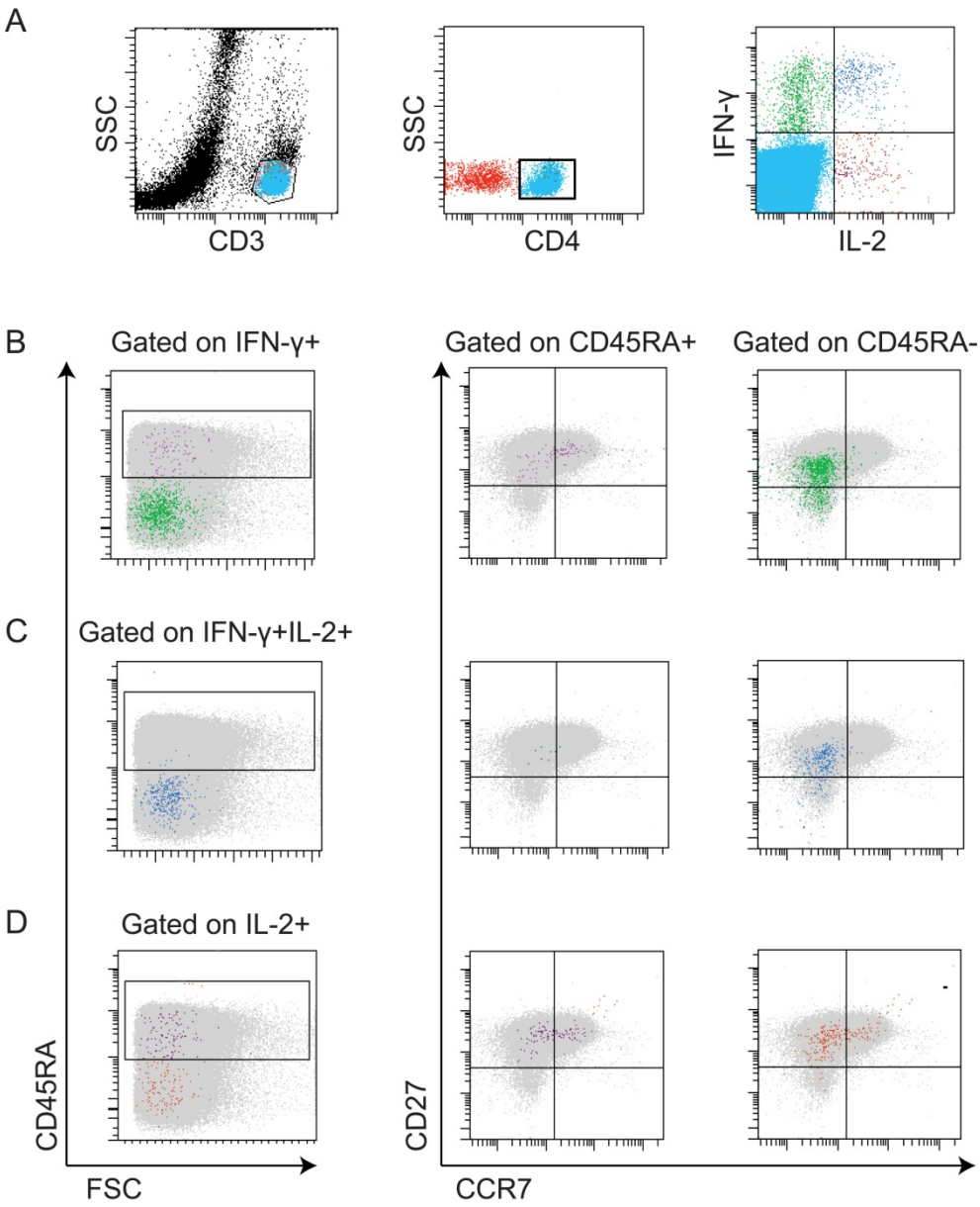

Figure 1. Phenotypic profiling of CD4+ Th1 cytokine expressing T cells in whole blood incubated with BCG for 12 hours. (**A**) Gating strategy to identify T cells by CD3 expression and T cell lineages by CD4 or CD8 expression. CD45RA expression on (**B**) IFN-γ+ (**C**) IFN-γ+IL-2+ and (**D**) IL-2+ subsets were analysed for the expression of CD45RA. CD45RA+ and CD45RA- populations were delineated according to CD27 and CCR7 expression. Cytokine expressing T cells (in colour; foreground) were overlaid onto a density plot (grey shading) of the total CD4+ T cell population.

4.4. Results

4.4.1. Newborn vaccination with BCG induces complex Th1 CD4+ and CD8+ T cell responses

Intracellular expression of three Th1 cytokines thought to be critical for protective immunity against mycobacteria, IFN-γ, IL-2 and TNF-α, were evaluated by incubating blood from 29 BCG-vaccinated infants with BCG for 12 hours. The median frequencies of CD4+ T cells expressing either IFN-γ or IL-2 or TNF-α were similar **(Figure 2)**. Lower frequencies of CD8+ T cells expressed IFN-γ, IL-2, or TNF-α, compared with CD4+ T cells **(Figure 2)**.

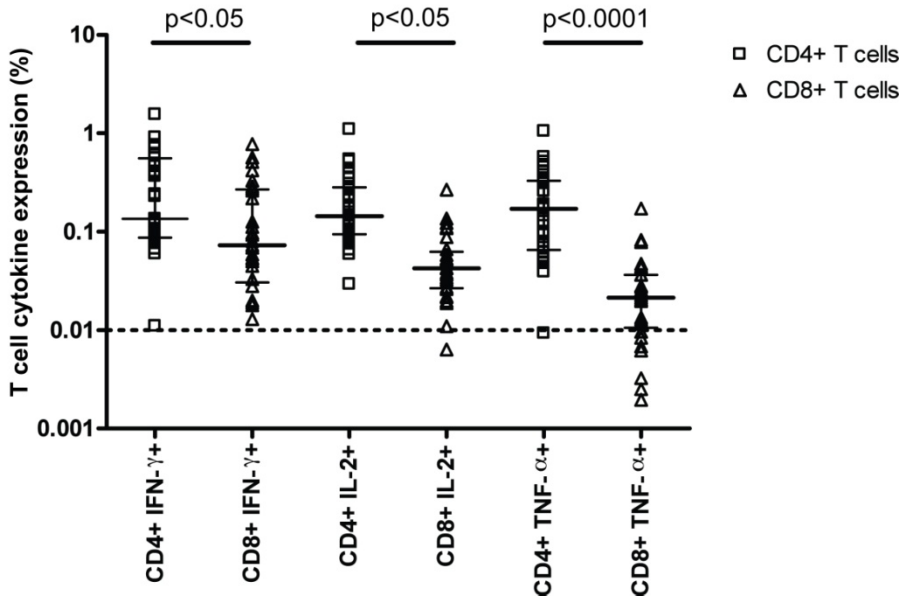

Figure 2. Individual Th1 cytokine expression of BCG-specific T cells. Frequency of CD4+ (□) and of CD8+ (△) T cells expressing individual Th1 cytokines following incubation of whole blood with BCG for 12 hours, in 29 infants. Responses above 0.01% were considered reliable. The horizontal line indicates the median and the whiskers the interquartile range. Differences were calculated using the Wilcoxon matched pair test.

A strong positive correlation between the frequencies of IFN-γ- or IL-2- or TNF-α-expressing CD4+ and CD8+ T cells was observed (r = 0.770, r = 0.879, r = 0.760, respectively, all $p < 0.0001$).

Analysis of simultaneous expression of IFN-γ, IL-2, and TNF-α on a single cell level revealed seven distinct Th1 cytokine-expressing CD4+ T cell populations **(Figure 3A)**. Among CD8+ T cells, the dominant population expressed IFN-γ only and three other populations were discernable **(Figure 3B)**. IFN-γ production is typically measured to diagnose latent infection (Mahomed et al., 2006; Janssens et al., 2007) or to describe human immune responses to TB vaccines (McShane et al., 2004). Importantly, a proportion of CD4+ T cells expressing IL-2 and/or TNF-α did not co-express IFN-γ **(Figure 3A)**. Similarly, among CD8+ T cells, a proportion of IL-2-expressing T cells did not co-express IFN-γ **(Figure 3B)**.

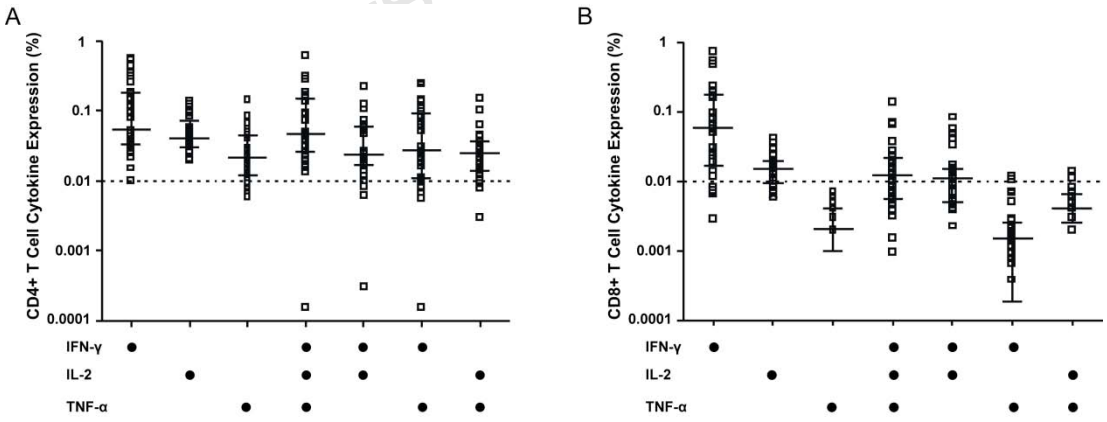

Figure 3. Th1 cytokine subsets induced by BCG vaccination. Frequency of BCG-specific (**A**) CD4+ and (**B**) CD8+ T cells T cells expressing different combinations of Th1 cytokines. The horizontal line indicates the median and the whiskers the interquartile range.

Total Th1 cell responses were dominated by IFN-γ expression **(Figure 4A and B)** and the frequency of IFN-γ expressing T cells correlated significantly with the total Th1 response (**Figure 4C and D**). However, measuring IFN-γ alone did not detect all Th1 cytokine-expressing T cells.

Taken together, these data show that BCG vaccination of newborns induces multiple Th1 cell subsets defined by expression of distinct cytokine combinations, including IFN-γ negative populations.

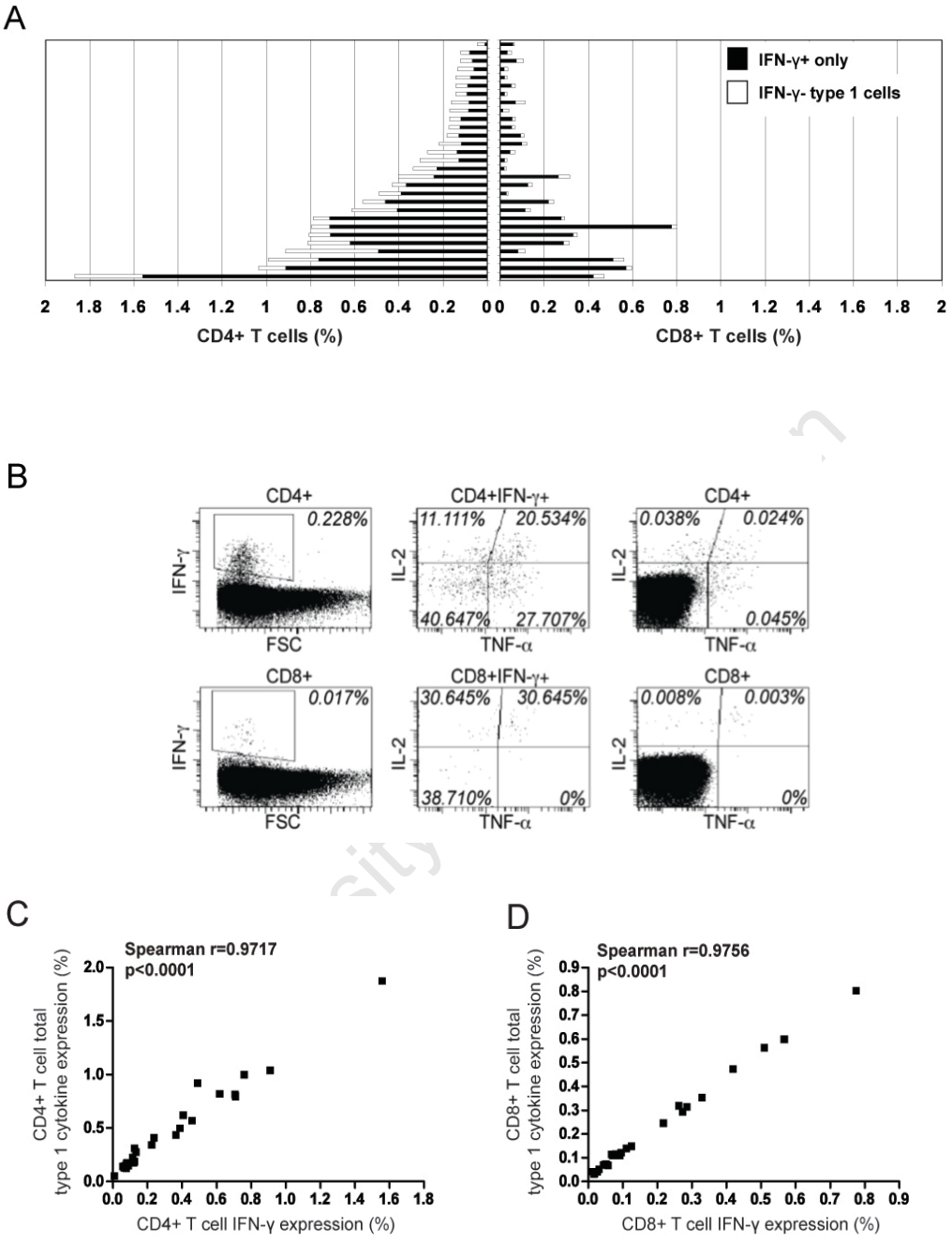

Figure 4. CD4+ and CD8+ T cells pre-dominantly express IFN-γ. (**A**) Comparison of frequency of CD4+ and of CD8+ T cells expressing IFN-γ+ only (■) T cells IFN-γ- expressing IL-2 and/or TNF-α (□), in 29 BCG-vaccinated infants. (**B**) Representative intracellular staining of Th1 cytokines in BCG-specific $CD4^+$ T cells and CD8+ T cells, from a single 10-week old infant. Relationship between the frequency of (**C**) CD4+ and (**D**) CD8+ T cells expressing IFN-γ alone, and cells expressing any Th1 cytokine. Correlation coefficients were determined by a Spearman test.

4.4.2. BCG-specific IL-10 and IL-4 intracellular cytokine expression

The frequency of T cells expressing IL-10 or IL-4 following incubation of whole blood with BCG was low **(Figure 5)**. Few donors had responses above 0.01%, but IL-10 and IL-4 production were consistently above the background expression levels found in NIL controls **(Figure 5A and D)**. CD4+ T cell expression of both cytokines was marginally higher than that of CD8+ T cells (data not shown). Intracellular IL-4 could be readily be detected in HICK-2 cytokine-expressing cells, which served as positive control **(Figure 5C)**.

These data show that intracellular IL-4 and IL-10 expression are not reliably detectable intracellularly after short-term incubation of whole blood with BCG.

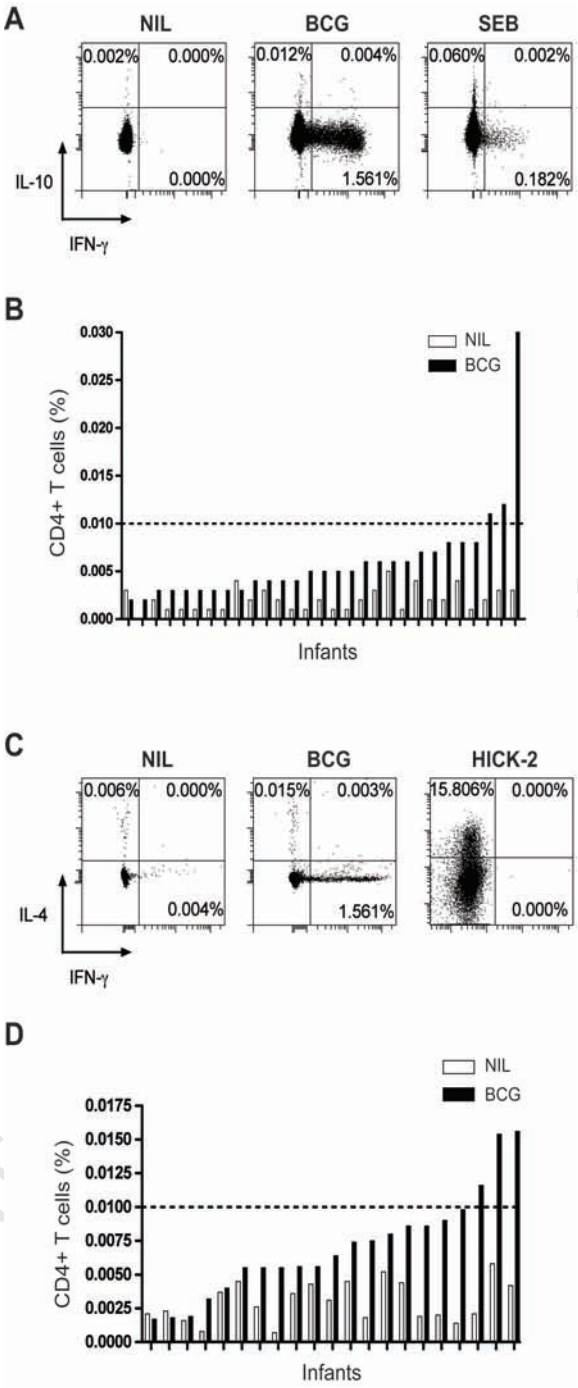

Figure 5. IL-10 and IL-4 frequencies were below the detection limit of this assay. Expression of (**A**) IL-10 or (**C**) IL-4 in CD4+ T cells in whole blood from a single, vaccinated infant. Frequency of CD4+ T cells expressing (**B**) IL-10 or (**D**) IL-4 after incubation of whole blood with co-stimulants only (□) or BCG (■) for 12 h in 29 infants.

4.4.3. Th1 and Th2 cytokines are detectable in plasma following incubation of whole blood with BCG

All three Th1 cytokines and IL-12p70 could also be detected in plasma **(Figure 6)**. There was a significant correlation between plasma levels of IFN-γ, IL-2, and TNF-α and frequencies of CD4+ T cells producing these cytokines **(Table 1)**. Plasma IFN-γ and IL-2 also correlated with frequencies of CD8+ T cells producing IFN-γ and IL-2, respectively **(Table 1)**.

IL-4, IL-10 and IL-13 were detected at low levels in plasma **(Figure 7)** and IL-13 correlated with IL-4 levels ($r=0.5924$, $p<0.001$). IL-5 was also measured, however levels of this cytokine were below the detection range of the assay.

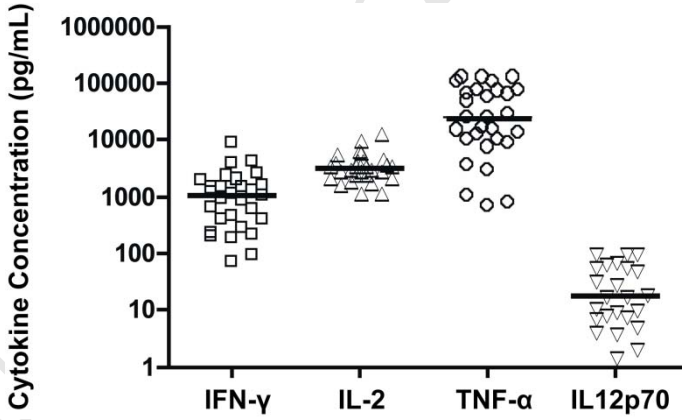

Figure 6. Th1 cytokines detected in plasma. Levels IFN-γ, IL-2, TNF-α and IL-12p70 detected by bead array in plasma from whole blood incubated with BCG for 7 hours. The horizontal line reflects the median. Background cytokine levels were subtracted.

Table 1. Association between Th1 cytokine levels in plasma, and frequencies of CD4+ or CD8+ T cells expressing these cytokines, after incubation of whole blood with BCG.

	CD4+ T cells expressing cytokine		**CD8+ T cells expressing cytokine**	
	r	***p***	***r***	***p***
Plasma IFN-γ	0.6793	<0.0001	0.6305	0.0004
Plasma IL-2	0.6441	0.0003	0.6013	0.0009
Plasma TNF-α	0.4034	0.01	0.3187	0.1052

A Spearman test was used to assess correlation in 29 infants.

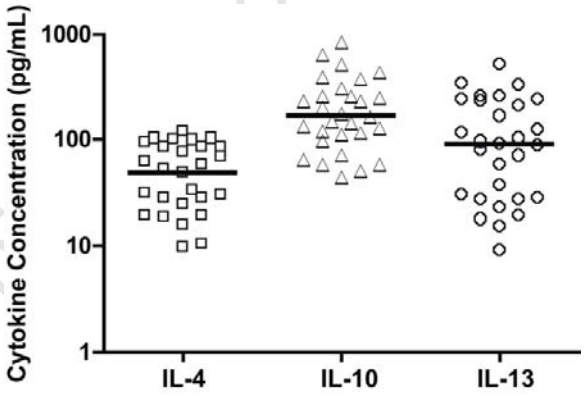

Figure 7. Th2 cytokines detected in plasma. Levels of the Th2 cytokines IL-4, IL-13, and IL-10, detected by bead array in plasma from whole blood incubated with BCG for 7 hours. The horizontal line reflects the median. Background cytokine levels were subtracted.

These data show that Th1 cytokines are detectable in plasma after short-term incubation of whole blood with BCG and correlate with intracellular cytokine expression. BCG vaccination of newborns also induces low levels of Th2 cytokines, which can be detected in plasma.

4.4.4. BCG vaccination of newborns induces Th1 CD4+ and CD8+ T cells with a predominantly T_{EM} phenotype

Studies in infectious disease and vaccine models have shown that distinct memory subsets of antigen-experienced T cells may be associated with long-lived protection (Combadiere et al., 2004; Zaph et al., 2004b). Therefore the phenotypic profiles of BCG-induced T cells were examined. Antigen-specific T cells were defined based on IL-2 and/or IFN-γ expression (**Figure 2**). BCG vaccination induced a heterogeneous Th1 CD4+ and CD8+ T cell response as described previously (data not shown). Five distinct T cell phenotypes were discerned among specific CD4+ T cells, based on expression of CD45RA, CCR7 and CD27 (**Figure 8**). The most common phenotype of both IFN-γ and IL-2 expressing CD4+ T cells was CD45RA-CCR7-CD27+ (**Figure 9**), a phenotype that has been reported to be characteristic of T_{EM}. The second most common phenotype among IFN-γ expressing CD4+ T cells was CD45RA-CCR7-CD27-, also characteristic of T_{EM} (**Figure 9**). Among IL-2 expressing CD4+ T cells, the latter population was significantly less frequent (**Figure 9**). IL-2+ cells were more likely to be CD45RA+CCR7-CD27+, CD45RA+CCR7+CD27+ and CD45RA-CCR7+CD27+ compared with IFN-γ expressing CD4+ T cells (**Figure 9**). CD4+ T cells that expressed both IFN-γ

and IL-2 were predominantly CD45RA–CCR7–CD27+ or CD45RA–CCR7+CD27+ (**Figure 9**).

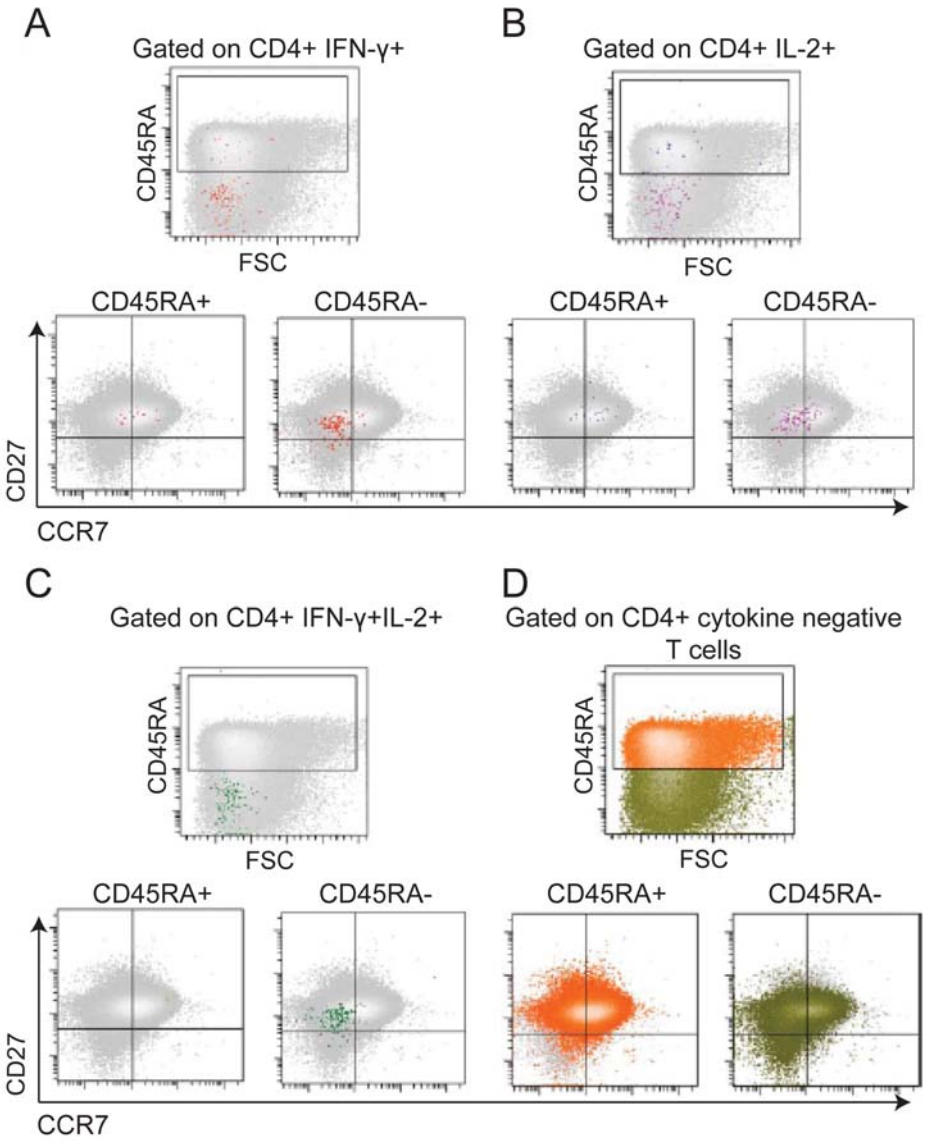

Figure 8. Phenotypic analysis of BCG-specific CD4+ T cells. Antigen-specific CD4+ T cells were identified as (**A**) IFN-γ+, (**B**) IL-2+ and (**C**) IFN-γ +IL-2+. Cytokine subsets were phenotyped according to CD45RA, CCR7 and CD27 expression. The plots illustrate the distribution of cytokine expressing cells (in colour; foreground) in relation to the entire CD4+ T cell population (grey; background, also shown in (**D**). CD8+ T cell phenotypes, not shown here, were evaluated in the same manner.

As a comparison, the expression of phenotypic markers among cytokine-negative CD4+ T cells are depicted in **Figure 9C**.

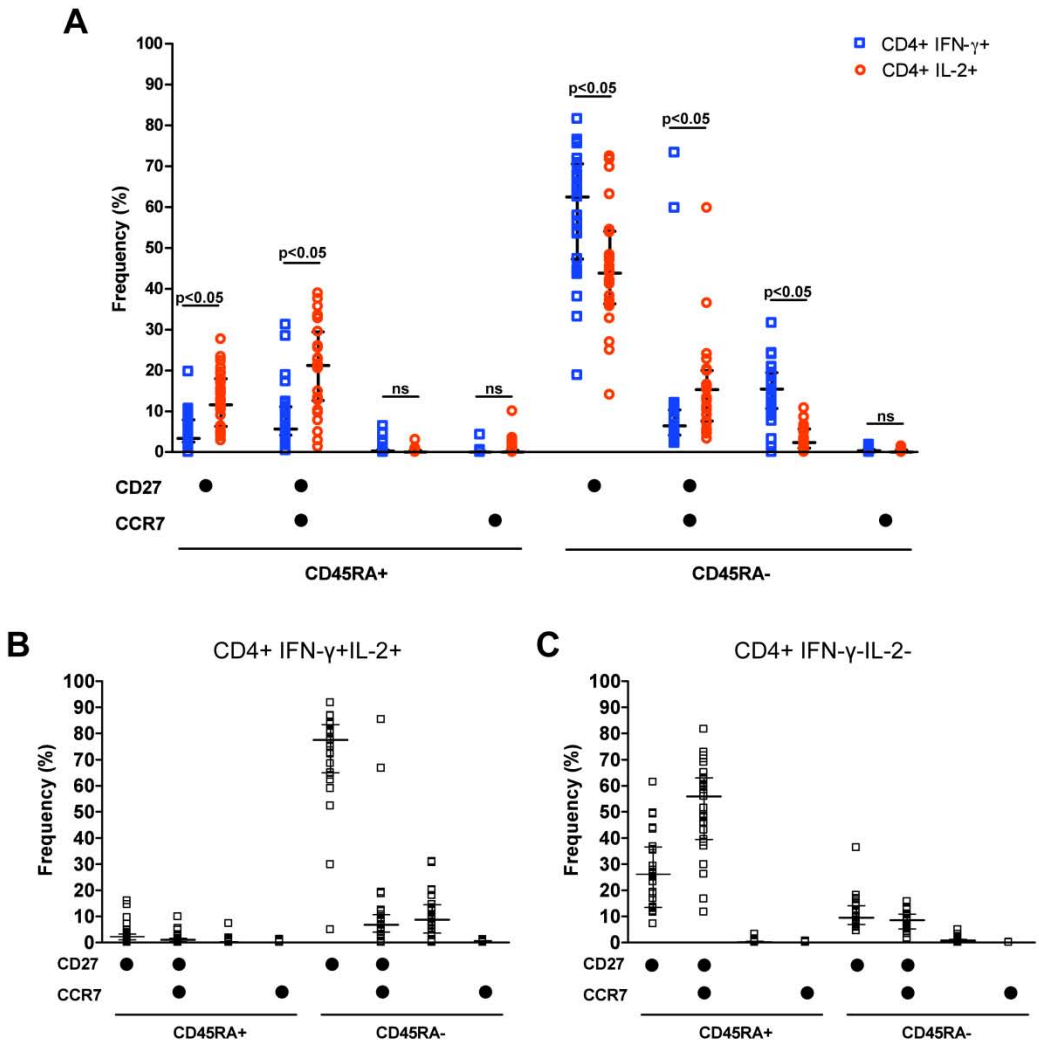

Figure 9. BCG specific CD4+ T cell memory subsets. Frequency of different subsets of BCG-specific CD4+ T cells, based on expression of CD45RA, CCR7 and CD27 among (**A**) IFN-γ+ and IL-2+ and (**B**) IFN-γ+IL-2+ expressing CD4+ T cells. (**C**) shows the frequency of these cells among cytokine-negative, i.e., mostly mycobacteria-non-specific, CD4+ T cells. The horizontal line represents the median and the whiskers the interquartile range. Differences were calculated using the Kruskal-Wallis test followed by the Wlcoxon matched pairs test. Ns; not significant.

CD4+IL-2+ T cells expressed significantly higher levels of CCR7 and CD45RA compared to CD4+ T cells expressing IFN-γ (**Figure 10**). No significant difference in CD27 expression was observed (data not shown).

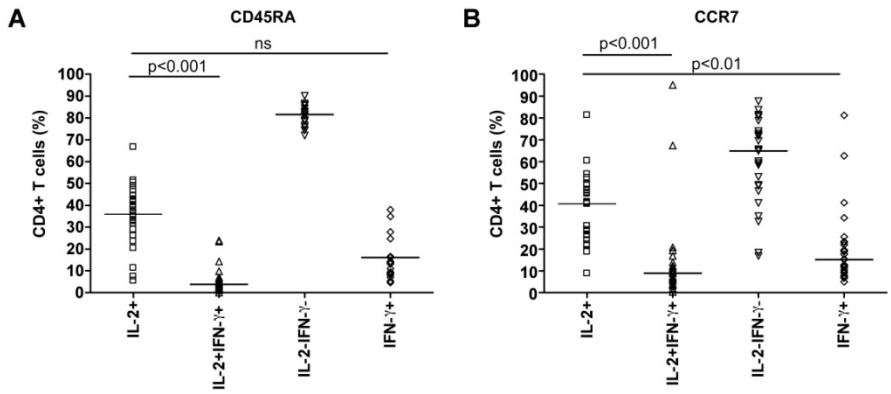

Figure 10. (**A**) CD45RA and (**B**) CCR7 expression of BCG specific CD4+ T cells. The horizontal line represents the median. Differences were calculated using the Kruskal-Wallis test followed by the Wilcoxon matched pairs test. Ns, not significant.

Phenotyping of BCG-specific CD8+ T cells was reliable only for IFN-γ expressing cells as the number of events available for analysis of other CD8+ T cell cytokine subsets were too low. CD8+IFN-γ+ T cells also displayed a pre-dominant CD45RA-CCR7-CD27+ effector phenotype (**Figure 11A**). The expression of phenotypic markers among cytokine-negative CD8+ T cells are depicted in **Figure 11B**.

Collectively, these data show that the majority of specific T cells induced by BCG vaccination of newborns have a T_{EM} phenotype, and that IL-2 expression is more likely to be associated with a T_{CM} phenotype.

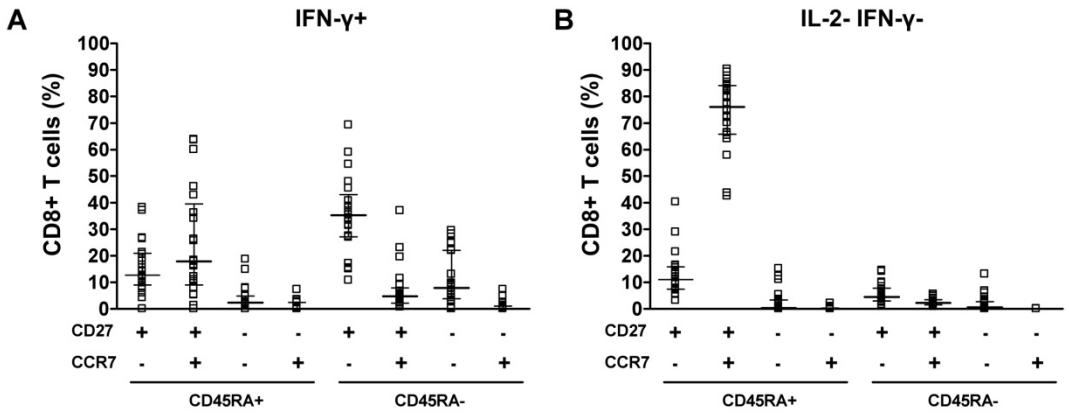

Figure 11. BCG-specific CD8+ T cell memory subsets. Frequency of BCG-specific CD8+ T cell subsets based on expression of CD45RA, CCR7 and CD27 among (**A**) IFN-γ+ and (**B**) cytokine negative CD8+ T cells. The horizontal line represents the median and the whiskers the interquartile range.

4.5. Discussion

Recent advances in flow cytometry have led to more comprehensive characterisation of antigen-specific T cell effector functions at the single cell level (Perfetto et al., 2004). In this study, PFC analysis revealed that BCG vaccination of newborns induces a diverse set of T cells delineated by distinct cytokine expression profiles. Prior to the work completed in this project, the polyfunctional (co-expression of multiple cytokines) nature of BCG-specific T cells after newborn vaccination had not been described.

As has been demonstrated previously, BCG-specific T cells produced mainly Th1 cytokines. However, an important observation was that Th1 cytokines were co-expressed in multiple combinations with a considerable number of IFN-γ negative CD4+ T cells expressing IL-2 and TNF-α. Similarly, a proportion of CD8+ T cells produced IL-2 in the absence of IFN-γ. This is an important observation as the most commonly used measure of mycobacteria-induced immunity is IFN-γ production, be this to diagnose latent infection via IFN-γ release assays (Mahomed et al., 2006; Janssens et al., 2007) or to describe human immune responses to novel tuberculosis vaccines (McShane et al., 2004) or BCG (Hussey et al., 2002; Davids et al., 2006). Consequently, the measurement of a single component of the immune response, such as IFN-γ expression has underestimated the magnitude and complexity of BCG-induced immunity. Additionally, experimental data suggest that measuring IFN-γ alone may not correlate with vaccination-induced protection against tuberculosis (Hovav et al., 2003; Hope et al., 2005; Majlessi et al., 2006). These findings strongly support measurement of multiple relevant cytokines on a cellular level to delineate mycobacteria-specific responses.

BCG-induced CD8+ T cells expressing Th1 cytokines were also readily detectable confirming previous observations that BCG is capable of inducing CD8+ T cell responses (Smith et al., 1999; Murray et al., 2006). Murine studies suggest that CD8+ T cells play an important role in control of *M. tb* infection and contribute substantially to total IFN-γ production (Lazarevic et al., 2005). Although BCG-induced CD8+ T cell responses have been described before (Smith et al., 1999; Murray et al., 2006), the data presented here show that the response is characterised by both IFN-γ and IL-2 producing subsets. BCG-specific CD8+ T cells expressed significantly less Th1 cytokines than CD4+ T cells. Antigen load has been shown to be a determinant of CD8 T activation following mycobacterial infection (Russel et al., 2007; Ryan et al., 2009). Therefore, low Th1 CD8+ T cell responses following BCG vaccination of infants may be attributed to reduced antigen load at the site of T cell priming (Ryan et al., 2009). The low levels of TNF-α expression by CD8+ T cells observed here contrast with previous published data. Smith, et al., detected similar frequencies of CD8+ T cells expressing TNF-α and IFN-γ following incubation of PBMC with BCG for 6 days (Smith et al., 1999). The contrasting low frequency of TNF-α expression observed in this study could be due to differences in assays, as cytokine expression was measured 12 hours after incubation of whole blood with BCG. In longer term assays such as those performed by Smith, et al., TNF-α production may be derived from newly differentiated effector T cells, whereas short term assays measure cytokine producing potential directly *ex vivo* (Schwendemann et al., 2005; Hanekom et al., 2008). The results presented here also contrast with data from HIV-infected adults, whose HIV-specific CD8+ T cells readily

express TNF-α in short-term intracellular cytokine assays (de Castro Cunha et al., 2005; Betts et al., 2006), implying that BCG-specific CD8+ T cells in newborns express little TNF-α.

PFC is a highly sensitive technique that allows analysis of rare events such as those measured within this study. Despite this, IL-4 and IL-10 could not be reliably detected intracellularly. Although IL-4 and IL-10 responses were below the reliable detection limit of the ICS assay, CD4+ T cell expression of these cytokines was in most infants greater than background frequencies. Soluble IL-4, IL-10 and IL-13 were however detectable in plasma by a sensitive multi-plex bead array. Expression of Th2 cytokines has been associated with a sub-optimal immune response to mycobacteria (Hernandez-Pando et al., 1996; Wedlock et al., 2003). For example, Ordway, *et al*., showed that long-term control of latent *M.tb* infection in humans appeared to be associated with optimal Th1 cytokine production and absence of detectable Th2 cytokine production (Ordway et al., 2004). This study showed that high percentages of IL-4 expressing CD8+ and γδ T cells in latently infected individuals were associated with ultimate development of TB disease. IL-4 expressing cells were detected after incubation of PBMC for 6 days, which contrasted with the 12-hour assay completed in this study. Longer-term assays may be required to detect these cells in the setting of BCG vaccination of the newborn. Intracellular expression of IL-10 could also be detected in this cohort, but was low. These cells are likely to represent induced Treg cells, which are expected to be present at low frequencies. Treg cells control conventional effector immune responses, are induced by infections (Grazia Roncarolo et al., 2006), and likely also by vaccination with BCG. The overall

levels of Th2 cytokine production was low in comparison to the dominant Th1 response, indicating that the Th2 cytokines potentially play a immuno-regulatory roll.

The data presented here clearly demonstrate the advantages of complex multiparameter analysis for deciphering vaccine-induced immune responses. The value of this type of analysis over more traditional techniques is that the quality of the immune response can be assessed. However, this technology is expensive and not readily available. It is therefore important to note that the measurement of intracellular IFN-γ alone appears to be a good surrogate measurement of the total Th1 response, as the frequency of CD4+ or CD8+ T cells expressing this cytokine alone correlated well with the frequency of total Th1 response. Also, when flow cytometry is not available, plasma levels of IFN-γ or IL-2 may serve as surrogates of T cell cytokine production. Plasma levels of Th1 cytokines correlated strongly with intracellular expression despite the fact that non-T cells also have the ability to make these cytokines (Esin et al., 2004; Feinberg et al., 2004). Importantly, TNF was a poor correlate and may not be a good surrogate. This poor correlation may be attributed to high quantities of TNF secreted by innate cells in response to BCG.

The heterogeneous nature of T cells based on cytokine expression profiles has been described in various models of infection and vaccination (Harari et al., 2004a; Harari et al., 2005; Beveridge et al., 2007; Precopio et al., 2007). Exploring the quality of T cell responses may be highly relevant as the induction of polyfunctional T cells, either through infection or vaccination, have been shown to be more accurate predictors of protective capacity (Darrah et

al., 2007). In models of chronic viral infection these cells have shown to be superior in controlling viral replication (Kannanganat et al., 2007a; Kannanganat et al., 2007b). For example, in HIV infection the presence of polyfunctional HIV-specific T cell populations is associated with better clinical outcome (Betts, 2006; Kannanganat et al., 2007b). In the mouse model of *Leishmania major* (*L. major*) infection, polyfunctional T cell induction is also associated with lower parasite burden (Darrah et al., 2007). These data suggest that measurement of these cells may also be important following BCG vaccination.

The memory phenotype of BCG-induced T cells after vaccination of infants has not been described. In this study, five phenotypically distinct subsets within BCG-specific Th1 cells were identified based on CD45RA, CCR7 and CD27 expression. A predominant CD45RA-CCR7-CD27+ effector phenotype was displayed by BCG-specific CD4+ and CD8+ T cells, irrespective of whether these cells produced IFN-γ or IL-2. This observation is of interest as CCR7- effector phenotypes are characteristic of antigen-specific T cells seen in chronic viral infections (Appay et al., 2002a; Harari et al., 2004a; Harari et al., 2004b). In contrast, antigen-specific T cells retain or re-express CCR7 and display a predominantly T_{CM} phenotype in vaccine models of cleared antigen such as TT and hepatitis B surface antigen (HBs) (Cellerai et al., 2007; Stubbe et al., 2008). The predominantly CCR7- phenotype observed in 10-14 week old BCG vaccinated infants may arise due to a number of factors. Firstly, exposure of infants to cross-reactive environmental mycobacteria or persistence of BCG following vaccination may contribute to activation of T cells thereby driving a more effector phenotype. Secondly, the assay used in

this study is a direct *ex vivo* measurement of antigen-specific cells and may not represent the entire BCG-specific memory response. For example, short-term assays are likely to measure T_{EM} responses as this subset displays immediate effector functions. However longer term assays are required to measure T_{CM} proliferation and cytokine expression (Combadiere et al., 2004; Hanekom et al., 2008). Thirdly, BCG-specific CCR7+ T cells may reside pre-dominantly in secondary lymphoid organs and would only be detectable when re-circulating through the peripheral blood. Lastly, although classification of memory T cell subsets according to CD45RA, CCR7 and CD27 expression is informative, these markers may not always accurately reflect the long-lived potential of T cells *in vivo*. In humans, long-term memory T cells specific for vaccinia virus have been shown to have a pre-dominantly T_{EM} (CD45RA-CCR7-CD27+) phenotype years after smallpox vaccination (Combadiere et al., 2004) and not the classical T_{CM} phenotype.

A significant proportion of BCG-specific CD4+ and CD8+ T cells had a phenotype traditionally regarded as naïve, i.e., CD45RA+CCR7+CD27+. In a recent study of children with tuberculosis, Caccamo et al. also described this population, identified as specific by MHC I pentamers of antigen-85A (Caccamo et al., 2006). The CD45RA+CCR7+ populations observed here might be indicative of an early antigen-specific population that has not yet lost CD45RA expression.

Distinct differences in phenotypes were observed amongst CD4+ IFN-γ and IL-2 expressing cells. IL-2 expressing cells were significantly more likely to express CCR7 compared with IFN-γ expressing cells. Therefore, a subset of IL-2 expressing T cells induced by BCG vaccination follows a pattern similar

to that previously described for purified human T_{CM} populations, which are likely to express IL-2 (Sallusto et al., 1999). T_{CM} persist *in vivo* in the absence of antigen, conferring long-term protection to the host (Sallusto et al., 1999; Sallusto et al., 2004; Zaph et al., 2004b). In the experimental *L. major* model of infection, T_{CM} (defined by CD62L expression) conferred protection by proliferating and differentiating into effector CD4+ T cells capable of IFN-γ production (Zaph et al., 2004b). Like *M. tb*, *L. major* is an intracellular pathogen and protection against *L. major* infection is associated with the development of a Th1 cytokine response (Sacks and Noben-Trauth, 2002). The IL-2 expressing T_{CM} subset observed following BCG vaccination of infants may confer long-term protection by maintaining memory cells capable of differentiating into Th1 effector cells upon antigen encounter (Zaph et al., 2004a; Bouneaud et al., 2005).

Collectively, these data indicate that BCG vaccination of newborns elicits polyfunctional Th1 cells, which predominantly express a T_{EM} phenotype. BCG vaccination also induces low level expression of type 2 cytokines and IL-10, that may play an immuno-regulatory role. The kinetics of BCG-specific T cell responses after vaccination of infants remains to be determined.

Chapter 5

Longitudinal characterisation of CD4+ and CD8+ T cells directly *ex vivo*.

5.1. Introduction

Characterising functional and phenotypic changes in T cells over the first year of life is important for understanding factors that influence the development of the immune system during infancy. T cells develop in the thymus from common hematopoietic lymphoid progenitors that migrate from the bone marrow. The main goals of lymphopoiesis are to generate a T cell repertoire with broad TCR diversity and to generate a sufficiently large enough clonal pool size of naïve T cells with a particular specificity. In the thymus naïve T cell diversity is established through numerous re-arrangements of gene segments encoding the variable portions of the TCR α and β chains (Shortman and Wu, 1996). Once naïve T cells exit the thymus, clonal population sizes are established through post-thymic proliferation (Hassan and Reen, 2001). Survival and maintenance of a stable peripheral T cell population is maintained through homeostatic proliferation driven by common γc cytokines, IL-7 and IL-15 (Lantz et al., 2000; Geginat et al., 2001; Bradley LM 2005; Fry and Mackall 2005).

Naïve T cells undergo functional and phenotypic changes, which are driven by numerous encounters with foreign antigens. Consequently, a progressive shift in the composition of the peripheral T cell pool from a naïve phenotype towards an antigen-experienced phenotype is observed from infancy to adulthood (Tsegaye et al., 2003; Saule et al., 2006; van Gent et al., 2009). In

addition, antigen exposure drives *in vivo* turnover of T cells which can be monitored by methodologies including Ki67 expression by T cells directly *ex vivo* or using *in vivo* DNA labels such as deuterated glucose or water. (Sachsenberg et al., 1998; Hellerstein et al., 1999; Miller et al., 2008; van Gent et al., 2009).

Age-related changes in memory T cell distribution has been demonstrated in a number of longitudinal studies analysing a large number of surface molecules including CD27, CD28, CCR7 and CD45RO expression (Tsegaye et al., 2003; Saule et al., 2006; van Gent et al., 2009). In this chapter, maturation of T cells over the first year of life was determined by assessing CD27 and CD28 expression. According to this model of differentiation, early differentiated antigen-experienced and naïve T cells defined by CD27 and CD28 co-expression, progress through an intermediate differentiation stage associated with the loss of CD27 on CD4+ T cells and CD28 on CD8+ T cells. Fully differentiated T cells lack expression of both CD27 and CD28 (Appay et al., 2002a; Kovaiou et al., 2005). Numerous studies have shown an association between distinct stages of T cell differentiation and functional capacity, such as proliferation and the expression of effector molecules. For example, T cells that express a highly differentiated or mature phenotype express cytotoxic molecules but show diminished proliferative capacity (Appay et al., 2002a; Appay et al., 2002c; Tomiyama et al., 2004). Given that cytotoxic molecules are stored in pre-formed granules within antigen-experienced T cells, these molecules could be analysed directly *ex vivo* without activating T cells with specific antigens (Appay et al., 2002c).

The objective of this chapter was to explore longitudinal changes in T cell turn

over, expression of differentiation markers and expression of cytotoxic molecules directly *ex vivo* over the first year of life.

5.2. Aims

Neonatal T cells predominantly express a naïve phenotype, however homeostatic mechanisms required for T cell survival and exposure of infants to foreign antigens contributes to functional and phenotypic changes within the peripheral T cell compartment. Characterising these changes over the first year of life, when infants are continuously exposed to foreign antigens through vaccination and environmental exposure, is important for understanding the development of the immune system. To assess longitudinal changes within the whole T cell compartment, the following aims were addressed:

1. Determine *ex vivo* T cell turnover kinetics over the first year of life.
2. Characterise changes in T cell cytotoxic molecule expression directly *ex vivo* during the first year of life.
3. Monitor longitudinal changes in T cell differentiation stage.

5.3. Materials and methods

5.3.1. Study participants and phlebotomy

The infants studied in this chapter were selected from the longitudinal cohort described in chapter 2, for which direct *ex vivo* samples had been collected. Direct *ex vivo* assessment of T cells was completed on samples collected from 47 infants. Cross-sectional and longitudinal analyses were done.

5.3.2. Sample processing and antibodies

Fresh whole blood was lysed and fixed with FACS lysing solution directly *ex vivo*, cryo-preserved and stained with the optimised "Cytotoxic Panel" shown in chapter 3 using the "one step staining method" described in chapter 2 (section 2.5.1.). Samples for all time points from each individual were stained and acquired in a single batch to reduce variability.

5.3.3. Data analysis

T cells were identified using a SSC vs. CD3 dotplot. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. Samples collected for the longitudinal study were cryo-preserved for lengthy periods of time (>1 year for some samples), which may result in the accumulation of cellular debris. Debris or dead cells tend to auto-fluoresce in the 490nm-560nm range and are identifiable as a diagonal line between the FITC and PE channel. When necessary, dead cells/debris were identified and excluded by gating using a Ki67-PE vs. granulysin-Alexa Fluor 488 dotplot. CD4+ and CD4- T cells were identified using a CD4 vs. Ki67 dotplot. CD8+ T cells were selected from the CD4-negative T cell population using a CD4 vs.

GrmB dotplot. A skewed gate was used to select CD8+ T cells due to data spread between the APC-H7 and Alexa Fluor 700 channels (**Figure 1A**). Boolean gating analysis was carried out once positive gates were established for functional parameters (**Figure 1B and C**). Single-stained mouse κ beads were used to calculate compensations for every run. Data were analysed with FlowJo software v 9.0.1, Pestle v 1.6.2 and Spice v 5.1 software (provided by M. Roederer, National Institutes of Health, Bethesda, MD). Statistical analyses were performed using GraphPad Prism v 4.0.

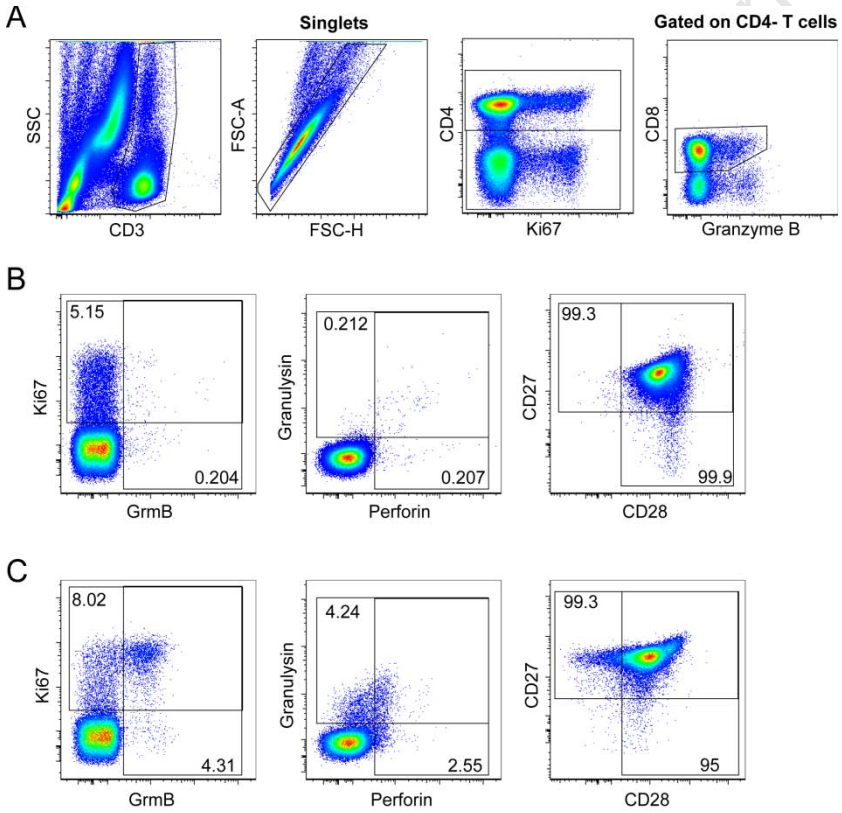

Figure 1. Flow cytometric analysis of T cell cytotoxic molecule expression and differentiation phenotypes. (**A**) Gating strategy from a representative 6-week old infant. Representative dotplots showing boolean analysis of Ki67, GrmB, granulysin, perforin and differentiation markers CD27 and CD28 on (**B**) CD4+ and (**C**) CD8+ T cells. Fresh whole blood was lysed and stained directly *ex vivo*.

5.4. Results

5.4.1. Longitudinal analysis of CD4+ and CD8+ T cell turnover

In vivo CD4+ and CD8+ T cell turnover was investigated by measuring the expression of Ki67 by T cells directly *ex vivo*. Cross-sectional analysis showed that Ki67 expression by CD4+ T cell peaked at 6 weeks of age (median, 6.85%) and remained at a relatively steady plateau over the first year of life (p=0.2463, Kruskal-Wallis; **Figure 2A and B**). The turnover kinetics and magnitude of CD8+ T cells differed to that observed for CD4+ T cells. At 3 weeks after vaccination, Ki67 expression on CD8+ T cells was similar to that observed in CD4+ T cells, but at all other time points more CD8+ T cells expressed Ki67 than CD4+ T cells. The frequency of Ki67+CD8+ T cells was higher at 6 weeks of age and thereafter compared with 3 weeks. The frequency of Ki67+CD8+ T cells peaked at 14 weeks (median, 16.06%) and thereafter gradually declined with age (**Figure 2B and C**). To corroborate the results obtained by cross-sectional analysis, results from individual infants were investigate longitudinally. Consistent with cross-sectional observations, longitudinal analysis of Ki67 expression in CD4+ and CD8+ T cells showed that a subset of T cells consistently express Ki67 over the first year of life and confirmed that the turnover kinetics of CD8+ T cells differed to that observed for CD4+ T cells (**Figure 2D and E**)

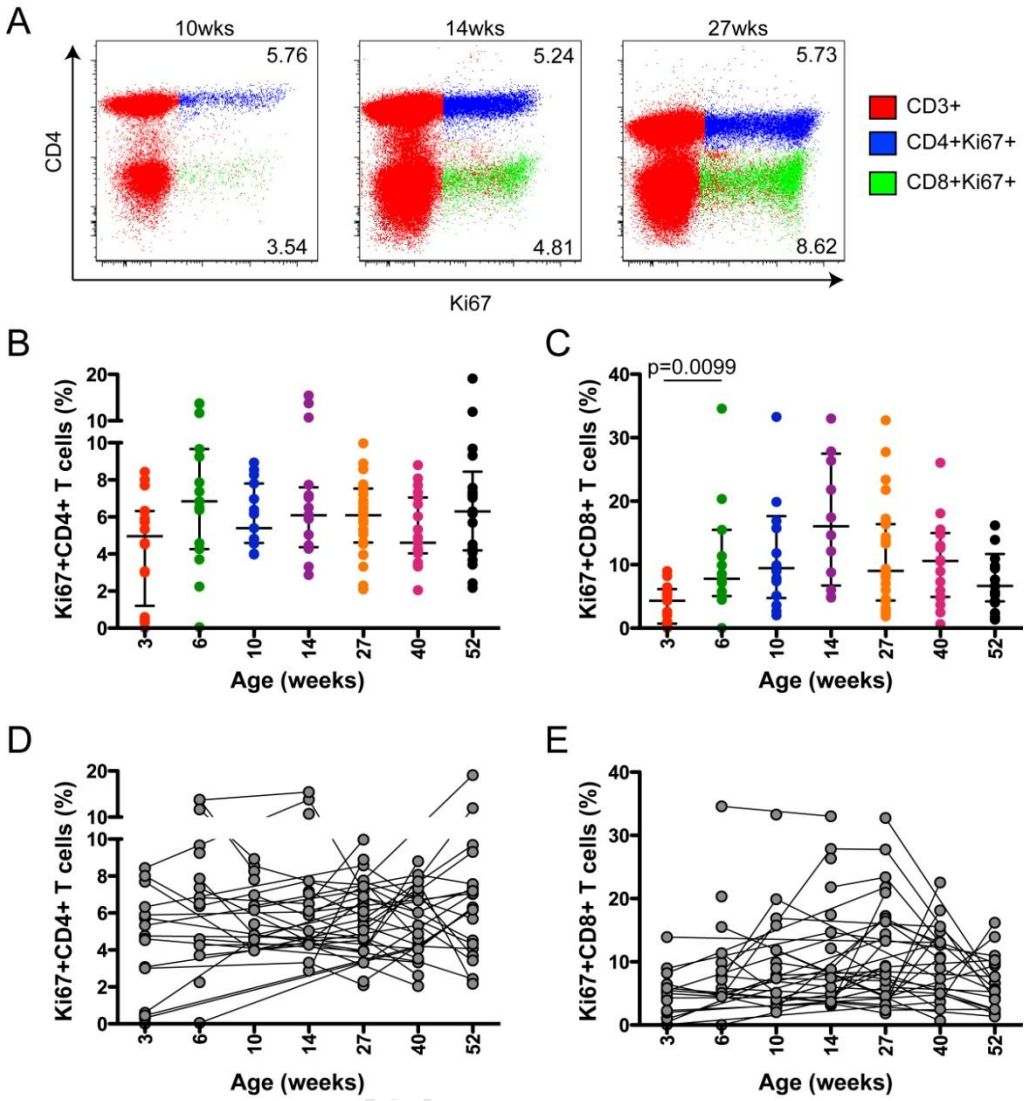

Figure 2. T cell turnover over the first year of life. (**A**) Representative dotplots showing longitudinal changes in the frequency of CD4+ (blue; foreground) and CD8+ (green; foreground) T cells expressing Ki67 in whole blood directly *ex vivo*. Dotplots are gated on CD3+ lymphocytes (red; background). This example is from a representative BCG-vaccinated infant at 10 weeks (wks), 14wks and 27wks of age. Values in each dotplot represent the frequency of Ki67+ T cells within the CD4+ (upper) and CD8+ (lower) T cell population. Cross-sectional analysis of Ki67 expression in (**B**) CD4+ and (**C**) CD8+ T cells. The horizontal line indicates the median and the whiskers the interquartile range. Differences were calculated using the Kruskal-Wallis test followed by a Mann Whitney U test. Longitudinal changes in Ki67 expression in (**D**) CD4+ and (**E**) CD8+ T cells for individual infants. Each line represents a different infant. Statistical analyses were not performed on longitudinal data. Please refer to the rationale at the end of the discussion section of this chapter.

These findings show that T cells remain in a constant state of *in vivo* proliferation throughout the first year of life and that turnover kinetics differ between CD4+ and CD8+ T cell.

5.4.2. Longitudinal analysis of T cell cytotoxic molecule expression directly ex vivo

Expression of cytotoxic molecules GrmB, granulysin and perforin was assessed cross-sectionally in CD4+ and CD8+ T cells. At 3 weeks of age both CD4+ and CD8+ T cells expressed low levels of all 3 cytotoxic molecules (**Figure 3A-D**). A gradual increase in the frequency of CD4+ T cells expressing GrmB, granulysin and perforin was observed from 3 to 52 weeks of age (**Figure 3A and C**). Frequencies of GrmB expressing CD4+ T cells were significantly higher at 14 weeks of age and all other time points thereafter compared to 3 weeks. Frequencies of perforin and granulysin expressing CD4+ T cells were significantly higher at 27 weeks of age and thereafter compared to 3 weeks (**Figure 3C**).

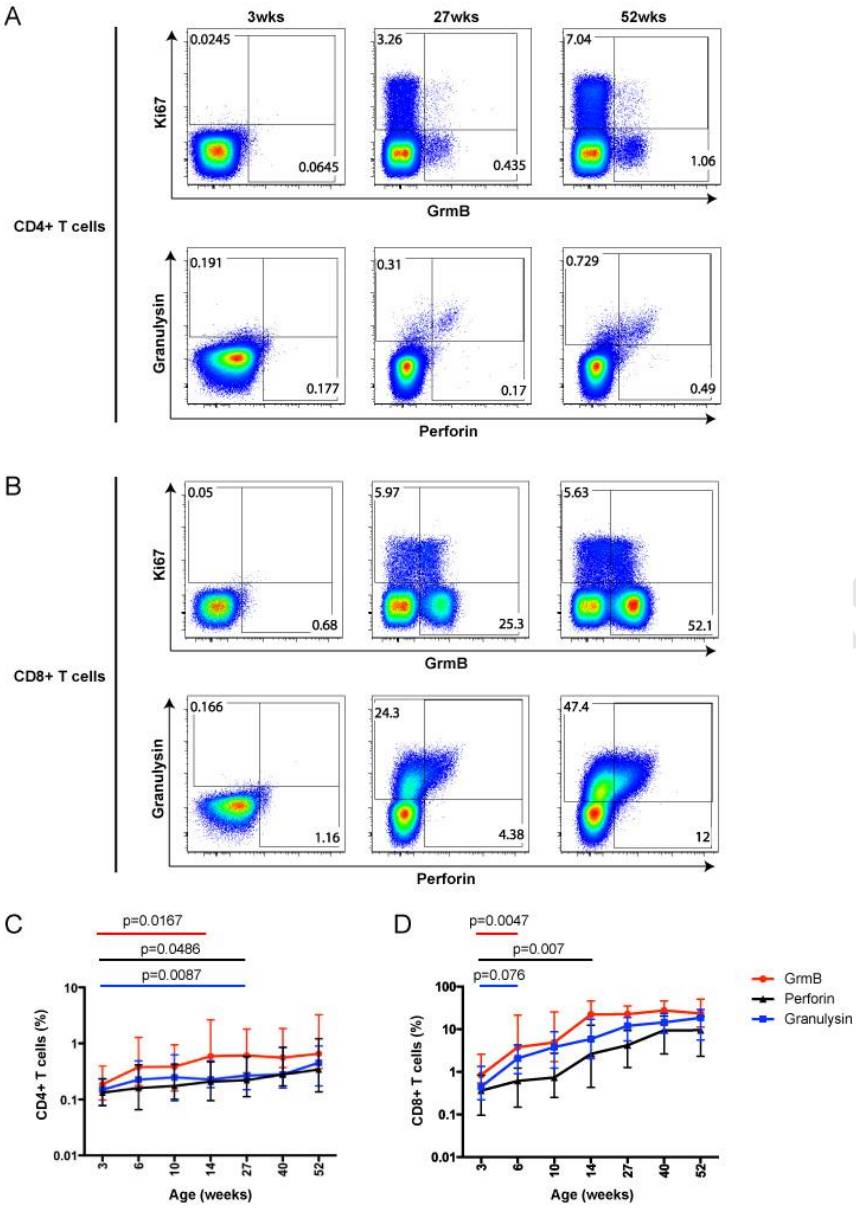

Figure 3. Cross-sectional analysis of T cell cytotoxic molecule expression directly *ex vivo*. Representative dotplots showing longitudinal changes in Ki67, GrmB, granulysin and perforin expression in (**A**) CD4+ and (**B**) CD8+ T cells. Whole blood was analysed directly *ex vivo* following lysis of red blood cells. Dotplots are from a representative BCG-vaccinated infant at 3 weeks (wks), 27wks and 52wks of age. Values in each boolean gate represent the frequency of CD4+ or CD8+ T cells expressing Ki67 or cytotoxic molecules. Kinetic changes in GrmB, granulysin and perforin expression in (**C**) CD4+ and (**D**) CD8+ T cells. The horizontal line indicates the median and the whiskers the interquartile range. Differences were calculated using the Kruskal-Wallis test followed by the Mann Whitney U test.

An increase in the frequency of CD8+ T cells expressing cytotoxic molecules was also observed over time (**Figure 3B and D**). This increased expression was more pronounced than that observed for CD4+ T cells and the median frequency of CD8+ T cells expressing cytotoxic molecules was higher at all time points compared with CD4+ T cells (**Figure 3A-D**). Frequencies of GrmB and granulysin expressing CD8+ T cells were significantly higher at 6 weeks of age and thereafter compared with 3 weeks, perforin expressing CD8+ T cells were significantly higher at 14 weeks of age and thereafter (**Figure 3D**). Longitudinal analysis showed a continuous increase in the frequency of CD4+ and CD8+ T cells expressing cytotoxic molecules over the first year of life and thus supported results obtained by cross-sectional analysis (**Figure 4A and B**).

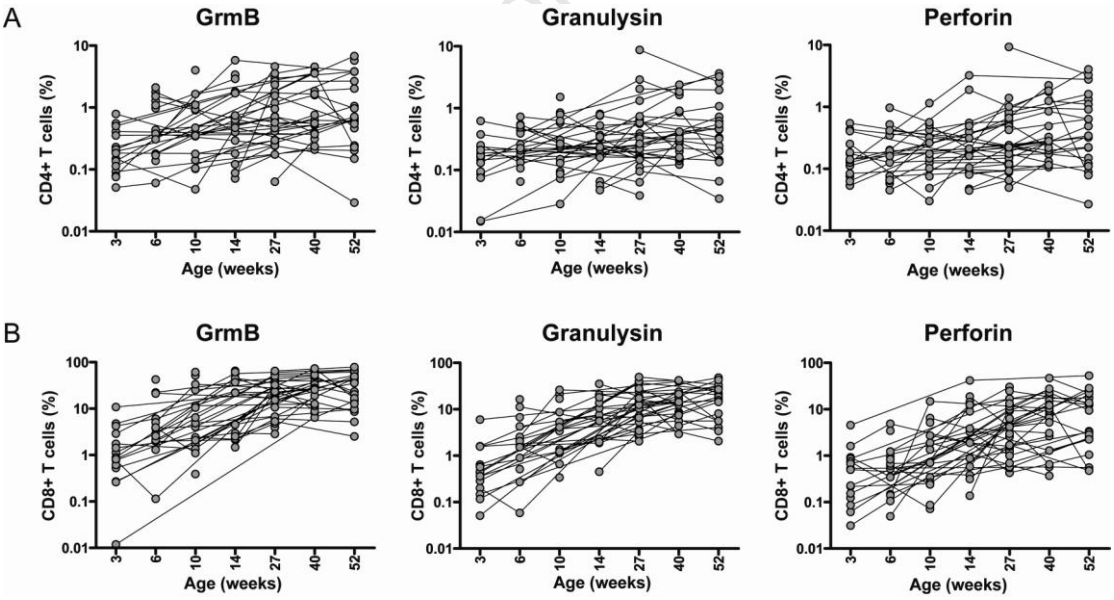

Figure 4. Longitudinal changes in T cell cytotoxic molecule expression directly *ex vivo*. Longitudinal changes in GrmB, granulysin and perforin expression in (**A**) CD4+ and (**B**) CD8+ T cells for individual infants. Each line represents a different infant. Statistical analyses were not performed on these data. Please refer to the rationale at the end of the discussion section of this chapter.

These data show that the frequencies of CD4+ and CD8+ T cells expressing cytotoxic molecules increases with age.

5.4.3. Qualitative changes in the total T cell compartment and in Ki67+ T cells

To determine changes in the quality of the T cell response, the proportions of T cells expressing cytotoxic molecules was monitored over time. Cross-sectional analysis showed that at 3 weeks, <10% of CD4+ T cells within the total CD4+ T cells compartment expressed cytotoxic molecules or Ki67 and remained low over the first year of life (**Figure 5A**). A similar trend was observed for Ki67+CD4+ T cells: only a small fraction of cycling CD4+ T cell expressing cytotoxic molecules. The majority of Ki67+CD4+ T cells expressed GrmB. (**Figure 5B**) By contrast, the proportion of CD8+ T cells expressing cytotoxic molecules within the total CD8+ T cell compartment gradually increased over the first year of life (**Figure 5C**). At 52 weeks, >25% of CD8+ T cells co-expressed three (3+) or (2+) cytotoxic molecules. The majority of CD8+ T cells expressed GrmB and the proportion of T cells expressing perforin or granulysin was similar. Perforin was always co-expressed with GrmB and/or granulysin but not with Ki67 (**Figure 5C**).

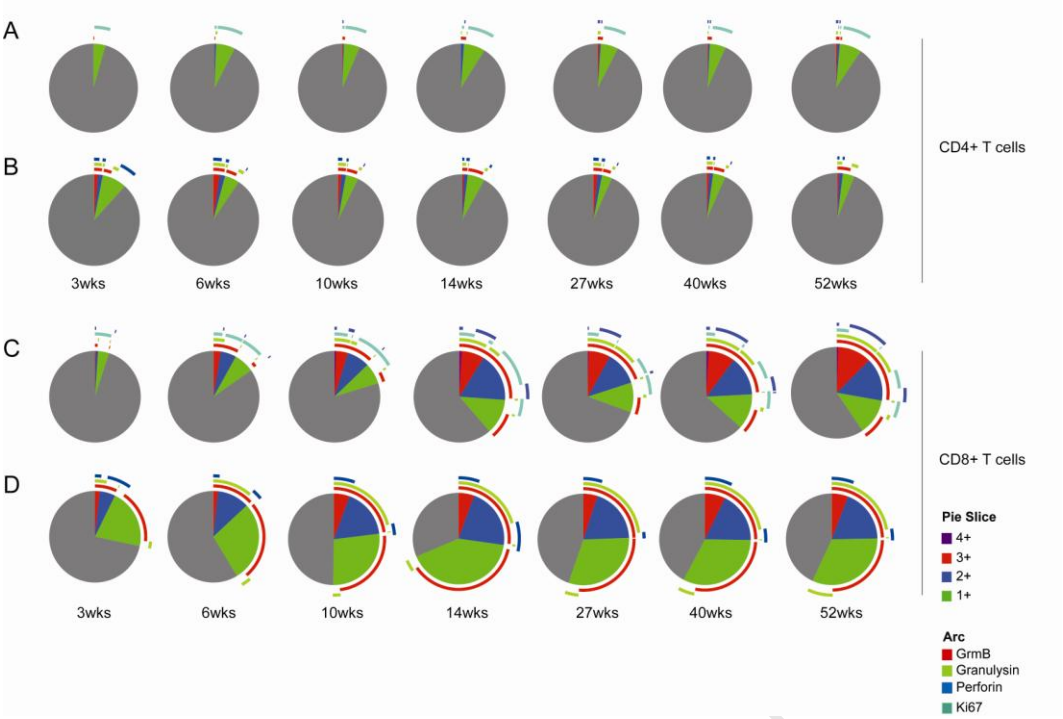

Figure 5. Changes in the proportion of T cells expressing cytotoxic molecules over the first year of life. Pie charts representing the proportions of T cells expressing a given number of markers within (**A**) the total CD4+ T cell compartment, (**B**) Ki67+CD4+ T cells, (**C**) total CD8+ T cells compartment and (**D**) Ki67+CD8+ T cells. Responses are grouped and colour-coded according to the number of markers expressed. The contribution of each marker to each pie slice is depicted by the outer, colour-coded arcs.

A similar trend was observed for Ki67+CD8+ T cells. The proportion of cycling CD8+ T cells expressing cytotoxic molecules progressively increased over the follow-up period (**Figure 5D**). At 52 weeks >50% of Ki67+CD8+ T cells expressed cytotoxic molecules. The majority of Ki67+CD8+ T cells expressed GrmB and/or granulysin (**Figure 5D**). Longitudinal data were consistent with results obtained by cross-sectional analysis (data no shown).

These data show that cytotoxic granule expression is predominantly restricted to CD8+ T cells and that the proportion of CD8+ T cells that express cytotoxic molecules progressively increases over time.

5.4.4. Changes in T cell differentiation phenotype

Differentiation stage of CD4+ and CD8+ T cells was defined according to the expression of CD27 and CD28. CD27+CD28+ (early differentiation stage) T cells were the pre-dominant CD4+ T cell subset in infants between 3 (median, 93.05%) and 6 weeks (median, 69.23%) of age (**Figure 6A and B**). From 10 weeks of age, most CD4+ T cells expressed an intermediate phenotype (CD27-CD28+; median, 88.56%) and retained this phenotype during the entire follow-up period (**Figure 6A**). Changes in the differentiation phenotype of CD4+ T cells undergoing cycling (Ki67+) were similar to those observed for the total T cell compartment (**Figure 6C and D**).

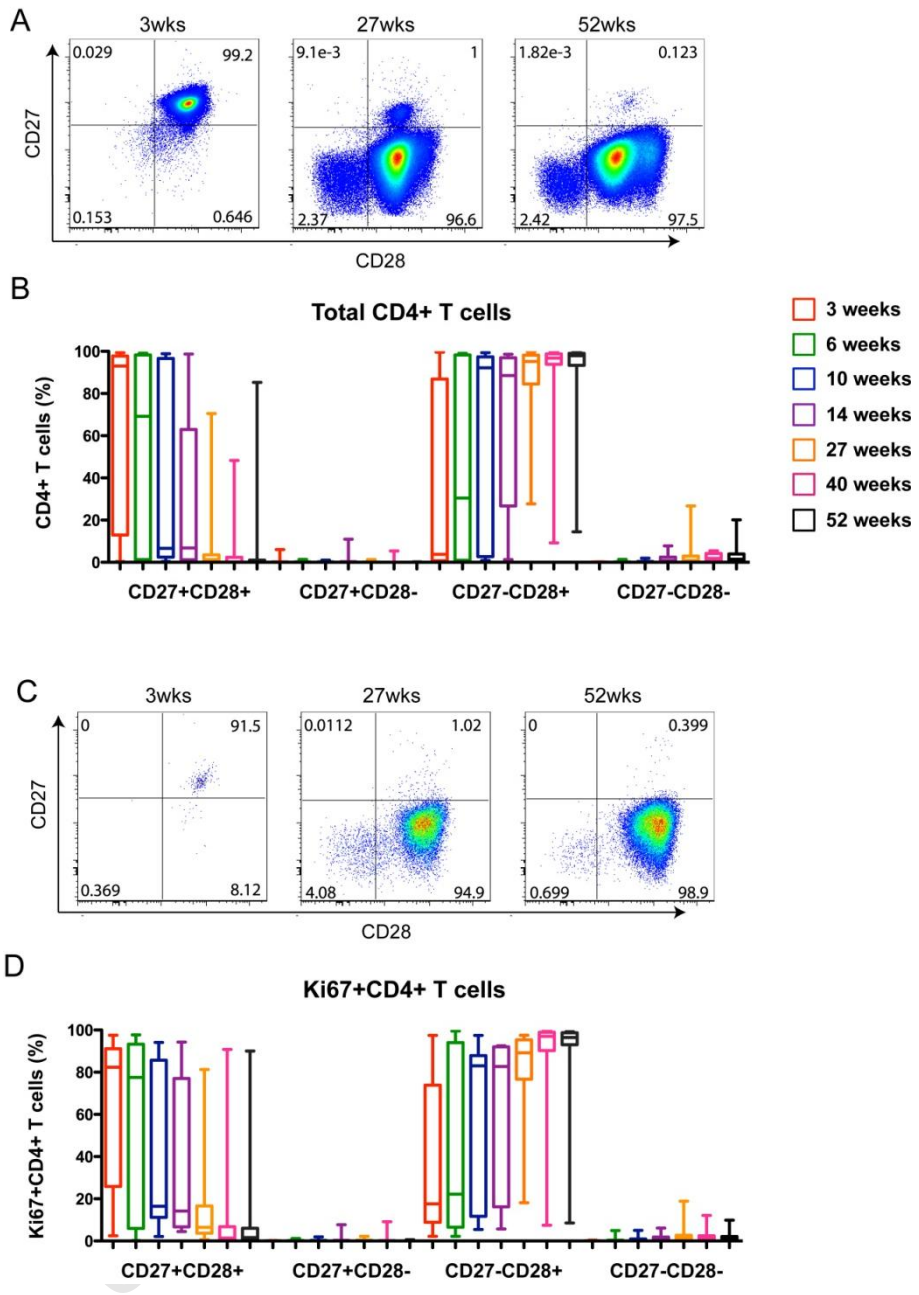

Figure 6. Changes in CD4+ T cell differentiation phenotype. Longitudinal changes in the differentiation phenotypes of CD4+ T cells based on CD27 and CD28 expression within the (**A**) total CD4+ T cell compartment and for (**C**) Ki67+CD4+ T cells. Dotplots are from a representative BCG-vaccinated infant at 3 weeks (wks), 27wks and 52wks of age. Changes in differentiation phenotypes of T cells within the (**B**) total CD4+ T cell compartment and (**D**) Ki67+CD4+ T cells. Whole blood was analysed directly *ex vivo* following lysis of red blood cells The horizontal line indicates the median, box the interquartile range and the whiskers the range.

Differentiation kinetics of CD8 T cells was comparable to that observed for CD4+ T cells. Among CD8+ T cells, CD27+CD28+ T cells were predominant between 3 (median, 87.91%) and 6 weeks (median, 78.65%) of age (**Figure 7A and B**). In contrast to CD4+ T cells, which predominantly retained CD28 expression over the follow-up period, a subset of CD8+ T cells progressively lost expression of both CD27 and CD28. By 52 weeks of age virtually all CD8+ T cells expressed an intermediate (CD27-CD28+; median, 70.84%) or late (CD27-CD28-; median, 28.90%) differentiation phenotype (**Figure 7B**). Changes in the differentiation phenotype of CD8+ T cells undergoing cycling (Ki67+) were similar to those observed for the total T cell compartment (**Figure 7C and D**). Longitudinal data were consistent with results obtained by cross-sectional analysis (data no shown).

These data show that the proportions of circulating CD4+ and CD8+ T cells with an intermediate and late differentiation phenotype appear at 10 weeks of age and progressively increase throughout the first year of life

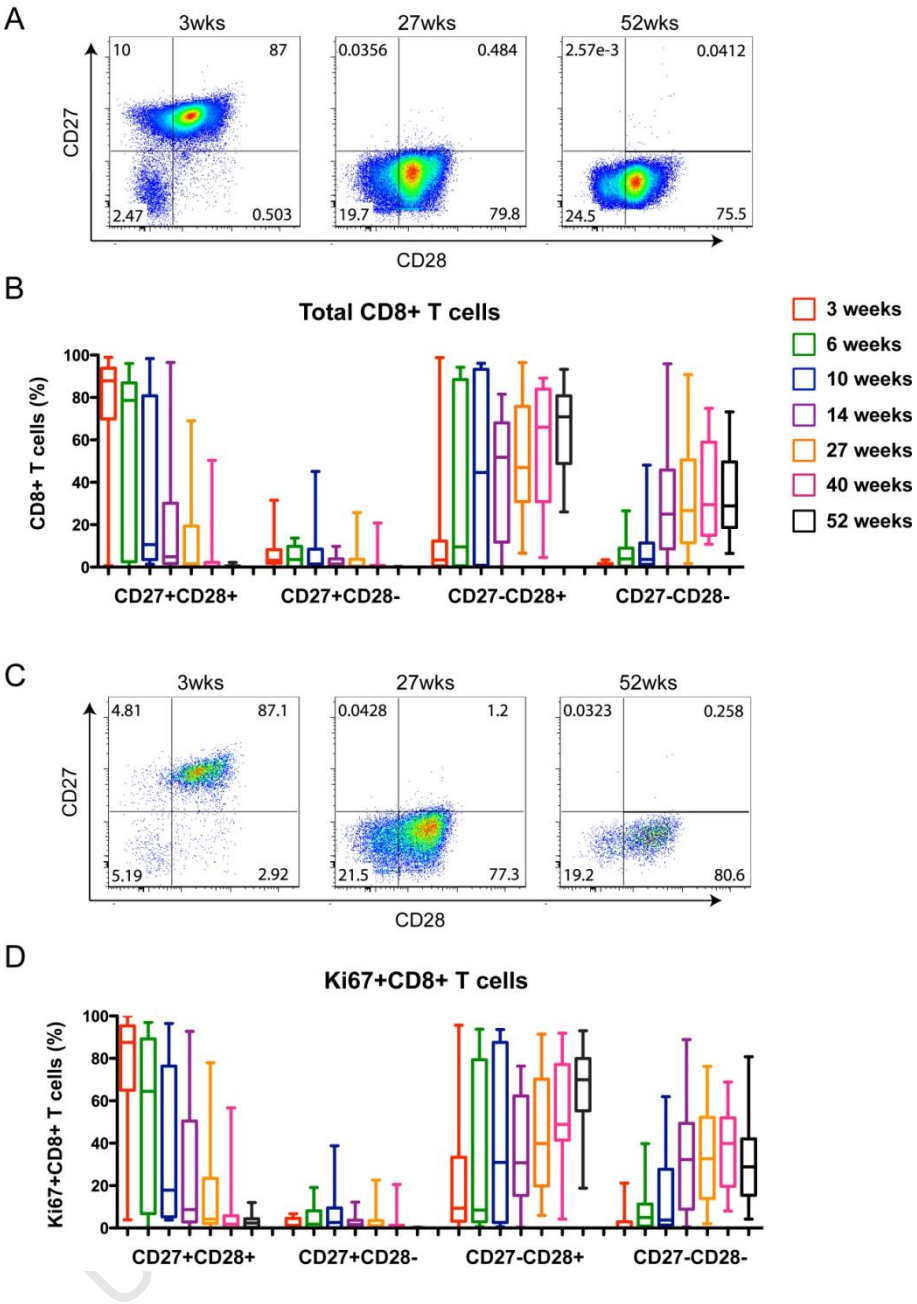

Figure 7. Changes in CD8+ T cell differentiation phenotype. (**A**) Longitudinal changes in the differentiation phenotypes of CD8+ T cells based on CD27 and CD28 expression within the (**A**) total CD8+ T cell compartment and for (**C**) Ki67+CD8+ T cells. Dotplots are from a representative BCG-vaccinated infant at 3 weeks (wks), 27wks and 52wks of age. Changes in differentiation phenotypes of T cells within the (**B**) total CD8+ T cell compartment and (**D**) Ki67+CD8+ T cells. Whole blood was analysed directly *ex vivo* following lysis of red blood cells The horizontal line indicates the median, box the interquartile range and the whiskers the range.

5.5. Discussion

During the first year of life the neonatal immune system is continuously exposed to foreign antigens, both pathogenic and benign, resulting in marked alterations of the T cell compartment. Studies in infants are important for assessing the modulatory effects of environmental exposure, infection and/or vaccination on T cell function and phenotype.

In this chapter, *in vivo* T cell turnover was examined by assessing the expression of the nuclear antigen, Ki67, which is expressed by all cycling T cells (Gerdes et al., 1984). The data presented here show that T cells remain in a constant state of turnover over the first year of life, which may result from the high demand for lymphopoiesis in infants. The generation of naïve T cells in infants is driven by two mechanisms, thymic output and peripheral T cell turnover (Schonland et al., 2003; Hazenberg et al., 2004; van Gent et al., 2009). Thymopoiesis is required for the generation of a diverse set of naïve T cells, while peripheral turnover is required for generation of sufficiently large clonal pools of a particular specificity. The contribution of the thymus versus homeostatic mechanisms in the establishment of the naive T cell pool can be determined by measuring T-cell receptor excision circles (TRECS) (Hazenberg et al., 2004; Naylor et al., 2005). TRECS are episomal DNA by-products generated through cleavage of DNA fragments during TCR α-chain rearrangement and are typically used as a marker of recent thymic emigrants (Hazenberg et al., 2000). Since TRECS are not replicated during cellular division, the frequency of naïve T cells in the periphery generated either in the thymus or through homeostatic proliferation can be determined (Al-Harthi et al., 2000). Although thymic generation of naive T cells is highest during

infancy, previous studies in young children have shown that TREC numbers remain relatively stable despite increasing numbers of naïve T cells. This data shows that post-thymic homeostatic proliferation contributes significantly to the establishment and maintenance of the naïve T cell pool in children (Schonland et al., 2003; Hazenberg et al., 2004; van Gent et al., 2009).

In addition, neonatal T cells are highly sensitive to common γc cytokines required for homeostatic proliferation, relative to adult T cells. In the absence of TCR triggering, naïve CD4+ and CD8+ T cells isolated from cord blood have greater proliferative capacity in response to IL-7 and IL-15, compared with adult T cells (Schonland et al., 2003). However, it is important to note that the high concentrations of common γc cytokines used in *in vitro* studies may not represent *in vivo* physiological levels. Nevertheless, neonatal naïve T cells express high levels of the IL-7R and higher levels of IL-7 are detected in serum compared with adults (Bolotin et al., 1999). Therefore the high demand for T cell generation and the intrinsic sensitivity of neonatal T cells to common γc cytokines potentially accounts for the fraction of Ki67+ T cells observed throughout the first year of life.

In addition to homeostatic mechanisms, exposure of naïve T cells to antigens through vaccination, infection and/or the environment are thought to contribute markedly to *in vivo* proliferation. *Ex vivo* levels of Ki67 expression in T cells increase transiently following immunisation of adults with vaccines that are effectively cleared (Cellerai et al., 2007; Miller et al., 2008). During chronic viral infections such as HIV, Ki67 is also elevated compared to healthy controls (Sachsenberg et al., 1998). For example, in healthy adults mean Ki67 expression by CD4+ T cells is 1.1% and for CD8+ T cells 1%. However,

during HIV infection a mean 6.5% of CD4+ T cells and 4.3% of CD8+ T cells express Ki67 (Sachsenberg et al., 1998). Infants with known chronic medical conditions were excluded from this study; however, we cannot exclude all possible co-variants. For example, mothers and infants were not screened for CMV infection. Previous studies in both young and elderly subjects have shown that CMV-specific T cells do not continuously cycle or show increased proliferative capacity *in vivo* (Wallace et al., 2010). Therefore, CMV infection would not explain the high Ki67 expression observed over the first year of life. Instead, the 6 scheduled EPI vaccines administered at 6, 10 and 14 weeks of age are likely to contribute significantly to the continuous turnover of T cells in an antigen-dependent manner. Alternatively, high Ki67 expression may be a consequence of other vaccine-related factors such as inflammation. Interestingly, Ki67 expression revealed different *in vivo* turnover kinetics for CD4+ and CD8+ T cells, suggesting that homeostatic or antigen driven turnover of these two subsets may be different. This is supported by studies showing that CD8+ T cells express higher levels of Ki67 than CD4+ T cells in early childhood and that accumulation of highly differentiated CD8+ T cells, but not CD4+ T cells, is observed in the elderly (Schonland et al., 2003; Saule et al., 2006; van Gent et al., 2009).

An increase in the frequency of both CD4+ and CD8+ T cells to express cytotoxic molecules was also observed over time. Although the frequency of CD4+ T cells expressing cytotoxic molecules increased with age, qualitative analysis revealed that the majority of CD4+ T cells do not express cytotoxic molecules. By contrast, a large proportion of CD8+ T cells within the total T cell compartment and the majority of cycling Ki67+CD8+ T cells expressed

cytotoxic molecules at 52 weeks. These data suggest that cytotoxic molecule expression is a dominant feature of CD8+ T cells and that CD4+ T cells are likely to mediate their effector functions through the expression of cytokines or other effector molecules not measured in this study.

The expression of cytotoxic molecules such as perforin and Grm has been shown to be predominantly restricted to CD4+ and CD8+ T cells with an effector or effector memory phenotype, whereas naïve T cells do not express these molecules (Appay et al., 2002a; Appay et al., 2002c; Takata and Takiguchi 2006). Therefore, the gradual increase in the frequency of T cells expressing cytotoxic molecules is most likely associated with an increase in the proportion of differentiated or antigen-experienced T cells over time. This observation is supported by the analysis of CD27 and CD28 expression, which showed an increase in T cells expressing intermediate and late differentiation phenotypes over time. The loss of CD27 and CD28 expression has been associated with the capacity of T cells to express cytotoxic molecules (Appay et al., 2002a; Appay et al., 2002c; Tomiyama et al., 2004; Takata and Takiguchi 2006; Makedonas et al., 2010).

Almost all T cells in the newborn have a naïve phenotype (Schonland et al., 2003; van Gent et al., 2009) and co-express CD27 and CD28 (Hamann et al., 1997; McFarland et al., 2000; van Gent et al., 2009). However a progressive loss of both molecules is observed from childhood to adulthood as T cells become more differentiated (Tsegaye et al., 2003; Saule et al., 2006; van Gent et al., 2009). In addition to TCR triggering, proliferation of T cells induced through homeostatic cytokines further contributes to T cell differentiation. For example, naïve CD8+ T cells down-regulate CD28 and

express a CD45RO+ memory phenotype after several rounds of antigen-independent replication induced by IL-15 (Kim et al., 2002; Schonland et al., 2003; Chiu et al., 2006). Importantly, neonatal CD4+ and CD8+ T cells retain a naïve CD45RO- phenotype following IL-7 dependent proliferation. Since IL-15 preferentially drives the proliferation of CD8+ T cells, the contribution of homeostatic turnover to CD4+ T cell differentiation may not be significant.

Despite the contribution of these mechanisms to T cell differentiation, one of the surprising findings of this study was the high proportion of T cells expressing an intermediate or late differentiation phenotype after 6 weeks of age. An accumulation of highly differentiated T cells has been observed in the elderly or under conditions of chronic infection and inflammation (Marchant et al., 2003; Tsegaye et al., 2003; Saule et al., 2006; Miles et al., 2008). Congenital CMV infection affects up to 3% of all infants born in developing countries and may have contributed to the differentiation of T cells observed in this study. Significantly higher proportions of both CD4+ and CD8+ T cells are observed in CMV-infected infants compared with healthy infants (Marchant et al., 2003; Miles et al., 2008). However, the EPI vaccines infants receive at 6 weeks of age is also likely to contribute significantly to the dramatic switch in T cell differentiation stage observed at 10 weeks of age. Booster vaccines administered at the scheduled EPI time points would further contribute to the progressive differentiation of T cells observed over the first year of life. Since T cells transiently down-regulate CD27 and CD28 after TCR triggering and in response to inflammatory and homeostatic cytokines, a proportion of T cells expressing this phenotype may represent a transient population rather than a stable population of highly differentiated cells (Bryl et

al., 2001; Kim et al., 2002; Ochsenbein et al., 2004; Chiu et al., 2006). Unfortunately the contribution of homeostatic cytokines and antigen-driven mechanisms to the differentiation of T cells could not be completely discerned in this study as CD27 and CD28 does not allow complete separation of naïve and memory T cells. In addition, the influence of vaccine antigens versus exposure of infants to environmental antigens on the functional and phenotypic differentiation of T cells could also not be discerned.

A statistical limitation was introduced into this study due to the small volumes of blood that could be safely drawn from infants over a specified period of time. As a result of this restriction, data was only collected at three to four of the seven scheduled time-points. Therefore we were unable to reliably apply traditional statistical methods to analyse longitudinal data due to loss of power and potential introduction of bias associated with missing data points. However, sample size determinations for each time point was such that reliable analysis of data could be completed both cross-sectionally (at each specific time point) and longitudinally for each infant while taking variability into account (chapter 2, section 2.7.2). More appropriate and advanced statistical methods which are unaffected by missing data, such as mixed effects models, will be carried out by a statistician at a later stage. Despite this statistical limitation, longitudinal trends were consistent with those obtained by cross-sectional analysis and the data presented here extends onto previously published findings, which report changes within the total T cell compartment in healthy children.

Chapter 6.

Novel application of Ki67 as a marker of antigen-specific *in vitro* lymphoproliferation.

6.1. Introduction

Proliferation and clonal expansion of antigen-specific T cells are critical functions for the mediation of protective immunity and immunological memory (Rosenberg et al., 1997). *In vitro* proliferation of antigen-specific human T cells is most commonly assessed by detecting incorporation of specific tags into actively dividing cells during long-term culture of whole blood or PBMC with antigen (Gratzner, 1982; Lyons and Parish, 1994; Mehta and Maino, 1997; Fulcher and Wong, 1999; Lyons, 2000; Magg and Albert, 2007). Measuring this important T cell function has numerous applications, including monitoring of vaccine immunogenicity (Davids et al., 2006; Beveridge et al., 2007; Miller et al., 2008), drug toxicity screening (Elattar and Virji, 2000; Hideshima et al., 2000) and exploring immune regulation in disease (Woo et al., 2002; Jagannathan et al., 2009; Sharma et al., 2009).

Until recently the standard method for detection of antigen-specific T cell proliferation was through incorporation of ^{3}H-thymidine during DNA replication of dividing cells (Payan et al., 1983; Marchant et al., 1999). This technique has largely been replaced by more powerful assays that do not require radioactive materials and rely on assessment of cell cycling by flow cytometry. Fluorescent dye dilution assays, utilising dyes such as 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) or its derivative, Oregon Green (OG) (Magg and Albert, 2007; Wallace et al., 2008; MacMillan et al., 2009), or the

use of the DNA intercalating agent, 5-bromo-2'-deoxyuridine (BrdU), detected by fluorochrome-conjugated antibody staining (Dolbeare et al., 1983; Houck and Loken, 1985; Rosato et al., 2001), are typical examples of these. These flow cytometry based proliferation assays also allow co-staining of other cellular markers, enabling cell sub-populations to be delineated by multiparameter analysis (Precopio et al., 2007). This includes immunophenotyping of antigen-responsive cells and assessment of other T cell functions following antigen stimulation (Lyons, 2000; Bachmann et al., 2005; Davids et al., 2006).

Ki67 is a nuclear protein that plays a role in the regulation of cell division and is used conventionally in cancer biology as an indicator of tumour cell proliferation (Gerdes, 1990; Scholzen and Gerdes, 2000). This protein is expressed during all active phases of cell division but is absent in quiescent cells and during DNA repair (Gerdes et al., 1984). Assessment of intracellular Ki67 expression directly *ex vivo* or after short-term cell culture has been utilised in a number of studies to monitor induction of specific T cell responses upon vaccination (Stubbe et al., 2006; Cellerai et al., 2007; Miller et al., 2008), or to measure T cell turnover in individuals with chronic viral infections, such as HIV (Sachsenberg et al., 1998; Doisne et al., 2004).

In this chapter, we determine whether antigen-specific lympho-proliferation, after long-term culture, can be identified through the analysis of Ki67 expression in T cells.

6.2. Aims

Proliferation of T cells if required to expand effector T cells of a particular specificity and is often used as a measure of T_{CM} cell responses following vaccination. Proliferation assays are often technically difficult and require the incorporation of dyes or DNA intercalating agents, which are toxic to cells. In this chapter, Ki67 was explored as a specific and quantitative indicator of *in vitro* T cell proliferation by establishing the following aims:

1. Determine the kinetics of Ki67 expression in T cells following long-term culture with antigen.
2. Compare Ki67 as an indicator of antigen-specific T cell proliferation with standard proliferation assays.
3. Compare the functional profiles of Ki67 expressing T cells with other markers of proliferation.
4. Longitudinally monitor vaccine-specific T cell proliferation with Ki67 expression.

6.3. Materials and methods

6.3.1. Study participants

Healthy adult donors were recruited as described in chapter 2 (section 2.1.1.) Healthy, 18 month old toddlers were recruited at SATVI clinic sites in the Western Cape, South Africa, before, and 11-13 days after their routine 18 month vaccination against TT. Enrolled toddlers had received all routine childhood vaccinations as set out by the WHO EPI. Heparinised venous blood from adults and toddlers was collected into BD Vacutainer CPT tubes (BD Biosciences) and immediately processed as outlined below. Blood was collected by qualified phlebotomists within the guidelines of written consent.

6.3.2. Whole blood BrdU incorporation assay

Whole blood (125µL diluted 1:10 in warm RPMI 1640) was incubated with antigens for 6 days at 37°C with 5% CO_2. Antigens were used at the following final concentrations: 1 x 10^5cfu/mL Danish BCG (Danish strain 1331; Statens Serum Institut), 1µg/mL TB10.4 protein (kindly provided by Tom Ottenhoff, Leiden University, Leiden, Netherlands), 2µg/mL *M.tb* PPD (Statens Serum Institut) and 0.16IU TT (Tetavax, Sanofi Pasteur). On day 6 (day 3 for PHA), 10µmol/L BrdU (Sigma-Aldrich) was added for the last 5 hours of culture. When intracellular cytokine expression was assessed, 10ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 1.5µg/mL ionomycin (Sigma-Aldrich) and 1.5µg/mL Brefeldin A were also added during the last 5 hours of culture. Control antigens included 1µg/mL PHA (positive control, Sigma-Aldrich), medium only (unstimulated, NIL, negative control) or, for intracellular cytokine assays, medium with PMA and ionomycin (NIL-PI). On day 6, cells

were harvested with 2mM EDTA and red blood cells lysed. White cells were stained with a viability dye (ViViD, LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen), fixed in BD FACS lysing solution according to manufacturer's instructions and cryopreserved until analysis.

6.3.3. PBMC isolation and the OG assay

PBMC were isolated by density gradient centrifugation and immediately stained with 10µg/mL of CellTrace Oregon Green 488 (Molecular Probes, Invitrogen) per $1X10^7$ cells and rested overnight at 37°C, 5% CO_2. Cells were either incubated with medium or stimulated with 1 x 10^5cfu/mL Danish BCG, 0.5µg/mL PPD, 1µg/mL TB10.4 protein or 0.05µg/mL SEB (positive control, Sigma-Aldrich), for 6 days at 37°C with 5% CO_2. On day 6 for some assays, PBMC were restimulated with 50ng/mL PMA, 250ng/mL ionomycin and 10µg/mL Brefeldin A for a further 5 hours. Finally, PBMC were stained with ViViD, fixed with BD FACS lysing solution and cryopreserved until analysis.

6.3.4. Antibodies

The following monoclonal antibodies were used for kinetic analysis of Ki67 expression by T cells:

CD3-QDot 605, CD4-PerCP, Ki67-PE.

The following monoclonal antibodies were used for phenotypic and/or intracellular cytokine staining:

CD3-QDot 605, CD8-PerCP-Cy5.5, Ki67-PE, IFN-γ-Alexa Fluor 700, TNF-α-PE-Cy7, IL-2-APC, anti-BrdU-FITC.

All antibodies were obtained from BD Biosciences except for CD3-QDot 605, which was from Invitrogen.

6.3.5. Data analysis

Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. Live T cells were selected using a ViVid versus CD3 parameters. In some experiments CD4+ T cells were gated as CD3+ CD8- lymphocytes, because PMA and ionomycin stimulation strongly down-regulates CD4 expression on T cells. Boolean gating analysis was carried out once positive gates were established for cytokine expression. Single-stained or unstained mouse κ beads were used to calculate compensations for every run. Data were analysed with FlowJo software v.8.8.6, Pestle v 1.6.2 and Spice v 4.3.2 software. Statistical analyses were calculated using GraphPad Prism v 4.0.

6.4. Results

6.4.1. Ki67 is a specific marker of in vitro lymphoproliferation

Ki67 is expressed by all cells undergoing cycling (Lopez et al., 1991; Scholzen and Gerdes, 2000). To investigate the kinetics of Ki67 expression in T cells cultured over 6 days, whole blood was either cultured in the absence of antigen (unstimulated, NIL), or in the presence of PPD or anti-CD3 and anti-CD28 (αCD3/αCD28). Expression of Ki67 was quantified each day. Ki67 expression was low in unstimulated CD4+ T cells on day 1 (24 hours, median, 0.62%), and by day 6, had decreased to <0.1% of CD4+ T cells (median, 0.08%, **Figure 1A**). PPD stimulation resulted in Ki67 expression levels above those in unstimulated cells between days 2 and 4; expression peaked on day 6 (**Figure 1A and B**). High expression of Ki67 following polyclonal T cell stimulation with αCD3/αCD28; Ki67 responses were high on day 1 already, peaked on day 3, and declined thereafter (**Figure 1A and C**).

Next, proliferation by Ki67 detection was assessed in whole blood from 15 healthy donors, after 6-day culture with no antigen (unstimulated, NIL), or with PPD. All donors had undetectable or very low frequencies of Ki67+CD4+ T cells in unstimulated blood (median, 0.07%). PPD stimulation resulted in higher frequencies of Ki67+CD4+ T cells in all donors (median, 46.1%, **Figure 1D**).

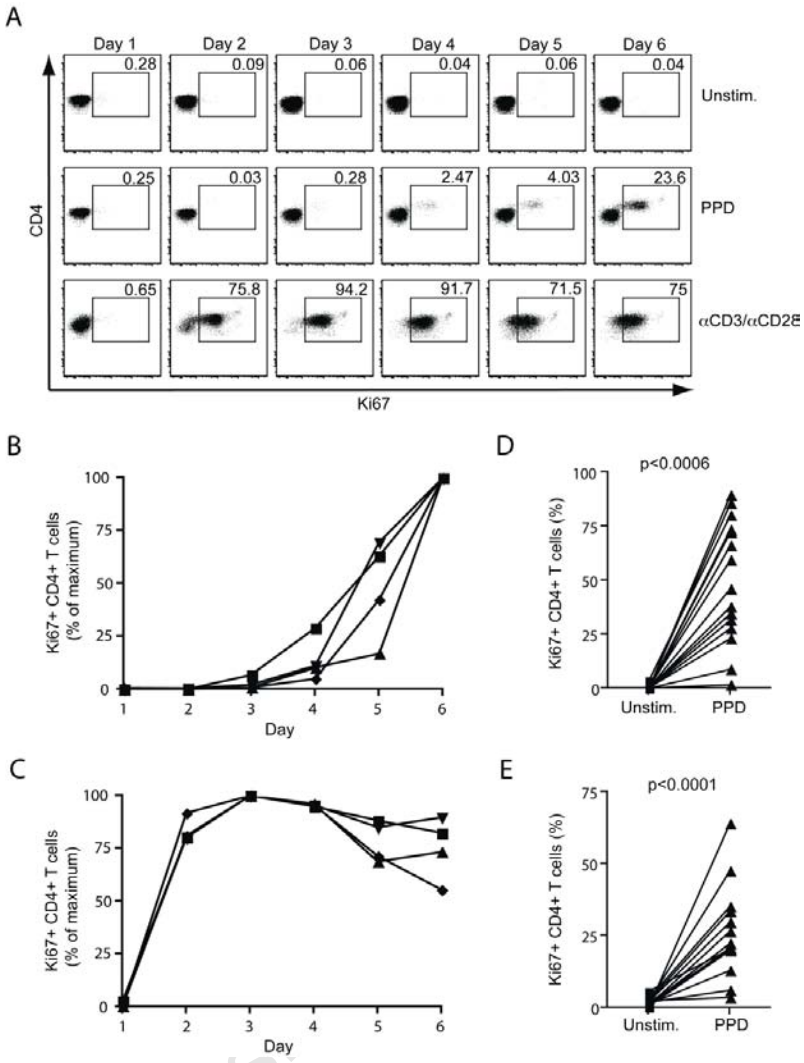

Figure 1. **Ki67 as a specific marker of *in vitro* lymphoproliferation.** Whole blood from healthy donors was incubated with the indicated antigens and Ki67 expression quantified on a daily basis over 6 days. (**A**) Representative example showing the frequencies of Ki67 expression by CD4+ T cells after incubation of whole blood with medium only (NIL), PPD or αCD3/αCD28 over 6 days. Dotplots were gated on live, CD3+ CD4+ lymphocytes. Ki67+ CD4+ T cell frequencies after (**B**) PPD stimulation or (**C**) αCD3/αCD28 stimulation in 4 donors. Data are expressed as a percentage of the maximum response. The frequency of Ki67+CD4+ T cells is indicated in each plot. (**D**) Frequencies of Ki67 expressing CD4+ T cells in whole blood from 15 donors after 6-day culture with medium only (NIL) or PPD. (**E**) Frequencies of Ki67+CD4+ T cells in PBMC from 14 donors. Differences were calculated using the Wilcoxon matched pairs test.

Ki67 expression was also assessed in PBMC. Again, Ki67 expression identified *in vitro* CD4+ T cell proliferation; frequencies of Ki67+ cells after PPD stimulation consistently exceeded those in unstimulated PBMC, at a median of 21.7% (**Figure 1E**).

These data suggest that in 6-day PBMC or whole blood culture with antigen, Ki67 expression is up-regulated in T cells undergoing *in vitro* proliferation.

6.4.2. Comparison of Ki67 expression with BrdU and OG assays

To determine how Ki67 performs in relation to traditional *in vitro* proliferation assays, detection of antigen-specific T cell proliferation by Ki67 expression in whole blood was compared with BrdU incorporation, and in PBMC with the dye dilution of OG (**Figure 2**).

BrdU is typically added during the last 2 to 24 hours of a proliferation assay; in this study BrdU was added for the last 5 hours of the 6-day culture. This marker is thus only incorporated into cells undergoing DNA synthesis at this stage of the culture period. Compared with the frequency of BrdU+ cells the frequency of Ki67+CD4+ T cells was significantly higher after whole blood stimulation with PPD or TB10.4 protein (**Figure 2A, B and C**). Importantly, all BrdU+ cells co-expressed Ki67, while Ki67 consistently identified a prominent population of proliferating, but BrdU-negative CD4+ T cells.

In contrast to BrdU, the OG and Ki67 assays yielded remarkably similar frequencies of proliferating, specific T cells; Ki67+ and OG^{low} CD4+ T cell frequencies were not significantly different in PPD or TB10.4-stimulated PBMC (**Figure 2D, E and F**).

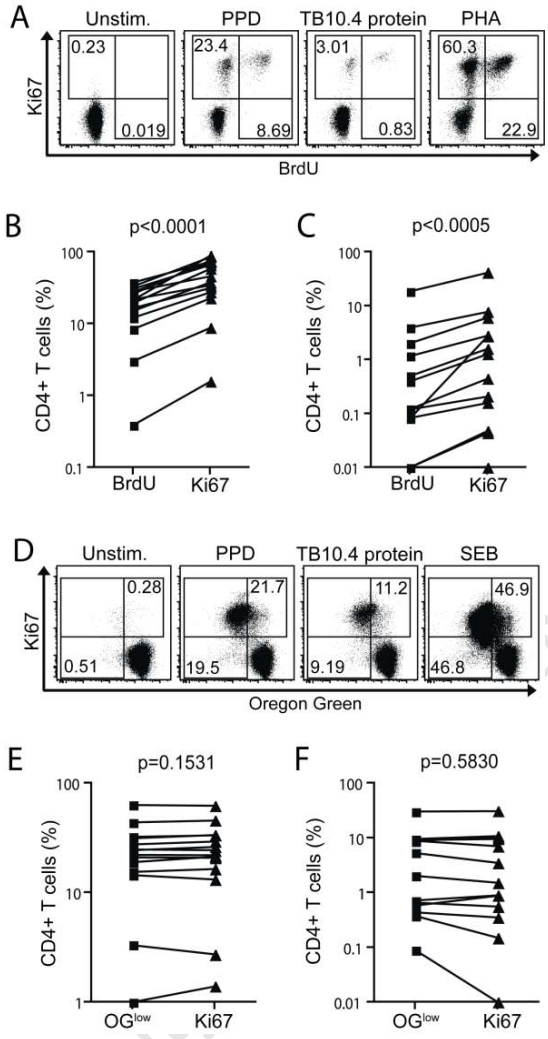

Figure 2. **Comparison of the Ki67 proliferation assay with the BrdU and OG proliferation assays**. (**A**) Representative dotplots showing Ki67 versus BrdU expression by CD4+ T cells in whole blood. Dotplots are gated on live, CD3+CD8- lymphocytes. Frequencies of (**B**) PPD- and (**C**) TB10.4-specific CD4+ T cell proliferation as detected by Ki67 expression or BrdU incorporation (n=15). CD4+ T cells are defined as CD3+CD8- T cells (see methods: Data analysis). (**D**) Representative dotplots showing Ki67 and dye dilution of OG by CD4+ T cells in PBMC. Dotplots are gated on live, CD3+CD8- lymphocytes. Frequencies of (**E**) PPD- and (**F**) TB10.4-specific CD4+ T cell proliferation as detected by Ki67 expression or dye dilution of OG (OG^{low}) in 14 donors. CD4+ T cells are defined as CD3+CD8- T cells (see methods: Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

Frequencies of Ki67+CD4+ T cells correlated strongly with BrdU+CD4+ T cell frequencies (PPD: r = 0.8036, p=0.0003; TB10.4 protein: r=0.9308, p<0.0001) (**Figure 3A and B)**. Similarly, a strong correlation was found between frequencies of antigen-specific Ki67+ and OG^{low} CD4+ T cells (PPD: r=0.9868, p<0.0001; TB10.4 protein: r=0.9473, p=0<0.0001) (**Figure 3C and D**).

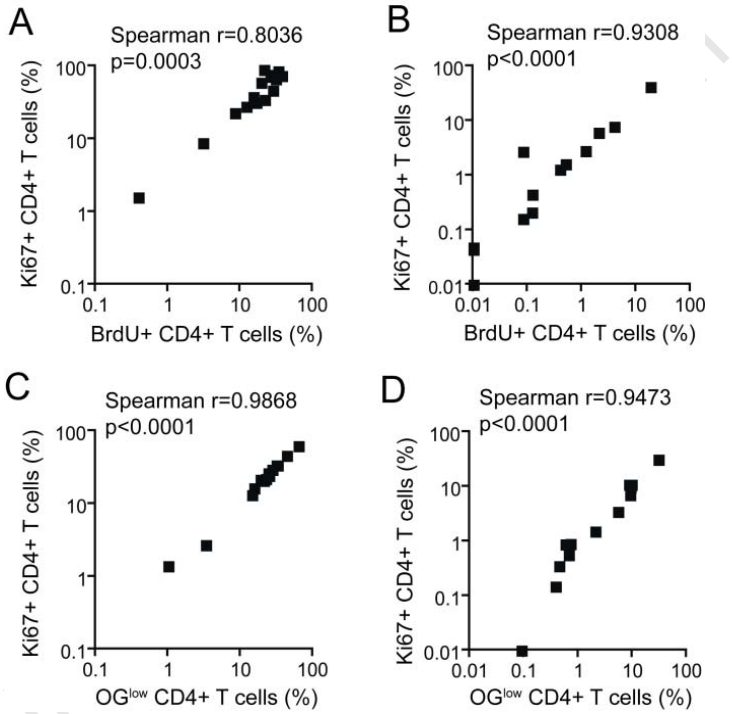

Figure 3. Correlations between Ki67+CD4+ T cell expression and BrdU incorporation or dye dilution of OG (OG^{low}). Whole blood was incubated with (**A**) PPD or (**C**) TB10.4 for 6 days (n=15). PBMC were incubated with (**B**) PPD or (**D**) TB10.4 for 6 days (n=14). Correlations were calculated using a Spearman's rank correlation coefficient.

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

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

6.4.3. Cytokine expression profiles of proliferating CD4+ T cells

The functional capacity of cells that have expanded during the 6-day culture can be assessed by short-term polyclonal re-stimulation with PMA and ionomycin on day 6. This allows detection of cytokine expression by intracellular cytokine staining. The expression of IFN-γ, IL-2 and TNF-α by Ki67+CD4+ T cells was compared with BrdU+ or OG^{low} CD4+ T cell populations. Similar expression of IFN-γ and TNF-α was observed in proliferating CD4+ T cell populations identified by BrdU and Ki67 (**Figure 4A and B**). However, expression of IL-2 was different amongst the distinct subsets. The highest proportion of IL-2 expressing cells was BrdU+, while CD4+ T cells positive for Ki67 only comprised the lowest proportion of IL-2 expressing cells (**Figure 4B**). When comparing the cytokine profiles of Ki67+ and OG^{low} CD4+ T cells, very similar expression profiles of IFN-γ, IL-2 and TNF-α were observed (**Figure 4C and D**).

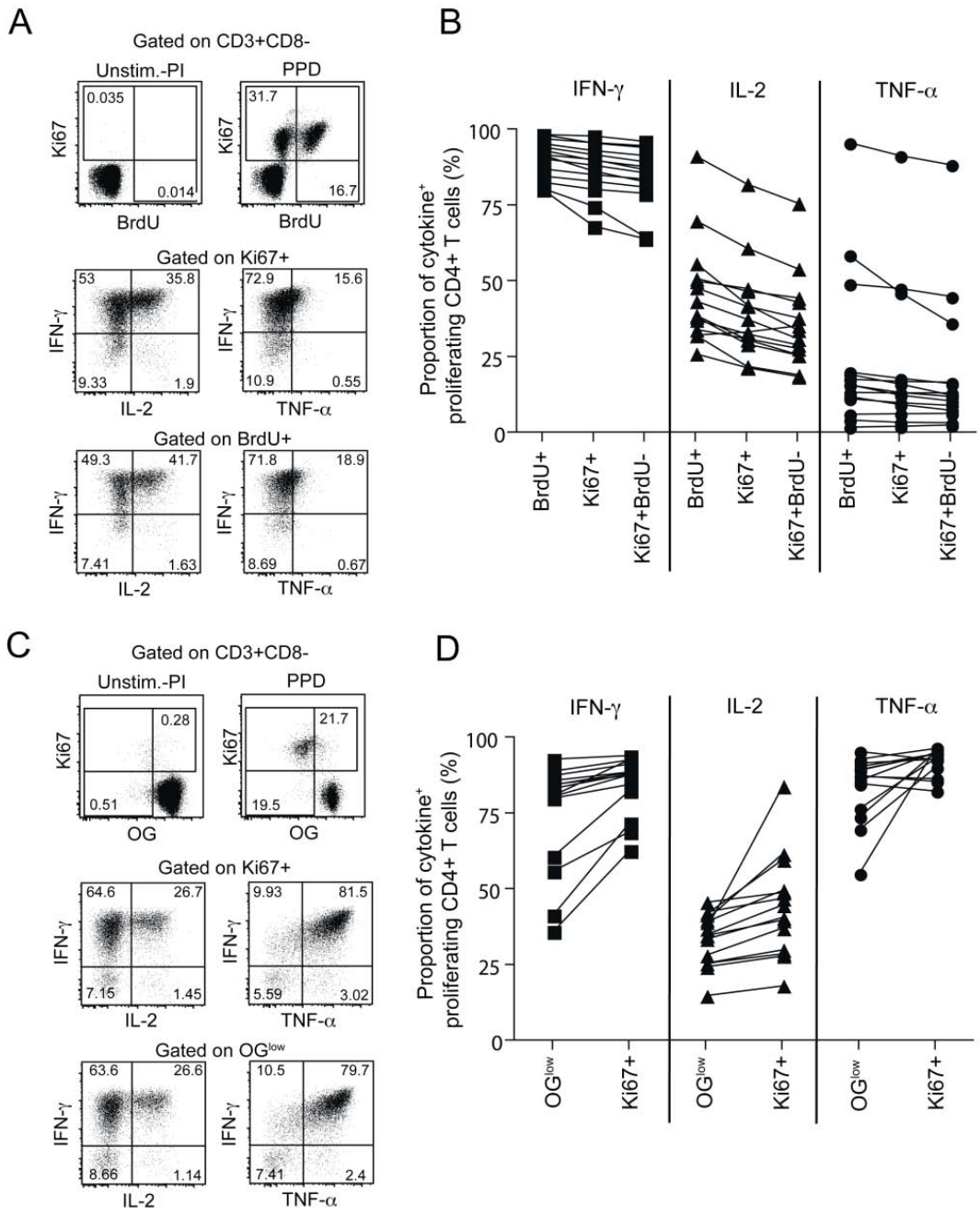

Figure 4. Cytokine expression profiles of proliferating CD4+ T cells. Whole blood or PBMC were cultured for 6 days with no antigen (NIL) or PPD. On day 6, cells were restimulated with PMA and ionomycin for 4 hours in the presence of Brefeldin A to detect cytokine expression by proliferating T cells.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

6.4.4. Intra-assay variability of Ki67 proliferation assay

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

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

PPD stimulation

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

PHA stimulation

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

6.4.5. Monitoring of vaccine-induced T cell proliferation

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

As expected, the frequency of *in vitro* proliferating, Ki67+CD4+ T cells observed pre-vaccination was low (median, 0.15%). After vaccination, TT-specific CD4+ T cell proliferation increased significantly (median, 3.77%, **Figure 5A and B**). To control for possible non-specific up-regulation of Ki67 after TT vaccination, we also quantified BCG-specific T cell proliferation pre- and post- vaccination. Frequencies of BCG-specific Ki67+CD4+ T cells before and after TT vaccination were not different (**Figure 5A and C**). We also compared the relative TT-induced increase in T cell proliferation between TT and BCG-specific Ki67+CD4+ T cells. TT vaccine boosting of TT-specific Ki67+CD4+ T cells was significantly higher than BCG-specific Ki67+CD4+ T cells (**Figure 5D**)

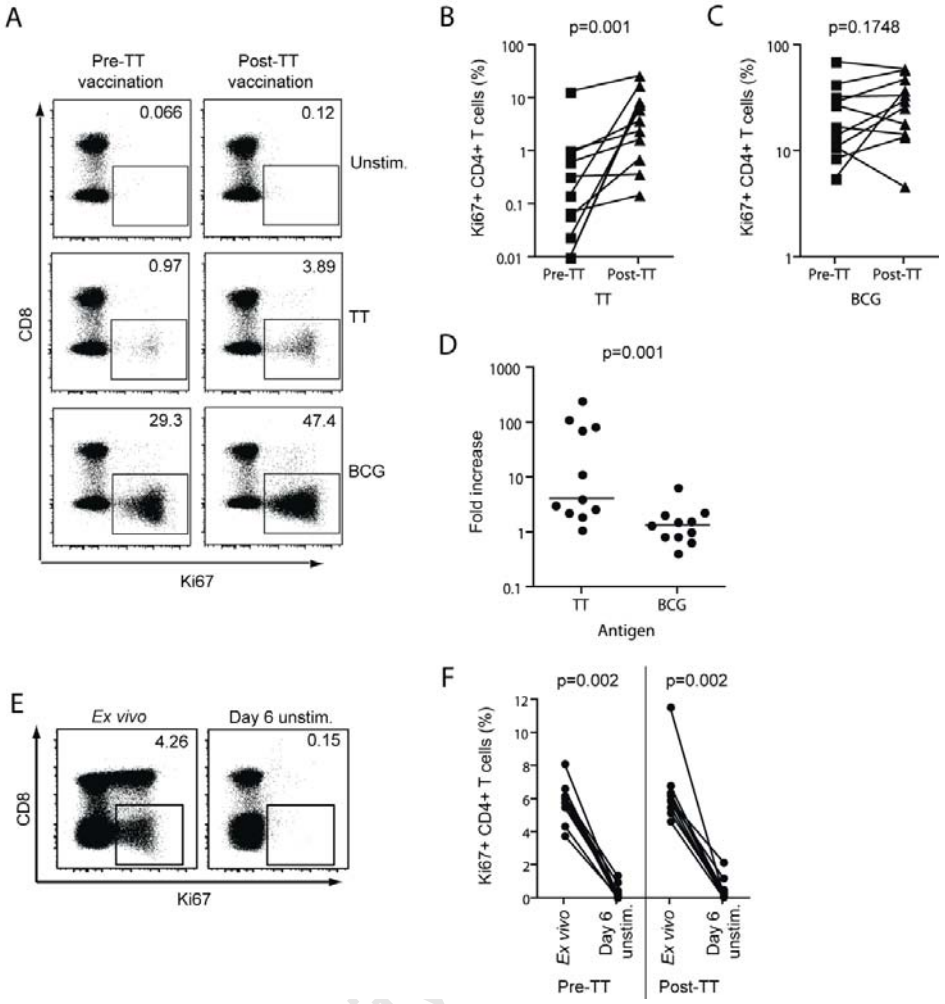

Figure 5. **Monitoring of vaccine-induced T cell proliferation.** (**A**) Dotplots showing Ki67 expression by CD4+ T cells from a representative 18 month old toddler before (pre-TT) and after TT vaccination (post-TT). Dotplots are gated on live, CD3+ lymphocytes. Values in each dotplot represent the frequency of Ki67+ T cells within the CD3+CD8- T cell population. Frequencies of (**B**) TT-specific and (**C**) BCG-specific CD4+ T cells pre- and post-TT vaccination in 11 toddlers. CD4+ T cells are defined as CD3+CD8- T cells (see methods: Data analysis). (**D**) Relative increase in TT-specific or BCG-specific CD4+ T cells pre- and post-TT. The lines represent the medians. (**E**) Dotplots depicting frequencies of Ki67+CD4+ T cells in whole blood directly *ex vivo* or after culture in the absence of antigen (unstim.) for 6 days. Values in each dotplot represent the frequency of Ki67+ T cells within the CD3+CD8- T cell population. (**F**) Frequencies of Ki67+ CD4+ T cells directly *ex vivo* or after culture for 6 days with medium (n=11). CD4+ T cells are defined as CD3+CD8- T cells (see methods: Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

To further examine the effects of vaccination-induced T cell proliferation on the Ki67 proliferation assay, Ki67 expression was quantified directly *ex vivo* in whole blood from toddlers before and 11-13 days after TT-vaccination. High *ex vivo* frequencies of Ki67+CD4+ T cells were readily detected before and after vaccination in all toddlers (**Figure 5E and F**). Importantly, after 6 days of culture with no antigen, Ki67 expression decreased markedly in all toddlers to background levels (**Figure 5E and F**).

These data show that *in vivo* T cell proliferation does not interfere with the specificity of the Ki67 proliferation assay. This assay therefore sensitively detects antigen-specific *in vitro* T cell proliferation.

6.5. Discussion

Proliferation is commonly measured as one of the critical indicators of T cell function. Intracellular Ki67 expression was assessed as a marker of *in vitro* proliferation in whole blood- or PBMC-based assays. This study shows that the Ki67 assay provides an alternative approach to measuring antigen-driven T cell proliferation, and compared this assay system to other proliferation assays currently in use.

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

The development of fluorescent dyes and tracking markers has enabled combined analysis of antigen-specific T cell proliferation, phenotyping and cytokine expression by flow cytometry (Johannisson and Festin, 1995; Mehta and Maino, 1997; Lyons and Doherty, 2004; Wallace et al., 2008). To date, whole blood BrdU and PBMC dye dilution assays have been the preferred flow cytometry based methods to assess lymphocyte proliferation.

Ki67 expression identified approximately double the frequency of proliferating CD4+ T cells detected by BrdU incorporation. The classical BrdU assay involves addition of BrdU during the last 2 to 24 hours of cell culture. During this time BrdU, a synthetic thymidine analog, is incorporated into newly synthesised DNA of actively dividing cells in the S-phase (Gratzner, 1982; Dolbeare et al., 1983). Incubation of cells with BrdU is limited to 24 hours or less because incorporated BrdU inhibits cell cycle progression. Therefore a major limitation of this assay is that it only detects cells that have progressed through the S-phase during this short period of time on the last day of culture. By contrast, Ki67 is expressed by cells in all active phases of the cell cycle, including the S-phase. Therefore, Ki67 is a more sensitive marker for the detection of rare T cell responses and reflects the extent of *in vitro* antigen-specific proliferation more accurately than BrdU incorporation.

Higher proportions of IL-2 expressing BrdU+ CD4+ T cells were observed compared with IL-2 expressing Ki67+ CD4+ T cells. We propose that BrdU and Ki67 identify T cell populations in different states of cellular differentiation. CD4+ T cells that recently incorporated BrdU are actively dividing at the time of Brefeldin A-mediated cytokine accumulation and presumably express greater levels of IL-2 to maintain the ongoing proliferation. By contrast, a

higher proportion of Ki67+CD4+ T cells may have completed multiple cell cycles and differentiated into an effector phenotype (Schwendemann et al., 2005) characterized by low IL-2 expression (Sallusto et al., 1999; Harari et al., 2005).

Cellular proliferation in PBMC is routinely evaluated by dye dilution methods, such as CFSE or derivatives thereof, such as OG (Robinson and Amara, 2005). The advantage of PBMC-based assays is the ability to perform the assay after PBMC cryopreservation, a practical strategy utilised in large-scale clinical settings. Limitations of many protein reactive dye compounds include cellular toxicity and sensitivity to pH and light (Wallace et al., 2008). The Ki67 proliferation assay provides remarkably similar outcomes to the dye dilution assays, while it requires no incubation or washing steps prior to or during the culture and exposure of cells to toxic compounds is eliminated. Additionally, since labelling of cells before antigen stimulation is not required, detection of Ki67 by flow cytometry can be performed on antigen-stimulated cells after cryopreservation.

A limitation of Ki67 as a proliferation marker is its inability to resolve the number of proliferation cycles that cells have undergone. Dye dilution assays allow enumeration of cell cycles because the mean fluorescence intensity of the dye is halved between daughter cells after each cell division (Parish, 1999; Lyons and Doherty, 2004). Enumeration of cell cycles enables calculation of the original precursor frequency of specific cells, since the number of cells and their respective number of divisions are known (Givan et al., 1999). Since Ki67 expression does not allow differentiation of individual cell cycles the precursor frequency of specific cells can not be calculated.

Overall, these data show that outcomes of the Ki67 assay correlate strongly with current flow based whole blood and PBMC proliferation assays. This assay is highly reproducible, versatile, and presents several practical advantages over current techniques. We propose Ki67 as a marker for quantifying antigen-specific T cell proliferation, and incorporating the Ki67 assay as a practical tool for monitoring T cell responses in large field studies or pediatric studies based on limited blood volumes. This assay will be implemented in a future longitudinal study that aims to assess changes in BCG-specific T cell proliferative capacity and cytokine expression over the first year of life.

Chapter 7

Longitudinal changes in BCG-specific T cell cytotoxic molecule expression and differentiation phenotype over the first year of life.

7.1. Introduction

CTL contribute to mycobacteria-specific immunity through their capacity to recognise and kill *M.tb* infected target cells (Lalvani et al., 1998a; Stenger et al., 1998; Hussey et al., 2002; Stegelmann et al., 2005; Murray et al., 2006; Bastian et al., 2008; Klucar et al., 2008). Numerous *in vitro* studies have shown that CTL from healthy TST positive individuals and TB patients inhibit *M.tb* growth *in vitro* and lyse infected target cells (Tan et al., 1997; Lalvani et al., 1998a; Canaday et al., 2001; Stegelmann et al., 2005; Bastian et al., 2008). Killing and inhibition of *M.tb* growth is mediated through cytotoxic effector molecules, such as granulysin and perforin, which are expressed by both CD4+ and CD8+ T cells (Stenger et al., 1998; Klucar et al., 2008; Bruns et al., 2009). Additionally, clinical data suggests that CTL may be critical for maintaining latency in *M.tb* infected individuals (Bruns et al., 2009).

In vaccinated adults and infants, BCG-specific CTL up-regulate multiple cytotoxic effector molecules, degranulate (Smith et al., 1999; Murray et al., 2006) and lyse *M.tb* infected cells (Hussey et al., 2002). Murray et al. demonstrated that BCG-specific CD8+ T cells from 10-week old vaccinated infants up-regulate granulysin, perforin and Grm. Granulysin, a saposin like lipid-binding protein, can directly trigger apoptosis and in combination with perforin kills intracellular *M.tb* (Stenger et al., 1998). The expression of GrmB

correlates with CTL lytic capacity as is required for the optimal induction of perforin dependent apoptosis (Pardo et al., 2002; Harari et al., 2009). Therefore, the induction of CTL through BCG vaccination may provide an important component to vaccine induced host responses.

All antigen-experienced CTL store cytotoxic effector molecules in cytoplasmic granules. Therefore, measuring up-regulation of cytotoxic molecules in antigen-specific T cells from pre-formed molecules by flow cytometry is challenging (Appay et al., 2002c; Harari et al., 2009; Makedonas et al., 2010). To overcome this limitation previous studies have combined CTL analysis with measurement of activation markers, tetramers or ICS to identify antigen-specific up-regulation or de novo synthesis of cytotoxic molecules (Murray et al., 2006; Harari et al., 2009; Makedonas et al., 2010).

Characterisation of T cells in different viral models has shown that the expression of cytotoxic effector molecules is closely related to cellular differentiation and maturity (Appay et al., 2002a; Marchant et al., 2003; Harari et al., 2009). In this chapter, CD27 and CD28 expression was analysed to monitor the differentiation of BCG-specific T cells over the first year of life and to assess the relationship between cytotoxic molecule expression and differentiation phenotype. As described in chapter 5, co-stimulatory molecules CD27 and CD28 identify functionally discordant T cell populations. T cells with proliferative capacity pre-dominantly have an early differentiation phenotype (CD27+CD28+) and express low levels of perforin (Appay et al., 2002a; Kovaiou et al., 2005). T cells expressing a late differentiation phenotype (CD27-CD28-) typically lack proliferative capacity and express high levels of

cytotoxic molecules (Appay et al., 2002a; Appay et al., 2002c; Marchant et al., 2003; Tomiyama et al., 2004).

While cross-sectional studies have shown that BCG-vaccination of infants induces CTL (Hussey et al., 2002; Murray et al., 2006), longitudinal studies are needed to describe the kinetics of T cell responses following vaccination. This type of data is critical for evaluating novel TB vaccine candidates and for optimising vaccination strategies based on a BCG-prime heterologous-boost regimen.

7.2. Aims

The kinetics of BCG-specific T cell responses following vaccination of infants has not been studied. Infants are a likely target population for novel TB vaccines, which aim to boost BCG-specific T cell responses therefore this type of data is critical for determining when to boost BCG-induced responses. The objective of this study was to determine kinetic changes in the expression of cytotoxic effector molecules and differentiation stage of specific T cells following BCG vaccination. Ki67, a nuclear protein expressed during cell cycling, was used to detect BCG-specific cells following incubation of whole blood with BCG for 3 days, followed by measurement of BCG-specific induction of perforin, granulysin and GrmB in Ki67+ cells. Progressive loss of CD27 and CD28 was assessed as a proxy for T cell differentiation. To achieve the objective of this study, blood was collected from infants at multiple time points over a one year period and the following sub-aims were set:

1. Characterise BCG-specific T cell cytotoxic molecule expression during the first year of life.
2. Monitor differentiation markers within the total T cell compartment and for BCG-specific T cells.
3. Assess the relationship between T cell differentiation stage and the expression of cytotoxic molecules.
4. Determine the optimal time for giving a new TB vaccine to boost the BCG-induce response.

7.3. Materials and methods

7.3.1. Study participants and recruitment

The infants studied in this chapter were selected from the longitudinal cohort described in chapter 2. Infants were selected based on those with the most complete longitudinal data sets. Cross-sectional and longitudinal analyses were completed on samples from 68 infants selected from a cohort of 90.

7.3.2. Cytotoxicity assay

Whole blood was stimulated with antigens for 3 days as described previously in chapter 2 (section 2.3.2).

7.3.3. Antibodies

BCG-specific T cell cytotoxic molecule expression and differentiation phenotypes were assessed using the "Cytotoxicity Panel" optimised in chapter 3 (section 3.4.8).

7.3.4. Data analysis

T cells were identified by a SSC vs. CD3 dotplot. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. Dead cells and debris were excluded from analysis using a Ki67-PE vs. granulysin-Alexa Fluor 488 dotplot. CD4+ and CD4- T cells were identified on a dotplot showing CD4 vs. Ki67. CD8+ T cells were selected out of the CD4- T cell population using a CD4 vs. GrmB dotplot. A skewed gate was used to select for CD8+ T cells due to data spread between the APC-H7 and Alexa Fluor 700 channels (**Figure 1 Panel A**). Boolean gating analysis was carried out

once positive gates were established for functional (**Figure 1 Panel B-C**) and phenotypic (**Figure 1 Panel D**) parameters.

The expression of cytotoxic molecules and differentiation phenotypes are reported as a frequency of BCG-specific T cells, defined as Ki67+, and for the total T cell compartment (comprising Ki67+/- populations). Evaluation of Ki67 as a marker of antigen-specific T cells after stimulation of whole blood with BCG is described in chapter 3 (section 3.4.7). T cell phenotyping and monitoring of qualitative changes over time (pie charts) was carried out on samples which had at least 50 Ki67+ events and in which the frequency of Ki67+ T cells after stimulation with BCG was at least three times above background Ki67 expression (NIL). Samples were excluded from analysis if the frequency of Ki67+ T cells after incubation with PHA was <3 median absolute deviations above median background (NIL) expression. Reported data have been corrected for background except for the phenotypic results. Single-stained mouse κ beads were used to calculate compensations for every run. Data were analysed with FlowJo software v 9.0.1, Pestle v 1.6.2 and Spice v 5.1 software. Statistical analyses were calculated using GraphPad Prism v 4.0.

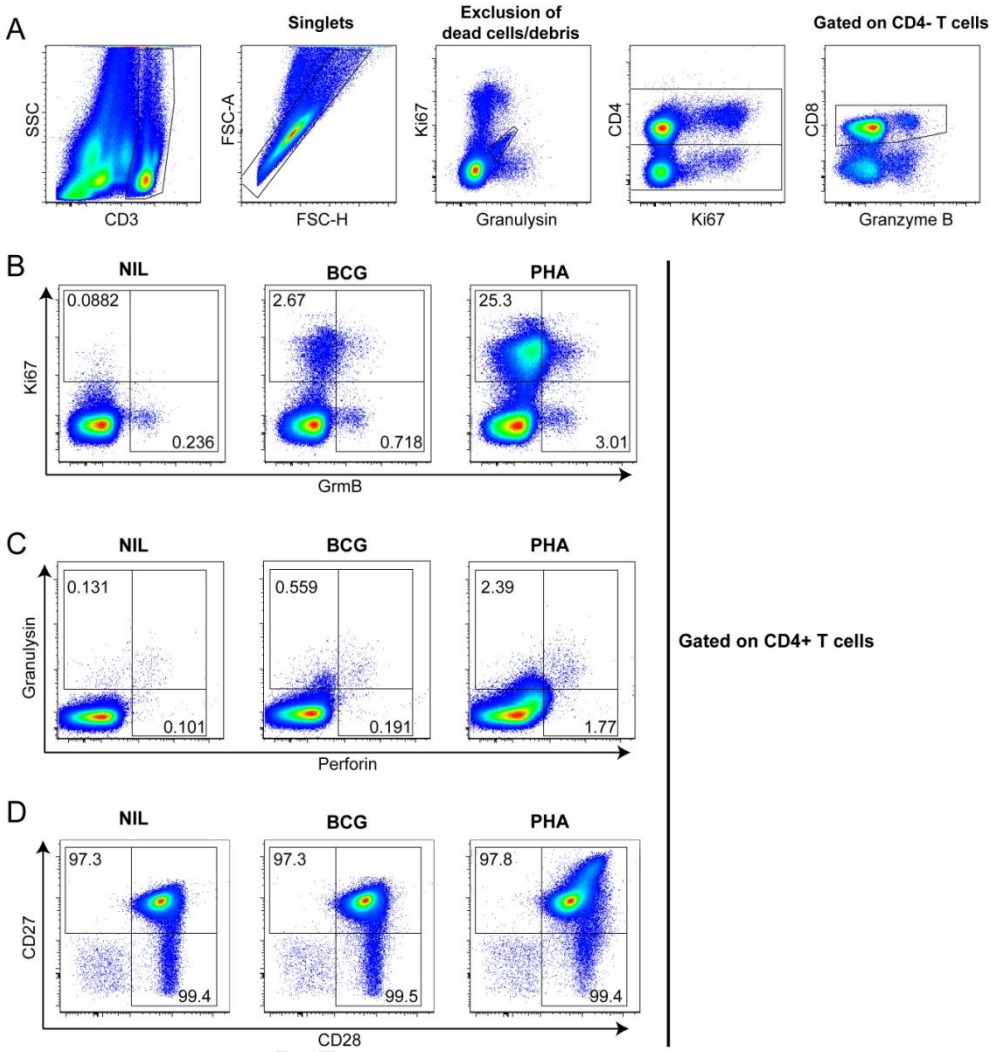

Figure 1. Flow cytometric analysis of T cell cytotoxic molecule expression and differentiation phenotypes. (**A**) Gating strategy from a representative infant donor. Whole blood was incubated with BCG for 3 days and then stained for (**B**) Ki67, GrmB (**C**) granulysin, perforin and differentiation markers (**D**) CD27 and CD28. Boolean gates for each condition were determined using unstimulated (NIL) controls. The positive control (PHA) is shown for each parameter measured.

7.4. Results

7.4.1. Longitudinal changes in Ki67 expression in CD4+ and CD8+ T cells following BCG vaccination of newborns

To determine the kinetics of BCG-specific T cell responses after vaccination, Ki67 expression was assessed cross-sectionally at multiple time points in CD4+ and CD8+ T cells from 3 weeks to 52 weeks of age, following incubation of whole blood with BCG for 3 days.

The frequency of Ki67+ T cells 3 weeks after BCG vaccination was low for CD4+ (median, 1.619%; **Figure 2A**) and CD8+ T cells (median, 0.5681%; **Figure 2B**). At 6 weeks, a significant expansion of Ki67+CD4+ T cells was observed (median, 7.198%), this was followed by the gradual contraction in the frequency of Ki67+ T cells (**Figure 2A**). At 52 weeks, Ki67+CD4+ T cell frequencies were significantly lower compared to 3 weeks (median, 0.2380%). The kinetics of Ki67+CD8+ T cells was similar to that of Ki67+CD4+ T cells. Ki67+CD8+ T cell responses peaked 6 weeks after vaccination (median, 0.8448%) and gradually waned with age (**Figure 2B**). At 6 weeks, the frequency of Ki67+CD8+ T cells was significantly higher compared to 52 weeks (median, 0.1183%; **Figure 2B**). Overall, the magnitude of the Ki67+CD8+ T cell response was lower at all time points compared with Ki67+CD4+ T cell responses. Longitudinal data for individual infants supported the results obtained by cross-sectional analysis (**Figure 2C and D**).

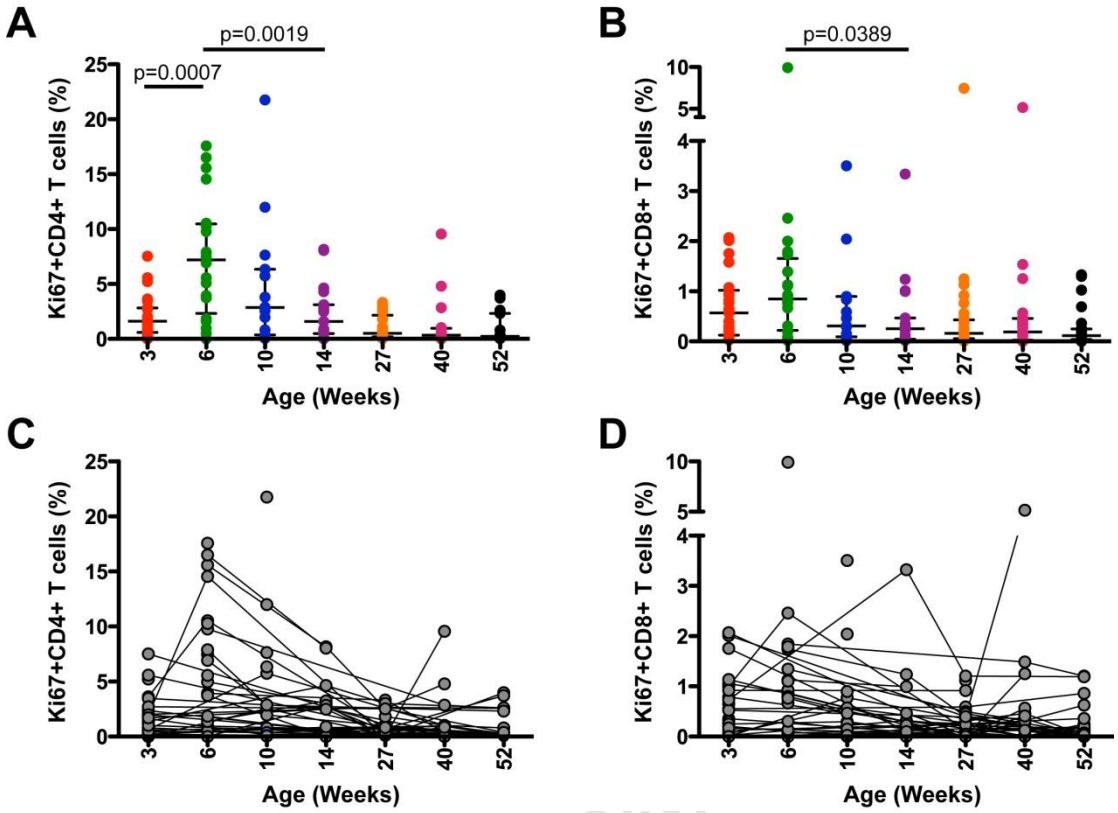

Figure 2. **Changes in Ki67 expression in T cells over the first year of life.** Cross-sectional analysis of Ki67 expression in (**A**) CD4+ and (**B**) CD8+ T cells after stimulation of whole blood for 3 days with BCG. The horizontal line indicates the median and the whiskers the interquartile range. Differences were calculated using the Kruskal-Wallis test followed by a Mann Whitney U test. Longitudinal changes in Ki67 expression in (**C**) CD4+ and (**D**) CD8+ T cells for individual infants. Each line represents a different infant. Statistical analyses were not performed on these data. Please refer to the justification at the end of the discussion section in chapter 5 (pg 128).

These data show that vaccination of infants with BCG predominantly drives the proliferation of CD4+ T cells and that T cell responses peak 6 weeks post vaccination and gradually wane over the first year of life.

7.4.2. Functional characterisation of cytotoxic T cell responses following BCG vaccination

To characterise T cell cytotoxic molecule expression after vaccination GrmB, perforin and granulysin expression by BCG-specific T cells were measured (**Figure 3A and B**). BCG-specific T cells were defined as Ki67+.

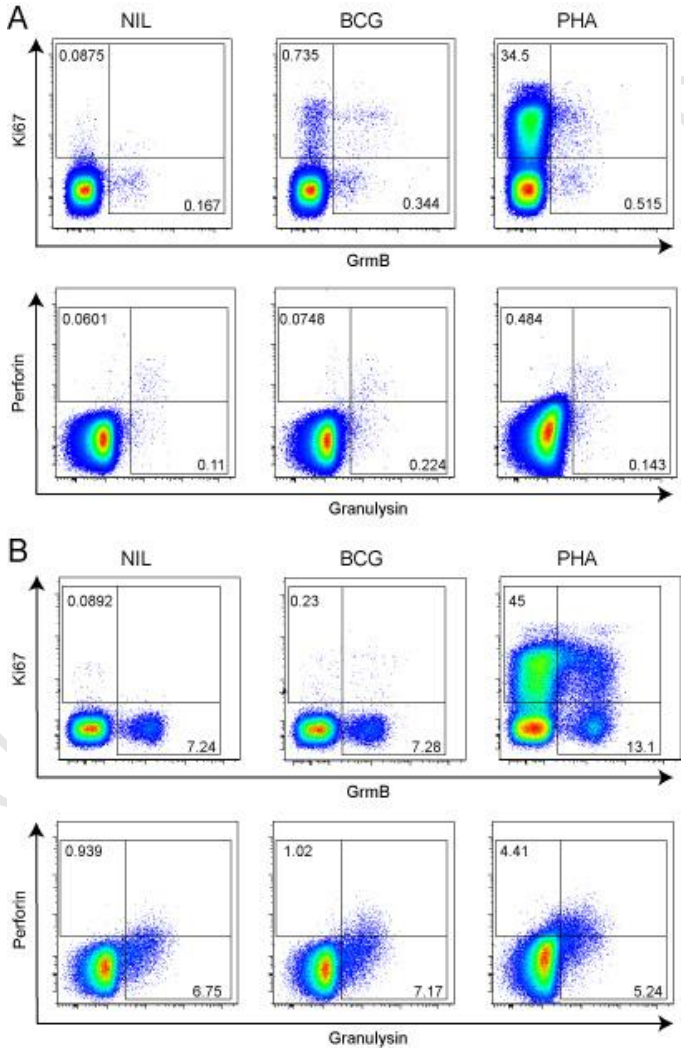

Figure 3. T cells up-regulate Ki67 and cytotoxic molecules in response to BCG. Representative dotplots showing Ki67, GrmB, granulysin and perforin expression in (**A**) CD4+ and (**B**) CD8+ T cells. Whole blood was stimulated for 3 days with medium only (NIL), BCG or PHA. Dotplots are from a representative 27-week old infant.

Cross-sectional analysis showed that granulysin and GrmB expression by Ki67+CD4+ T cells was detectable at low levels as early as 3 weeks after vaccination (**Figure 4A**).

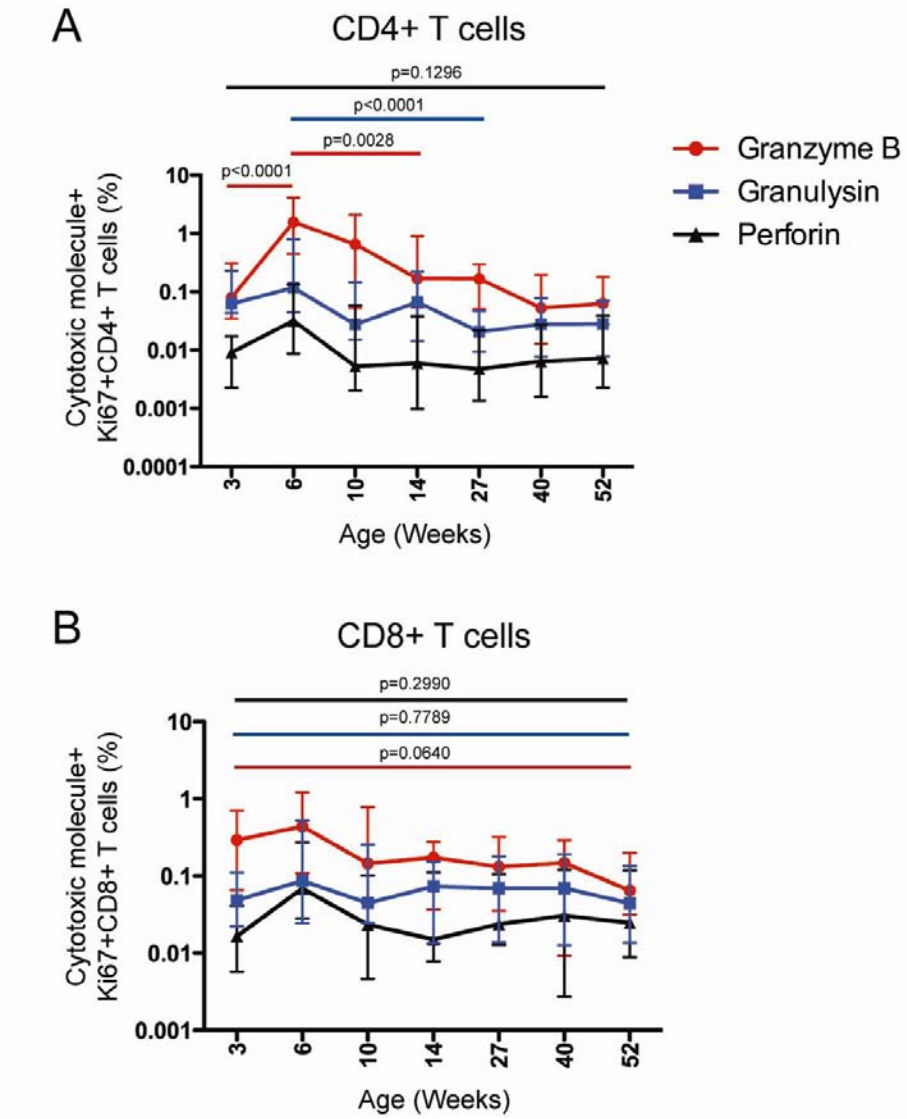

Figure 4. Changes in BCG-specific T cell cytotoxic molecule expression. Kinetic changes in GrmB, granulysin and perforin expression in BCG-specific (**A**) CD4+ and (**B**) CD8+ T cells. Summary data are reported as a frequency of CD4+ and CD8+ T cells. The horizontal line indicates the median and the whiskers the interquartile range. Differences were calculated using the Kruskal-Wallis test followed by the Mann Whitney U test.

Perforin expressing Ki67+CD4+ T cells remained below the sensitivity level of the assay for most of the follow-up period. The frequencies of BCG-specific T cells expressing GrmB, perforin and granulysin peaked 6 weeks after vaccination and gradually waned over time. Only frequencies of Ki67+CD4+ T cells expressing GrmB were significantly higher at 6 weeks compared with 3 weeks (**Figure 4A**).

Ki67+CD8+ T cells displayed similar kinetics to Ki67+CD4+ T cells. Expression of cytotoxic molecules by specific cells peaked 6 weeks after BCG vaccination and gradually waned over time (**Figure 4B**).

No significant changes in the frequency of BCG-specific CD8+ T cells expressing perforin, granulysin or GrmB were observed over time (**Figure 4B**).

GrmB expression at 6 weeks was significantly lower in Ki67+CD8+ T cells compared with Ki67+CD4+ T cells (p=0.223; Mann Whitney U test). BCG-specific CD8+ T cells expressed significantly more perforin than specific CD4+ T cells from 27 to 52 weeks of age (27 weeks, p=0.0003; 40 weeks, p=0.0368; 52 weeks, p=0.0281; Mann Whitney U test).

Longitudinal comparisons of cytotoxic profiles supported cross-sectional observations (**Figure 5A and B**), however changes in cytotoxic molecule expression patterns over the first year of life were clearer on cross-sectional analysis.

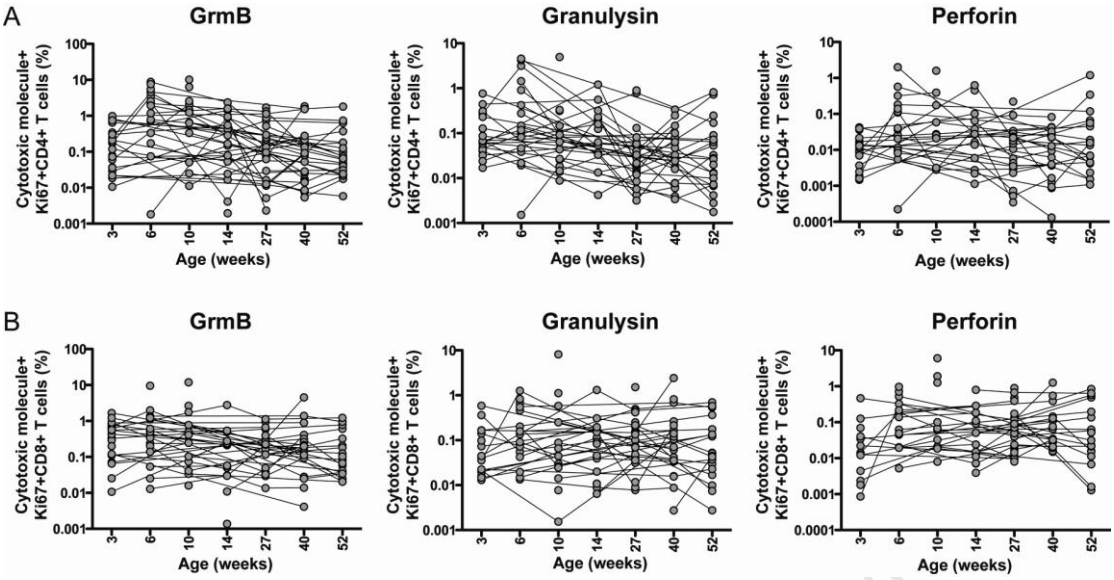

Figure 5. Longitudinal changes in BCG-specific T cell cytotoxic molecule expression. Longitudinal changes in GrmB, granulysin and perforin expression in BCG-specific (**A**) CD4+ and (**B**) CD8+ T cells for individual infants. Each line represents a different infant. Statistical analyses were not performed on these data. Please refer to the justification at the end of the discussion section in chapter 5 (pg 128).

These data show that BCG vaccination up-regulates the expression of cytotoxic molecules in CD4+ and CD8+ T cells. BCG-specific cytotoxic T cell responses peak 6 weeks post-vaccination and gradually decline with age.

7.4.3. Qualitative changes in BCG-specific T cell populations

To determine changes in the qualitative nature of the BCG-specific T cell response, the proportions of Ki67+ T cells expressing cytotoxic molecules was monitored over time. Cross-sectional analysis showed that three weeks after vaccination approximately 20% of Ki67+CD4+ T cells expressed one (1+) or two (2+) cytotoxic molecules (**Figure 6A and B**). The proportion of T

cells expressing GrmB only and co-expressing GrmB and granulysin gradually increased over time and by 52 weeks of age approximately 45% of Ki67+CD4+ T cells expressed cytotoxic molecules (**Figure 6B**). Ki67+CD4+ T cells predominantly expressed GrmB throughout the follow-up period (**Figure 6B**).

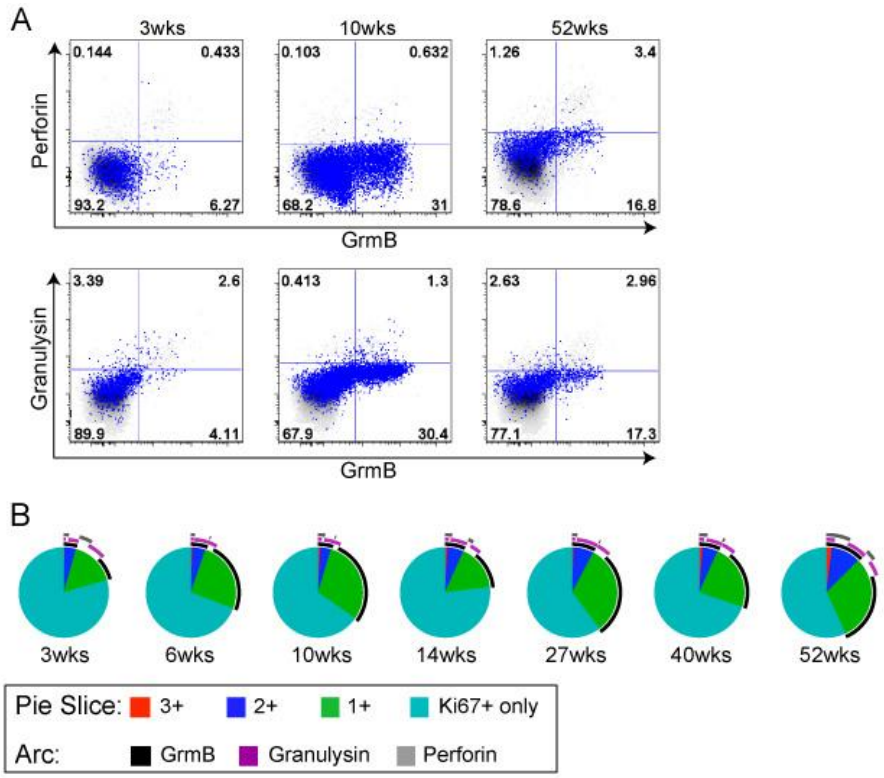

Figure 6. Changes in the proportion of BCG-specific CD4+ T cells expressing cytotoxic molecules over time. (**A**) Representative flow cytometric plots show changes in GrmB, perforin and granulysin expression by BCG-specific CD4+ T cells over time. Ki67+ T cells (blue events) were overlaid onto a density plot (grey shading) of the total CD4+ T cell population. Frequencies within specific quadrants reflect expression of cytotoxic molecules as a percentage of Ki67+CD4+ T cells. (**B**) Pie charts representing the proportions of the total Ki67+CD4+ response expressing a given number of cytotoxic molecules. Responses are grouped and colour-coded according to the number of cytotoxic molecules expressed. The contribution of each cytotoxic molecule to each pie slice is depicted by the outer, colour-coded arcs.

Overall, a greater proportion of Ki67+CD8+ T cells expressed cytotoxic molecules compared with CD4+Ki67+ T cells (**Figure 7A and B**).

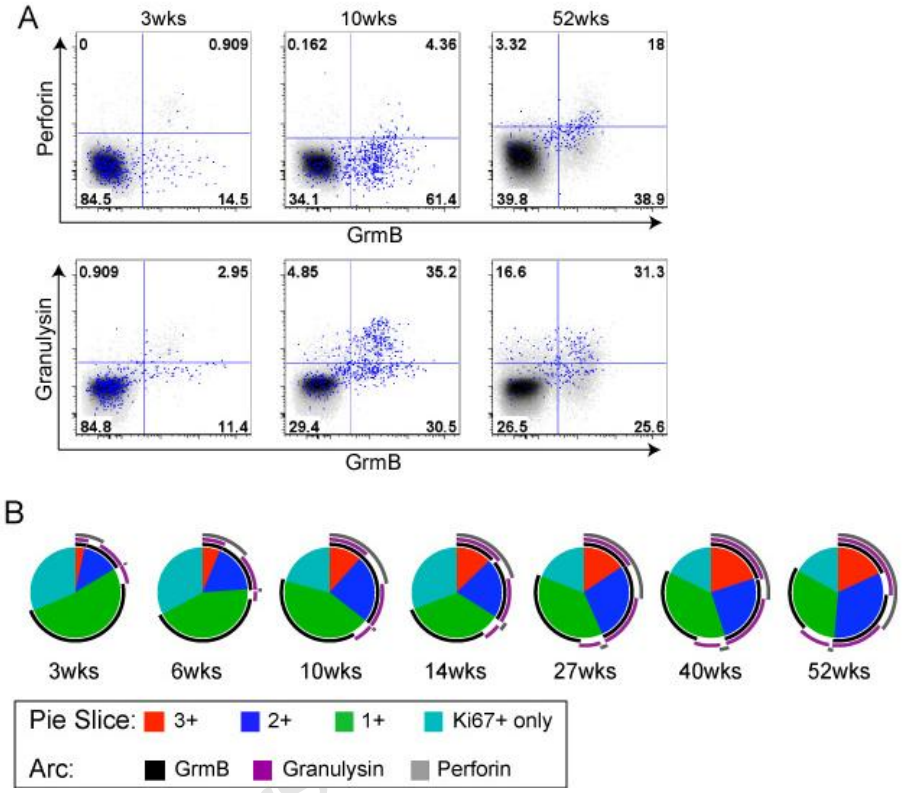

Figure 7. Changes in the proportion of BCG-specific CD8+ T cells expressing cytotoxic molecules over time. (**A**) Representative flow cytometric plots show changes in GrmB, perforin and granulysin expression by BCG-specific CD8+ T cells over time. Ki67+ T cells (blue events) were overlaid onto a density plot (grey shading) of the total CD8+ T cell population. Frequencies within specific quadrants reflect expression of cytotoxic molecules as a percentage of Ki67+CD8+ T cells. (**B**) Pie charts representing the proportions of the total Ki67+CD8+ response expressing a given number of cytotoxic molecules. Responses are grouped and colour-coded according to the number of cytotoxic molecules expressed. The contribution of each cytotoxic molecule to each pie slice is depicted by the outer, colour-coded arcs.

At 3 weeks of age, approximately 70% of CD8+Ki67+ T cells expressed cytotoxic molecules with GrmB only or GrmB and granulysin co-expressing T

cells dominating the response (**Figure 7B**). The proportion of Ki67+CD8+ T cells expressing 1+ or 2+ cytotoxic molecules increased with age. By 52 weeks of age, approximately 50% of CD8+Ki67+ T cells expressed 2+ or 3+ cytotoxic molecules (**Figure 7B**). This was mainly attributable to an increase in Ki67+CD8+ T cell expression of perforin and granulysin over time. Perforin and granulysin was mostly co-expressed by CD8+ T cells expressing other cytotoxic molecules. Longitudinal data were consistent with results obtained by cross-sectional analysis (data no shown).

These data show that the proportion of BCG-specific CD4+ and CD8+ T cells expressing cytotoxic molecules increases with age. Additionally, BCG-specific CD8+ T cells become progressively more polyfunctional (expressing multiple cytotoxic molecules) during the first year of life as they acquire the capacity to express granulysin and perforin.

7.4.4. Differentiation of BCG-specific T cells over time

To examine T cell maturation over time, surface expression of CD27 and CD28 was measured. The total CD4+ T cell population predominantly expressed both CD27 and CD28 (total CD4+ T cell population; 3 week median, 97.6%) and retained this phenotype throughout the follow-up period (52 week median, 95.9%; **Figure 8A and B**) The same phenotypic profile was observed for CD4+ T cells stimulated with medium only (NIL, data not shown). BCG-specific T cells were CD27+CD28+ 3 weeks after vaccination (median, 81.9%; **Figure 8A and C**). The proportion of Ki67+CD4+ T cells expressing this phenotype gradually declined during the follow-up period (52 weeks

median, 58.72%) and an increase in T cells expressing an intermediate phenotype (CD27-CD28+) was observed (**Figure 8C**).

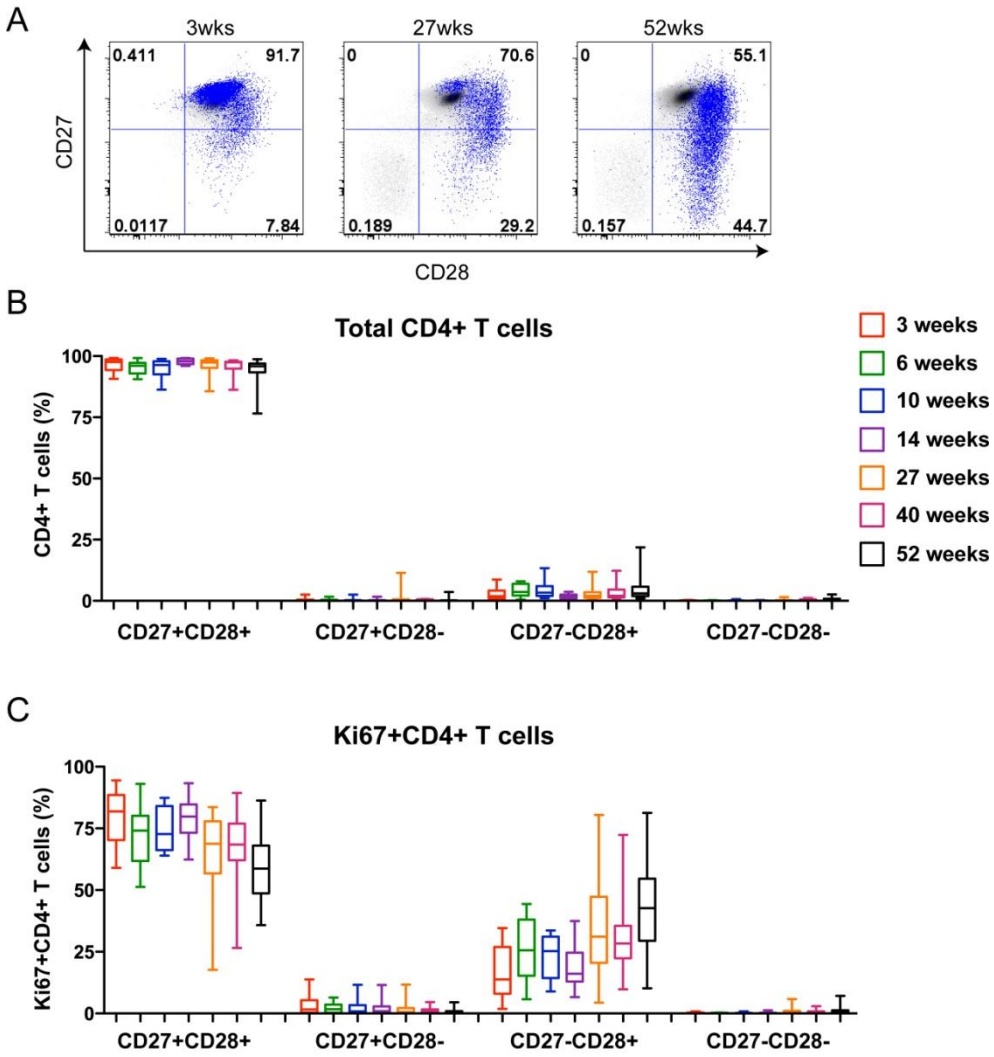

Figure 8. Changes in BCG-specific T cell differentiation phenotype over time. (**A**) Changes in the differentiation phenotypes of BCG-specific T cells (Ki67+, blue) based on CD27 and CD28 expression over time. Ki67+ T cells were overlaid onto a density plot of the total CD4+ T cell population (grey shading). Frequencies reflect the percentage of Ki67+CD4+ T cells within specific quadrants. Changes in differentiation phenotypes of (**B**) total CD4+ T cells and (**C**) Ki67+CD4+ T cells following stimulation of whole blood with BCG for 3 days. The horizontal line indicates the median, box the interquartile range and the whiskers the range.

At 3 weeks, total CD8+ T cells were CD27+CD28+ (3 week median, 97.6%) and the proportion of cells expressing this phenotype gradually decreased over time (**Figure 9A and B**).

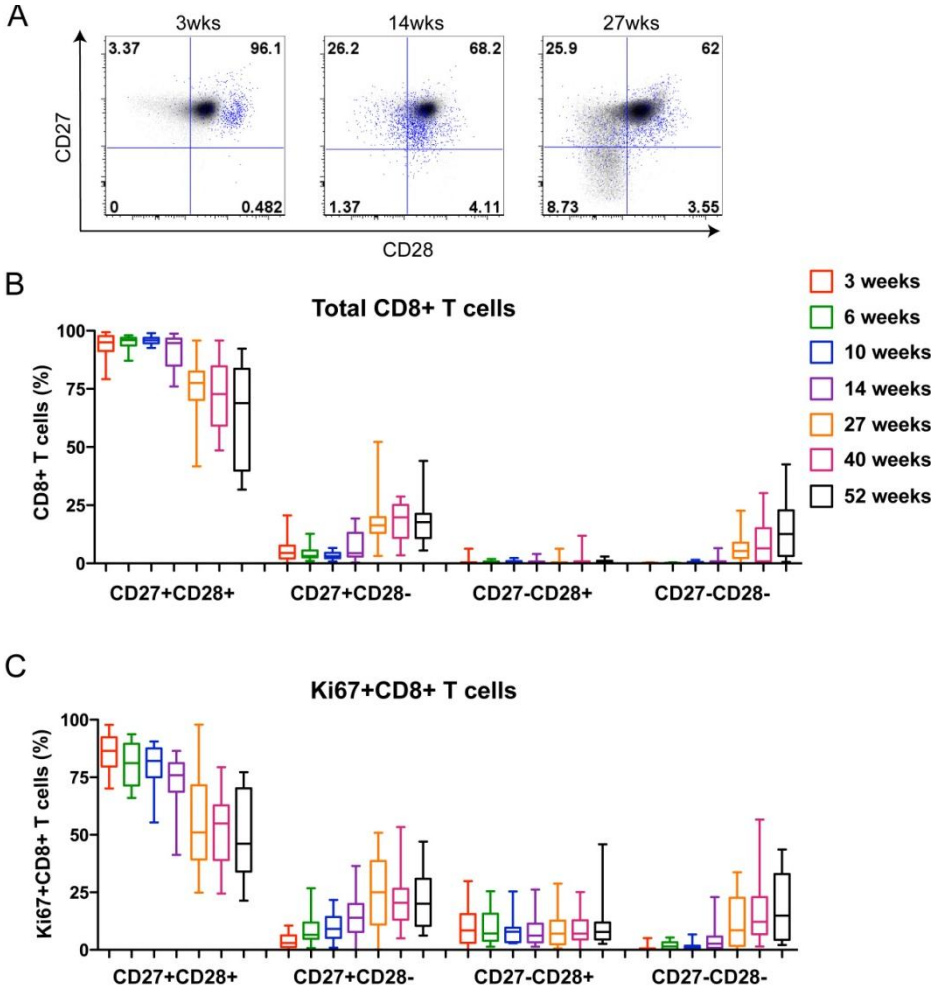

Figure 9. Changes in BCG-specific T cell differentiation phenotype over time. (**A**) Changes in the differentiation phenotypes of BCG-specific T cells (Ki67+, blue) based on CD27 and CD28 expression over time. Ki67+ T cells were overlaid onto a density plot of the total CD8+ T cell population (grey shading). Frequencies reflect the percentage of Ki67+CD8+ T cells within specific quadrants. Changes in differentiation phenotypes of (**B**) total CD8+ T cells and (**C**) Ki67+CD8+ T cells following stimulation of whole blood with BCG for 3 days. The horizontal line indicates the median, box the interquartile range and the whiskers the range.

A small population of CD8+ T cells expressed a CD27+CD28- or CD27-CD28- phenotype increased throughout the first year (**Figure 9B**).

Changes in the differentiation phenotype of BCG-specific CD8+ T cells was similar to that observed for the total T cell compartment (**Figure 9A and C**). However, the increase in the proportion of cells expressing intermediate (CD27+CD28-, CD27-CD28+) and late (CD27-CD28-) differentiation phenotypes was more pronounced compared with the total CD8+ T cell compartment (**Figure 9B and C**). The increase in the proportion of Ki67+CD8+ T cells expressing more mature phenotypes was predominantly due to the gradually loss of CD28 expression. Longitudinal data were consistent with results obtained by cross-sectional analysis (data no shown).

These data show that the proportion of BCG-specific CD4+ and CD8+ T cells expressing intermediate and late differentiation phenotypes progressively increase with age.

7.4.5. Relationship between cytotoxic granule profiles and differentiation phenotype of BCG-specific T cells

Having observed an increase in the proportion of BCG-specific T cells expressing cytotoxic molecules and changes in differentiation phenotype of these cells over time, we next examined the relationship between different cytotoxic profiles and differentiation phenotypes. The down-regulation of CD27 and CD28 is associated with acquisition of cytotoxic molecule expression (**Figure 10A-B**).

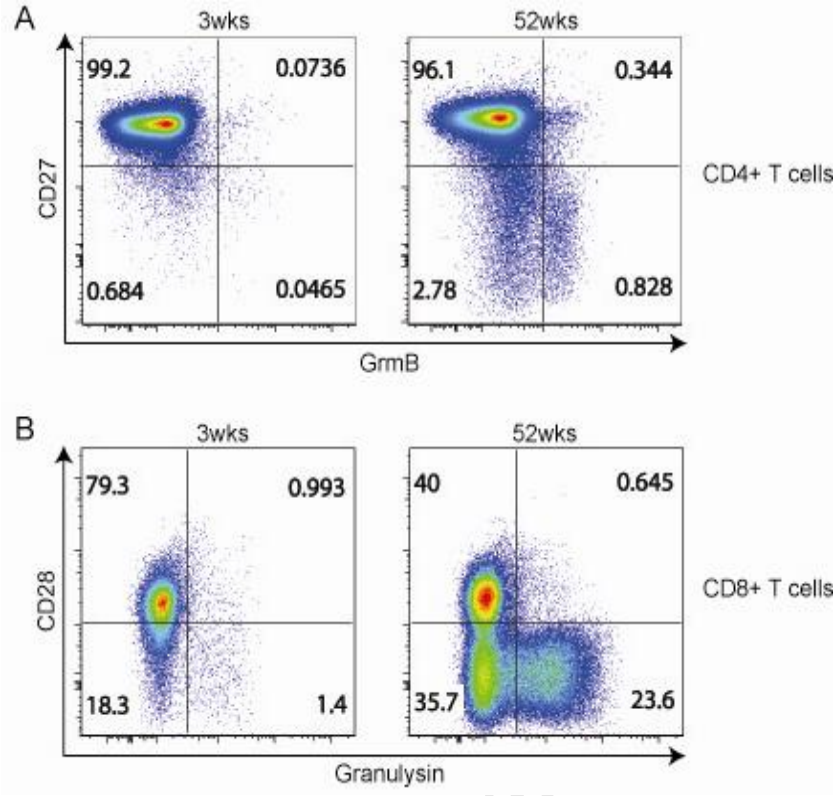

Figure 10. Loss of CD27 and CD28 and the acquisition of cytotoxic molecule expression. Representative dotplots showing the (**A**) loss of CD27 and acquisition of GrmB expression over time in CD4+ T cells (total CD4+ T cell compartment) and (**B**) the loss of CD28 and acquisition of granulysin expression in CD8+ T cells (total CD8+ T cell compartment).

A weak positive correlation was observed between frequencies of BCG-specific CD27-CD28+ cells and GrmB expressing CD4+ T cells (**Figure 11A and Table 1**). Perforin expressing CD4+ T cells were not analysed as expression levels for this molecule was low. The subset of CD4+ T cells expressing a late differentiation phenotype (CD27-CD28-) was small and therefore also not analysed.

For BCG-specific CD8+ T cells, CD27+CD28- and CD27-CD28- T cell populations positively correlated with granulysin (**Figure 11B and Table 2**) and perforin expressing T cells (**Table 2).**

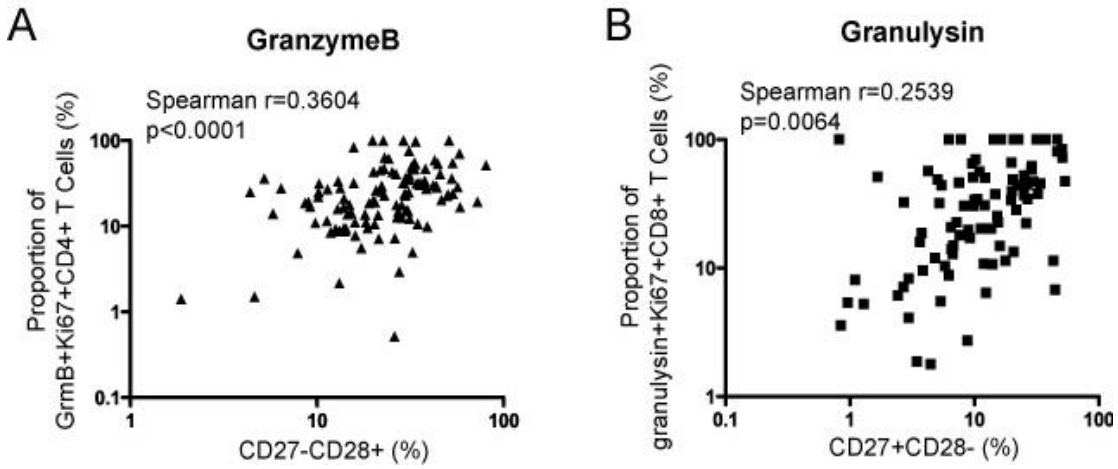

Figure 11. Correlations between BCG-specific (**A**) CD4+ and (**B**) CD8+ T cell subsets expressing cytotoxic molecules and an intermediate differentiation phenotype. Data included for analysis are from all time points. Correlations were calculated using a Spearman's rank correlation co-efficient.

An inverse relationship was found between the early differentiation stage CD27+CD28+ populations and GrmB and perforin expressing Ki67+CD4+ T cells (**Table 1**) and granulysin and perforin expressing Ki67+CD8+ T cells (**Table 2**).

Table 1. Relationship between the proportions of BCG-specific CD4+ T cells expressing cytotoxic molecules and differentiation phenotype.

	GrmB		**Granulysin**	
	r	**p**	**r**	**p**
CD27+CD28+	-0.3474	**<0.0001**	-0.101	0.2625
CD27-CD28+	0.3604	**< 0.0001**	0.1264	0.1601
CD27+CD28-	-0.1095	0.2241	-0.15	0.095

A Spearman test was used to assess correlations.

Table 2. Relationship between the proportions of BCG-specific CD8+ T cells expressing cytotoxic molecules and differentiation phenotype.

	GrmB		**Granulysin**		**Perforin**	
	r	**p**	**r**	**p**	**r**	p
CD27+CD28+	-0.1742	0.0638	-0.3201	**0.0005**	-0.3598	**<0.0001**
CD27-CD28+	0.07854	0.4062	0.05633	0.5517	0.05486	0.5621
CD27-CD28-	0.1191	0.2069	0.2479	**0.0078**	0.3046	**0.001**
CD27+CD28-	0.1146	0.2249	0.2539	**0.0064**	0.3156	**0.0006**

A Spearman test was used to assess correlations.

These data suggest that the differentiation of BCG-specific T cells into more mature phenotypes over time is associated with an increase in the capacity of these cells to express cytotoxic molecules.

7.5. Discussion

A number of novel TB vaccine candidates that have entered clinical trials are based on boosted BCG-primed T cell responses. Since infants are a likely target population for heterologous prime-boost regimens, determining the kinetics of T cell responses following BCG vaccination is critical for future vaccination strategies. In this study, the kinetics and magnitude of BCG-specific T cell responses following vaccination of infants were monitored over the first year of life. BCG-specific expression of Ki67 and cytotoxic effector molecules, GrmB, perforin and granulysin by T cells peaked 6 weeks after vaccination. This observation is highly significant for the rational design and optimisation of heterologous prime boost regimens based on BCG-primed T cell responses. Highly activated effector T cells are more susceptible to activation induced cell death (AICD), compared with naïve or resting memory T cells (Lynch et al., 1995). Therefore, vaccine boosting of BCG-specific T cell responses during the peak effector phase may result in increased apoptosis of vaccine-specific cells. The data presented here suggest that BCG-specific responses should ideally be boosted after 14 weeks of age following the contraction of specific T cells.

The number of detailed kinetic studies of mycobacteria-specific T cell responses following vaccination with BCG is limited and have predominantly been done in adults (McShane et al., 2004; Nabeshima et al., 2005). In these studies, T cell kinetic changes were monitored by measuring IFN-γ responses to *M.tb* PPD by ELISA or cultured ELISPOT. McShane et al. showed that antigen-specific IFN-γ secreting T cells peaked 4 weeks after BCG vaccination of BCG naïve adults. Nabeshima et al. observed peak responses

8 weeks after BCG re-vaccination of tuberculin skin test negative health care workers. Unlike adult participants in these studies, the infants recruited into this study were mycobacterially naïve when immunised with BCG (Hussey et al., 2002). Since pre-existing immunity to mycobacteria modulates T cell responses to BCG (Fine, 1995; Black et al., 2002), the differences in peak responses between this study and those done in adults may be related to prior exposure to mycobacteria. Different assay systems and antigens used for re-stimulation may also contribute to the discrepancy between results.

However, a common observation between all studies is that the peak BCG-specific T cell response is delayed relative to that following vaccination with live viral vaccines (Miller et al., 2008). Murine TB aerosol models suggest that the late onset of mycobacteria-specific T cell responses is attributed to delayed transport of mycobacteria from the site of challenge to the site of T cell priming, the lymph nodes (Gallegos et al., 2008; Wolf et al., 2008). Delayed adaptive immunity is also evident in humans as measured by TST, which is only detectable 4 – 8 weeks after BCG vaccination of mycobacteria naïve individuals (Menzies, 2000).

Multiple reports have demonstrated that CTL contribute to mycobacteria-specific protective immunity through their capacity to recognise and kill infected target cells. BCG vaccination of infants induces CTL, which kill and lyse *M.tb* infected target cells (Hussey et al., 2002; Murray et al., 2006), however detailed characterisation of BCG-specific CTL has not been done.

This study shows that BCG vaccination of infants up-regulates the expression of cytotoxic effector molecules in both CD4+ and CD8+ T cells. Previous reports have described the capacity of CD4+ CTL, which recognise

mycobacteria-specific antigens, to target and lyse *M.tb* infected cells (Bastian et al., 2008; Klucar et al., 2008). Bastian et al. showed that CD4+ T cells from BCG vaccinated (PPD+ESAT6-) and latently infected (PPD+ESAT6+) healthy adults respond to crude *M.tb* cell wall extract, which contains a broad spectrum of mycobacterial glyco-lipids and lipo-peptides (Bastian et al., 2008). When stimulated with a chloroform-methanol extract (CME) of *M.tb*, CD4+ T cells proliferated, expressed Th1 cytokines and up-regulated the expression of perforin, granulysin and GrmB. In addition, CME expanded CD4+ T cells recognised and killed *M.tb* infected macrophages. Klucar et al. generated CD4+ T cell clones specific for a single CFP-10 epitope from healthy PPD reactive donors and analysed anti-mycobacterial effector function. CFP-10 epitope specific clones targeted H37Rv infected autologous dendritic cells. This was dependent on granlysin and perforin because neutralising these cytotoxic molecules partially inhibited killing capacity (Klucar et al., 2008). These *in vitro* studies demonstrate that CD4+ T cells from BCG vaccinated and *M.tb* infected individuals play a role in cytotoxic mediated killing of intracellular *M.tb* and that this mechanism is dependent on cytotoxic effector molecules such as granulysin and perforin. The increased incidence of latent TB reactivation, a major complication in individuals treated with anti-TNF therapy for inflammatory rheumatic diseases (Gardam et al., 2003), has also provided clinical data that supports the role of CTL in mediating control of *M.tb* infection. Bruns et al. demonstrated that lymphocyte expression of perforin and granulysin decreased significantly within two weeks of rheumatoid arthritis and ankylosing spondylitis patients starting anti-TNF therapy. Decrease in the expression of these cytotoxic molecules was associated with

a loss of CD8+ T_{EMRA} cells, which had the highest lysing capacity against *M.tb* infected monocytes. These data suggest that CTL play a role in maintaining latent infection in healthy *M.tb* infected individuals and that induction of CTL through vaccination contributes to protective immunity.

Qualitative analysis of Ki67+ T cells revealed that the proportion of BCG-specific T cells with the capacity to express cytotoxic effector molecules increases with age. Despite this, a large subset of BCG-specific CD4+ T cells did not express cytotoxic molecules throughout the follow-up period. This subset most likely represents a population of T cells that express Th1 cytokines or effector/regulatory molecules not measured in this study. Although the majority of CD4+ T cells did not express perforin, the proportion of CD8+ T cells expressing this cytolytic protein increased with age. It has previously been shown that the expression of perforin correlates directly with CTL lysing capacity (Harari et al., 2009). The secretion of serine proteases or granulysin together with perforin, which facilitates the delivery of these molecules into target cells, is required for maximum cytotoxic activity (Stenger et al., 1998; Shi et al., 2005). An important question that was not addressed in this study was whether BCG-specific CTL can degranulate and if the observed increase in cytotoxic molecule expression over time is associated with improved killing capacity.

It is important to note that a novel mechanism of perforin-mediated killing independent of the granule exocytosis pathway was recently described (Makedonas et al., 2009). Makedonas et al. demonstrated that following degranulation, perforin is up-regulated in the absence of T cell proliferation and traffics directly to the immunological synapse. Newly synthesised perforin

can be detected by using an antibody (δG9 clone) that detects both granule-associated and granule-independent forms of perforin (Hersperger et al., 2008). The antibody (B-D48 clone) used in this study only detects granule-associated perforin and not newly synthesised perforin, which may define a novel correlate of cytotoxic capacity (Hersperger et al., 2008; Hersperger et al., 2010; Makedonas et al., 2010).

Previous studies, which combined phenotypic classification of T cells with the analysis of cytotoxic molecule expression, have shown a relationship between expression of cytotoxic molecules and T cell differentiation. Expression of cytotoxic molecules was associated with T cells of a more mature phenotype, whereas naïve T cells did not express cytotoxic molecules (Kovaiou et al., 2005; Chowdhury and Lieberman, 2008; Chattopadhyay et al., 2009). In agreement with previous studies, the expression of cytotoxic molecules in our study was associated with the loss of CD27 and CD28. Early after vaccination most BCG-specific T cells did not express cytotoxic effector molecules. Together these data imply that changes in cytotoxic expression profiles over time are related to a phenotypic shift from an early to a more mature differentiation stage.

As discussed in chapter 5, differentiation of T cells is driven by numerous mechanisms including TCR triggering, co-stimulation, cytokine levels, homeostatic turnover and inflammation. Memory T cell populations specific to antigens that are efficiently cleared, such as influenza and TT, predominantly retain an early differentiation phenotype (Stubbe et al., 2008; Harari et al., 2009). T cells specific to antigens that persist or to viruses that are poorly controlled, such as HIV, predominantly express a mature (or late

differentiation) phenotype (Appay et al., 2002a; Marchant et al., 2003; Stubbe et al., 2008; Harari et al., 2009). The accumulation of highly differentiated CD4+ and CD8+ T cells within the total T cell compartment is also observed in infants infected with CMV compared to healthy controls (Marchant et al., 2003; Miles et al., 2008). These data suggest that one of the main factors driving T cell differentiation is antigen persistence. Thus, the question arises whether the observation of BCG-specific T cells with a more differentiated phenotype is driven by continuous TCR triggering, either through the persistence of BCG or through exposure to cross-reactive environmental mycobacteria. This question is still difficult to address as all infants in South Africa are BCG vaccinated within 48 hours of birth. Therefore an unvaccinated control cohort in which environmental mycobacteria can be studied is not available. Interestingly, the accumulation of differentiated T cells was observed within the total CD8+ T cell compartment in addition to BCG–specific T cells. This observation suggests that other factors such as homeostatic T cell turnover, inflammation, exposure to other vaccine or environmental antigens and age-associated accumulation of more differentiated CD8+ T cells contribute to this observation (Geginat et al., 2003; Schonland et al., 2003; Chiu et al., 2006; van Gent et al., 2009).

Numerous studies have characterised the BCG-specific T cell response in humans, however this is the first comprehensive study monitoring quantitative and qualitative longitudinal changes following vaccination of infants. The results presented here provide novel insights into the kinetic of cytotoxic effector molecule expression and differentiation phenotypes of T cells following BCG vaccination. This data is instrumental for guiding novel TB

vaccination strategies based on boosting BCG-specific responses in infants and for comparative studies of potential vaccine candidates.

Chapter 8

8.1. General discussion

BCG is the most widely administered vaccine on the EPI schedule. Despite this, our knowledge of T cell responses induced after BCG vaccination of infants is limited. Since infants will be targets of novel TB vaccines a better understanding of the immune response induced by BCG is critical for developing novel strategies to improve vaccination against TB. Previous studies that have characterised BCG-specific T cell responses in infants have been limited, often because the methodologies employed did not allow for detailed characterisation of specific T cell responses. For example, the memory phenotype of BCG-specific Th1 cells was not known (Vekemans et al., 2001; Ota et al., 2002; Davids et al., 2006). The availability of new fluorescent dyes and development of PFC has facilitated detailed dissection of cell-mediated immunity induced through vaccination and infection (Harari et al., 2004; Harari et al., 2005; Beveridge et al., 2007; Precopio et al., 2007). Based on these studies it has become clear that previous methods used to assess T cell function have underestimated the complexity of antigen-specific responses. To characterise T cell responses following BCG vaccination in detail, we developed and optimised novel PFC reagent panels to address many gaps in our knowledge of T cell mediated immune responses. These included BCG-induced T cell cytokine and cytotoxic molecule expression, T cell memory phenotype and differentiation stage. These methodologies were also applied to characterising kinetics of specific T cell responses over the first year of life following BCG vaccination in infancy.

As described in chapter 4, cross-sectional analysis of a 10-14 week old infant cohort showed that BCG vaccination induces distinct T cell subsets that co-express Th1 cytokines in multiple combinations. This is an important finding because IFN-γ production is typically measure to diagnose latent infection via IFN-γ release assays (Mahomed et al., 2006; Janssens et al., 2007) or to describe human immune responses to novel TB vaccines (McShane et al., 2004). Furthermore, previous studies have shown that polyfunctional T cells display enhanced effector functions and are associated with slow progression of disease or no progression in individuals with chronic viral infections (Betts et al., 2006; Kannanganat et al., 2007b). Despite these observations, recent studies in mice and humans have shown that the frequency of mycobacteria-specific polyfunctional Th1 cells in peripheral blood does not correlate with protection against TB (Tchilian et al., 2009; Kagina et al., 2010). The findings in our study strongly support measurement of additional T cell cyokines and functions that may contribute to vaccine-mediated protection (Combadiere et al., 2004; Murray et al., 2006).

In addition to Th1 cytokines, T cells with cytolytic capacity contribute to mycobacteria-specific protective immunity through their capacity to kill infected target cells and have been suggested to play a role in maintaining latent infection in healthy *M.tb* infected individuals (Andersson et al., 2007; Bruns et al., 2009). The data in chapter 7 confirm previous findings that BCG vaccination of infants induces CTL that have the capacity to up-regulate GrmB, granulysin and perforin. In addition, the proportion of BCG-specific T cells expressing cytotoxic molecules increased over time and this was associated with the differentiation of T cells towards a more mature

phenotype. These findings are consistent with previous reports showing that the expression of cytotoxic effector molecules positively correlates with cellular differentiation and maturity (Appay et al., 2002a; Marchant et al., 2003; Chattopadhyay et al., 2009; Harari et al., 2009; Cellerai et al., 2010). An important question still to be addressed is whether BCG-specific CTL are functional and if the observed increase in cytotoxic molecule expression over time translates into improved killing capacity. As discussed in chapter 7, higher expression of perforin and GrmB correlates with the enhanced ability of virus-specific T cells to lyse infected target cells (Migueles et al., 2002; Harari et al., 2009; Hersperger et al., 2010).

Based on the data discussed above we can conclude that BCG vaccination induces cytokines and cytotoxic molecules which have been shown to control *M.tb*, and are thus likely to contribute to protective efficacy of BCG observed during infancy. However, most adults vaccinated during infancy will not be protected against pulmonary TB suggesting that protection mediated by BCG-specific memory T cells gradually wanes with age (Donald et al., 2010). The primary goal of vaccination is to induce long-lived antigen-specific T cells that respond rapidly upon pathogen re-encounter. Many factors influence the development of long-lived memory T cells, antigen load and duration of antigen exposure may be the most significant (Iezzi et al., 1998; Wherry et al., 2004). The development and stability of long-lived memory T cell populations following transient antigen exposure has been clearly demonstrated in experimental models of infection and following immunisation of humans with live viral vaccines (Hammarlund et al., 2003; Wherry et al., 2003; Wherry et al., 2003; Combadiere et al., 2004; Wherry et al., 2004; Cellerai et al., 2007;

Miller et al., 2008). Studies from three highly effective vaccines against tetanus, yellow fever and smallpox have shown that a stable long-lived memory T cell population may be established as early as 30 days after vaccination (Cellerai et al., 2007; Miller et al., 2008). The work completed in chapter 4 showing that BCG-specific T cells express a pre-dominantly T_{EM} phenotype 10-14 weeks after vaccination suggests that antigen may persist. We hypothesise that mycobacteria-specific T cell responses measured at 10-14 weeks of age are likely to be BCG-specific, because exposure to environmental mycobacteria is typically low before 10-14 weeks of age (Kagina et al., 2009). In mice, BCG induces a chronic infection that results in persistent immune activation (Dudani et al., 2002). Persistence of BCG in the murine host thus establishes a mycobacteria-specific CD4+ and CD8+ T cell response that expresses a predominantly T_{EM} phenotype. Only a small proportion of specific cells express a T_{CM} phenotype in this model (Dudani et al., 2002; Henao-Tamayo et al., 2010). Therefore, we propose that the low protective efficacy of BCG against pulmonary disease in adults may relate to diminished memory T cell quality and/or population size. Orme and colleagues have proposed that the primary T_{EM} pool established following BCG vaccination is able to confer protection to the host for a few decades (Orme, 2010). However, age-related T_{EM} attrition or exhaustion, driven by BCG persistence or through repeated exposure to cross-reactive mycobacteria, leads to a gradual decline in vaccine-mediated protection (Henao-Tamayo et al., 2010; Orme 2010). These effects are thought to be one of the contributing factors that result in ineffective immunity against *M.tb* eventually leading to reactivation of *M.tb* infection in the adult population. The

kinetic studies reported in chapter 7 support this “repeated antigen exposure” hypothesis. BCG-specific T cells clearly differentiated towards a more mature phenotype over the first year of life. This has important implications for prime-boost strategies, because differentiation of T_{CM} or T_{EM} subsets are governed by antigen load and duration of antigen exposure (Masopust et al., 2006; Wirth et al., 2010). T_{CM} are typically established following a brief period of antigenic stimulation during priming (Cellerai et al., 2007; Miller et al., 2008). By contrast, excessive antigenic stimulation favours commitment of T_{EM} and impairs the development of long-lived memory cells (Champagne et al., 2001; Kaech et al. 2002; Wherry et al., 2004; Golden-Mason et al., 2006; Wherry et al., 2007; Lv et al., 2010). Whether the accumulation of differentiated T cells over the first year of life is driven by persistence of BCG or through cross-reactive environmental mycobacteria is not known.

Other mechanisms such as homeostatic proliferation and vaccine-induced or infection mediated inflammation are likely to contribute to the differentiation of T cells. The direct *ex vivo* studies of T cell differentiation in chapter 5 suggest that these mechanisms contribute substantially to the marked functional and phenotypic alterations of T cells over the first year of life. Direct *ex vivo* analysis of CD27 and CD28 expression revealed that after 6 weeks of age a large proportion of T cells in infants expressed a highly differentiated phenotype. The 6 EPI vaccines infants receive at 6 weeks is likely to contribute significantly to the dramatic shift in T cell differentiation phenotype at 10 weeks of age. These results contrast with a previous report showing that >80% of all CD4+ and CD8+ T cells retain CD27 expression during infancy (van Gent et al., 2009). CD27 expression was assessed on cryo-preserved

PBMC therefore differences between our study and van Gent et al. may be attributed to cryo-preservation of cells which modulates the expression of cellular surface markers (Costantini et al., 2003). As shown in chapter 7, a large proportion of T cells re-express CD27 and CD28 after 3-days in culture. Re-expression of CD27 and CD28 was antigen-independent suggesting that *in vitro* conditions and manipulation of cells substantially influences expression of these molecules. Thus, the direct *ex vivo* analysis of fixed whole blood in our study is likely to be a more accurate representation of T cell phenotypes *in vivo.*

Since the onset of mycobacteria-specific T cell responses following vaccination or infection is relatively delayed compared to live viral vaccines, the transition of T_{EM} into T_{CM} may occur after 10-14 weeks of age (Miller et al., 2008; Wolf et al., 2008). Additional longitudinal studies are currently underway in our group to establish if and when a long-lived memory T cell pool is established following BCG vaccination. Whole blood samples from the same cohort were stimulated and cryo-preserved to assess changes in T cell proliferative capacity, cytokine expression and memory phenotype over the first year of life. The Ki67 assay described in chapter 6 was specifically optimised to assess longitudinal changes in T cell proliferation, a hallmark of memory T cells and mechanism of vaccine-mediated protection (Sallusto et al., 1999; Combadiere et al., 2004). Markers of activation and those associated with long-lived memory T cells will be analysed in whole blood following short-term stimulation with BCG and TT. In addition to the classical memory T cell markers, CCR7 and CD45RA, other markers associated with long-lived T cells will also be included in this longitudinal analysis. The

rationale for this is based on the observation that CCR7 and CD45RA expression may not accurately define a true memory T cell population. For example, Cellerai and colleagues assessed CD45RA and CCR7 in addition to BCL-2 and CD127 following re-immunisation of adults with TT. Based on CD45RA and CCR7, 1-2 years after vaccination equal proportions of IL-2 expressing TT-specific cells displayed T_{EM} and T_{CM} phenotypes whilst IFN-γ expressing cells predominantly displayed a T_{EM} phenotype. By contrast, analysis of IL-2 and IFN-γ expressing T cells using BCL-2 and CD127 revealed that all cytokine expressing TT-specific T cells displayed a long-lived memory phenotype, based on dual expression of BCL-2 and CD127 (Cellerai et al., 2007).

Heterologous prime-boost vaccination regimens aim to boost the BCG-primed response with novel subunit vaccines. These approaches aim to enhance vaccine mediated protective efficacy. Boosting T cell responses during the effector phase of the vaccine response may lead to T cell exhaustion and death or may drive the generation of predominantly short-lived antigen-specific effector cells. Expansion of a long-lived T_{CM} population under conditions of excessive antigenic stimulation and inflammation is unlikely (Wherry 2003; Dey et al., 2009). Antigen-specific responses should ideally be boosted after a stable memory T cell population has been established (Wherry et al., 2004; Wrammert et al., 2009). In chapter 7, longitudinal analysis of the frequency of BCG-specific cells and cytotoxic molecule expression showed that the expansion and contraction of primary BCG-specific T cell responses occurs within the first 3 months of life and that specific responses peak 6 weeks after vaccination. Although the transition of

T_{EM} into T_{CM} was not measured, the kinetic data discussed above suggest that we can reasonably conclude that BCG-specific responses could ideally be boosted with heterologous vaccines after 14 weeks of age, following the contraction phase (**Figure 1**). McShane and colleagues reported that the magnitude of the specific T cell response in adults is not influenced by the time interval between BCG-prime and MVA85A-boost (Pathan et al., 2007). Vaccine-specific responses were measured by IFN-γ ELISPOT only therefore, the effects of boosting BCG-primed responses at different intervals on the expression of other relevant cytokines and on differentiation of appropriate memory T cell subsets could not be determined.

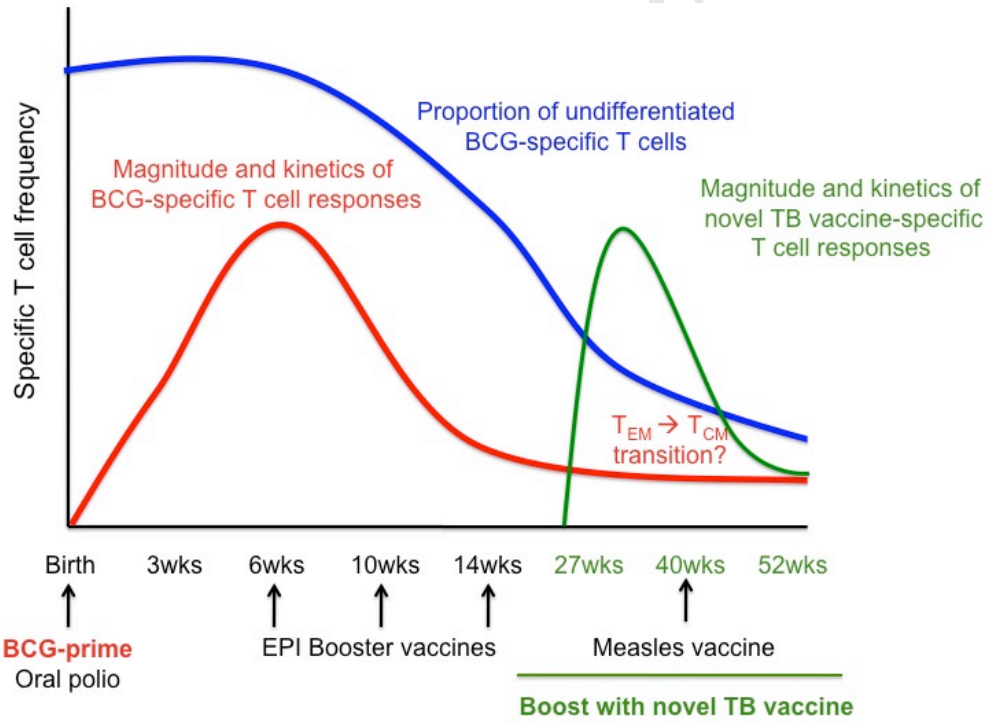

Figure 1. Proposed model describing specific T cell kinetics based on a BCG-prime novel vaccine-boost strategy.

A potential caveat in the interpretation of the BCG-speific kinetic data in our study is related to the analysis of Ki67 following stimulation of whole blood for

3 days. In chapter 6, quantification of the kinetics of Ki67 expression following antigen-stimulation demonstrated that a small subset of T cells began to cycle and express Ki67 after 3 days. The observed T cell kinetics may be different if a different assay system, such as a short-term whole blood assay, was used. The kinetics of vaccine-specific T cell responses in adults have been previously measured by assessing markers of activation and Ki67 directly *ex vivo* (Cellerai et al., 2007; Miller et al., 2008), as shown in chapter 5. However, using a similar approach to monitor antigen-specific T cell responses in infants is challenging due to increased *in vivo* T cell turnover over the first year of life.

Collectively, the data presented in this thesis serves as a basis for understanding the development of BCG-specific immunity in humans. These data provide a framework for optimising prime-boost vaccination strategies and are useful as a comparative model for assessing T cell responses following immunisation with novel TB vaccine candidates. Importantly, longitudinal changes in BCG-specific T cell differentiation phenotype suggest that low-level antigen exposure through persistence of BCG, or exposure to cross-reactive mycobacteria, results in continuous activation of specific T cells. This mimics chronic infection, which prevents the development of long-lived memory T cells. These observations support the hypothesis recently proposed by Orme and colleagues, suggesting that BCG vaccination is a poor inducer of long-lived memory T cells (Henao-Tamayo et al., 2010; Orme, 2010) and have important implications for optimising BCG-based vaccination strategies. A potential solution for this limitation would be to treat infants with antibiotics a few weeks after BCG vaccination to eliminate persisting bacteria

thus promoting the transition of T_{EM} into long-lived T_{CM} (Kamath et al., 2006). Colleagues from SATVI recently proposed a novel vaccination regimen based on priming with non-replicating viral-vectored or subunit vaccines and boosting with BCG at a later time point (**Figure 2**) (Hatherill et al., 2010).

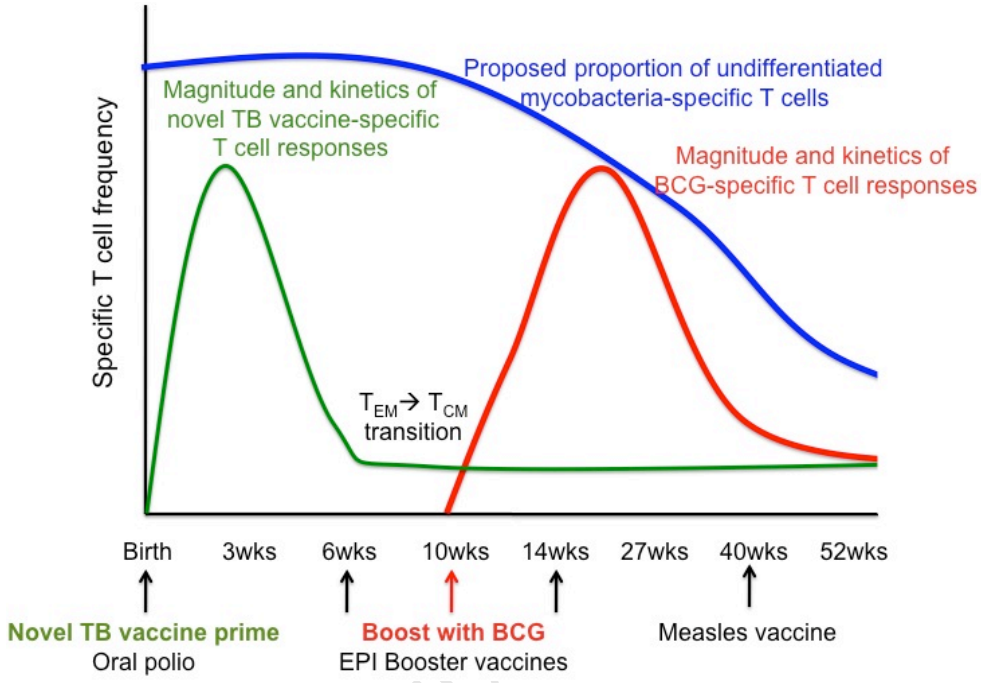

Figure 2. Proposed model describing specific T cell kinetics based on a novel TB vaccine-prime BCG-boost strategy.

This approach represents a safer option for HIV infected infants who have an increased risk of developing BCG-disease and other complications following BCG vaccination (Hesseling et al., 2007). These vaccination strategies based on priming with a novel subunit vaccine confer a similar level of protection in experimental TB challenge models compared with BCG-prime heterologous boost (Skinner et al., 2005; Romano et al., 2006). This alternative approach not only represents a feasible strategy to circumvent complications arising from BCG persistence in HIV-infected infants but may also represent a novel

solution for the induction of long-lived mycobacteria-specific immunity (Hesseling et al., 2007; Hesseling et al., 2008; Hatherill et al., 2010).

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Appendices

Appendix A

Instrument configuration. Fluorochromes assigned to each laser line and PMT on the SATVI LSR II. Fluorochrome Maximum emission wavelengths of fluorochromes, filter and mirror combinations for each PMT.

Laser excitation line (nm)	Fluorochrome	Maximum emission wavelength (nm)	Detector	Bandpass filter	Dichroic longpass mirror
488 (Blue)	PE-Cy7	785	A	780/60	735
	PerCP-Cy5.5	694	B	695/40	685
	PerCP	675	C	670/14	635
	Alexa 610 PE	628	D	610/20	600
	PE	578	E	575/26	550
	FITC	520	F	530/30	505
	Alexa Fluor 488	520	F	530/30	505
	SSC		G	488/10	Blank
	-		H	Blank	Blank

Appendix A (continued)

Instrument configuration. Fluorochromes assigned to each laser line and PMT on the SATVI LSR II. Fluorochrome Maximum emission wavelengths of fluorochromes, filter and mirror combinations for each PMT.

Laser excitation line (nm)	Fluorochrome	Maximum emission wavelength (nm)	Detector	Bandpass filter	Dichroic longpass mirror
633 (Red)	APC-Cy7	767	A	780/60	735
	Alexa Fluor 700	723	B	720/40	690
	APC	660	C	660/20	Blank
	Alexa Fluor 647	660	C	660/20	Blank
405 (Violet)	QDot 655	655	A	655/8	630
	QDot 605	605	B	605/12	505
	Amcyan	491	C	525/50	Blank
	Pacific Blue	455	C	440/40	Blank

Appendix B

Weeks	Frequency	Percentage	Cumulative
3 + 6	6	9.09	9.09
3 + 10	16	24.24	33.33
3 + 14	16	24.24	57.58
6 + 10	6	9.09	66.67
6 + 14	16	24.24	90.91
10 + 14	6	9.09	100.00
Total	66	100	

Weeks	Frequency	Percentage	Cumulative
27 + 40	22	33.33	33.33
27 + 52	22	33.33	66.67
40 + 52	22	33.33	100
Total	66	100	

Appendix C

The following tables outline the random allocation of infant participants to specific bleeding schedules (time given in weeks) and proposed volumes of blood drawn at each time point.

Participant	Bleed 1 (Week)	Bleed 1 blood Volume (mL)	Bleed 2 (Week)	Bleed 2 blood Volume (mL)	Bleed 3 (Week)	Bleed 3 blood Volume (mL)	Bleed 4 (Week)	Bleed 4 blood Volume (mL)	Maximum cumulative volume (mL)
1	3	6	14	10	40	30	52	30	76
2	3	6	10	6	40	30	52	30	72
3	6	6	10	6	27	21	40	30	63
4	3	6	10	6	27	21	40	30	63
5	3	6	6	6	27	21	52	30	63
6	3	6	10	6	40	30	52	30	72
7	6	6	14	10	27	21	40	30	67
8	3	6	10	6	27	21	52	30	63
9	3	6	6	6	27	21	40	30	63
10	3	6	10	6	40	30	52	30	72
11	10	6	14	10	40	30	52	30	76
12	3	6	14	10	27	21	52	30	67
13	6	6	14	10	27	21	52	30	67
14	6	6	14	10	27	21	40	30	67
15	10	6	14	10	27	21	52	30	67
16	3	6	10	6	40	30	52	30	72

Participant	Bleed 1 (Week)	Bleed 1 blood Volume (mL)	Bleed 2 (Week)	Bleed 2 blood Volume (mL)	Bleed 3 (Week)	Bleed 3 blood Volume (mL)	Bleed 4 (Week)	Bleed 4 blood Volume (mL)	Maximum cumulative volume (mL)
17	6	6	14	10	27	21	52	30	67
18	3	6	14	10	40	30	52	30	76
19	3	6	14	10	27	21	40	30	67
20	6	6	14	10	27	21	52	30	67
21	3	6	14	10	27	21	40	30	67
22	6	6	14	10	27	21	52	30	67
23	3	6	6	6	27	21	52	30	63
24	6	6	14	10	40	30	52	30	76
25	3	6	10	6	27	21	52	30	63
26	3	6	14	10	40	30	52	30	76
27	6	6	14	10	27	21	52	30	67
28	6	6	10	6	27	21	52	30	63
29	3	6	14	10	27	21	40	30	67
30	3	6	10	6	27	21	40	30	63
31	10	6	14	10	27	21	40	30	67
32	3	6	10	6	40	30	52	30	72
33	3	6	10	6	27	21	40	30	63
34	6	6	14	10	27	21	52	30	67
35	3	6	10	6	40	30	52	30	72
36	3	6	10	6	40	30	52	30	72

Participant	Bleed 1 (Week)	Bleed 1 blood Volume (mL)	Bleed 2 (Week)	Bleed 2 blood Volume (mL)	Bleed 3 (Week)	Bleed 3 blood Volume (mL)	Bleed 4 (Week)	Bleed 4 blood Volume (mL)	Maximum cumulative volume (mL)
37	6	6	14	10	27	21	40	30	67
38	3	6	6	6	27	21	40	30	63
39	3	6	14	10	27	21	40	30	67
40	10	6	14	10	40	30	52	30	76
41	3	6	14	10	40	30	52	30	76
42	6	6	10	6	27	21	52	30	63
43	6	6	14	10	27	21	52	30	67
44	6	6	14	10	40	30	52	30	76
45	3	6	14	10	40	30	52	30	76
46	3	6	10	6	27	21	52	30	63
47	6	6	14	10	40	30	52	30	76
48	3	6	10	6	40	30	52	30	72
49	3	6	10	6	40	30	52	30	72
50	6	6	10	6	40	30	52	30	72
51	6	6	14	10	27	21	40	30	67
52	6	6	14	10	27	21	40	30	67
53	10	6	14	10	27	21	52	30	67
54	3	6	14	10	27	21	40	30	67
55	3	6	14	10	27	21	40	30	67
56	3	6	6	6	27	21	52	30	63

57	3	6	10	6	40	30	52	30	72
Participant	Bleed 1 (Week)	Bleed 1 blood Volume (mL)	Bleed 2 (Week)	Bleed 2 blood Volume (mL)	Bleed 3 (Week)	Bleed 3 blood Volume (mL)	Bleed 4 (Week)	Bleed 4 blood Volume (mL)	Maximum cumulative volume (mL)
58	3	6	14	10	27	21	52	30	67
59	3	6	14	10	27	21	40	30	67
60	6	6	14	10	27	21	52	30	67
61	3	6	14	10	27	21	40	30	67
62	6	6	10	6	40	30	52	30	72
63	10	6	14	10	27	21	52	30	67
64	3	6	14	10	27	21	40	30	67
65	3	6	6	6	27	21	52	30	63
66	6	6	10	6	27	21	40	30	63
67	3	6	14	10	40	30	52	30	76
68	3	6	10	6	40	30	52	30	72
69	6	6	10	6	27	21	40	30	63
70	3	6	10	6	27	21	40	30	63
71	3	6	6	6	27	21	52	30	63
72	3	6	10	6	40	30	52	30	72
73	6	6	14	10	27	21	40	30	67
74	3	6	10	6	27	21	52	30	63
75	3	6	6	6	27	21	40	30	63
76	3	6	10	6	40	30	52	30	72

77	10	6	14	10	40	30	52	30	76
Participant	Bleed 1 (Week)	Bleed 1 blood Volume (mL)	Bleed 2 (Week)	Bleed 2 blood Volume (mL)	Bleed 3 (Week)	Bleed 3 blood Volume (mL)	Bleed 4 (Week)	Bleed 4 blood Volume (mL)	Maximum cumulative volume (mL)
78	3	6	10	6	27	21	52	30	63
79	3	6	6	6	27	21	40	30	63
80	3	6	10	6	40	30	52	30	72
81	10	6	14	10	40	30	52	30	76
82	3	6	14	10	27	21	52	30	67
83	6	6	14	10	27	21	52	30	67
84	6	6	14	10	27	21	40	30	67
85	10	6	14	10	27	21	52	30	67
86	3	6	10	6	40	30	52	30	72
87	6	6	14	10	27	21	52	30	67
88	3	6	14	10	40	30	52	30	76
89	3	6	14	10	27	21	40	30	67
90	6	6	14	10	27	21	52	30	67

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Bacillus Calmette-Guérin Vaccination of Human Newborns Induces T Cells with Complex Cytokine and Phenotypic Profiles[1]

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The immune response to vaccination with bacillus Calmette-Guérin (BCG), the only tuberculosis vaccine available, has not been fully characterized. We used multiparameter flow cytometry to examine specific T cell cytokine production and phenotypic profiles in blood from 10-wk-old infants routinely vaccinated with BCG at birth. Ex vivo stimulation of whole blood with BCG for 12 h induced expression of predominantly IFN-γ, IL-2, and TNF-α in CD4$^+$ T cells in seven distinct cytokine combinations. IL-4 and IL-10 expression was detected in CD4$^+$ T cells at low frequencies and only in cells that did not coexpress type 1 cytokines. Specific CD8$^+$ T cells were less frequent than CD4$^+$ T cells and produced mainly IFN-γ and/or IL-2 and less TNF-α, IL-4, and IL-10. Importantly, many mycobacteria-specific CD4$^+$ and CD8$^+$ T cells did not produce IFN-γ. The predominant phenotype of BCG-specific type 1 T cells was that of effector cells, i.e., CD45RA$^-$CCR7$^-$CD27$^+$, which may reflect persistence of *Mycobacterium bovis* BCG in infants until 10 wk of age. Among five phenotypic patterns of CD4$^+$ T cells, central memory cells were more likely to be IL-2$^+$ and effector cells were more likely to be IFN-γ^+. We concluded that neonatal vaccination with BCG induces T cells with a complex pattern of cytokine expression and phenotypes. Measuring IFN-γ production alone underestimates the magnitude and complexity of the host cytokine response to BCG vaccination and may not be an optimal readout in studies of BCG and novel tuberculosis vaccination. ***The Journal of Immunology,*** **2008, 180: 3569–3577.**

Nearly one-third of the global population is latently infected with *Mycobacterium tuberculosis* and ~2 million people die of tuberculosis (TB)[3] disease every year (Tuberculosis Fact Sheet, 2005, World Health Organization, Geneva, Switzerland). Bacillus Calmette-Guérin (BCG), the only vaccine against TB currently available, has variable efficacy in preventing pulmonary disease (1), but 80% efficacy in preventing childhood miliary disease and meningitis (2). Our knowledge of immunity induced by BCG vaccination is incomplete, particularly after human newborn vaccination. However, infants will be targets of novel, safer, and more efficacious TB vaccines in the future and a better understanding of the immune response induced by newborn BCG vaccination is likely to facilitate development of improved vaccines.

Experimental evidence suggests that both CD4$^+$ and CD8$^+$ T cells are important for protection against mycobacteria (3–6). In humans, the role of CD4$^+$ T cells has been highlighted by a increased risk of disease after infection with *M. tuberculosis* when CD4$^+$ T cell numbers decline in HIV-infected persons (7). A primary function of both CD4$^+$ and CD8$^+$ T cells is to produce the type 1 cytokine IFN-γ. The critical role of this cytokine has been demonstrated by characteristic severe mycobacterial disease in patients with mutations of the IFN-γ receptor (8, 9). Other type 1 cytokines, such as TNF-α and IL-2, may also be important in protection against TB: the role of TNF-α has been underscored by high rates of reactivation of latent TB following treatment of rheumatoid arthritis patients with specific inhibitors of this cytokine (10, 11); T cell IL-2 expression has been associated with long-term memory (12, 13), which is the aim of protective immunity. BCG vaccination of human newborns does indeed induce specific CD4$^+$ and CD8$^+$ T cells capable of producing IFN-γ (14–16) Previous studies have also shown that BCG vaccination of infants induces TNF-α, which is detectable in plasma by ELISA (17). However, the pattern of production of all type 1 cytokines, on a single cell basis, has not been delineated.

Our aim was to also describe expression of type 2 cytokines such as IL-4, thought to reflect a suboptimal immune response to mycobacteria (18, 19). Although BCG vaccination of infants has been shown to induce low levels of type 2 cytokines (15, 16, 20), the detection was in plasma, and cell-associated expression has not

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[3] Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; SEB, streptococcal enterotoxin B.

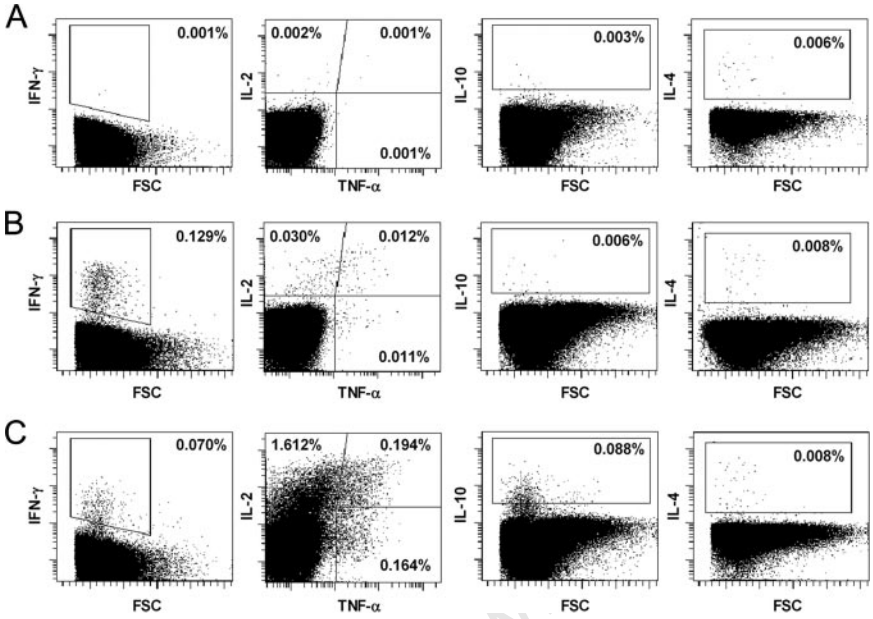

FIGURE 1. Flow cytometric detection of $CD4^+$ T cell cytokine expression in whole blood incubated with BCG for 12 h from a single 10-wk-old BCG-vaccinated infant. The cutoff gates for cytokine expression were determined using unstimulated T cells from whole blood incubated with costimulants only (*A*). Cytokine expression in $CD4^+$ T cells from whole blood incubated with BCG (*B*) and with SEB (*C*) is shown. IL-2/TNF-α subset gating was based on patterns of cytokine expression in SEB-stimulated whole blood. Dot plots are gated on $CD3^+$ $CD4^+$ T cells and are representative of 29 infants.

been reported. We also wished to assess T cell IL-10 expression, since this cytokine is likely to be an important regulator of effector T cell responses against TB (21) and is induced by newborn BCG vaccination (15, 16, 22).

The memory phenotype of T cells induced by BCG vaccination of newborns has not been described. Ag-experienced cells may be categorized based on expression of surface markers (13, 23–26). Central memory cells express CCR7 but not CD45RA and are likely to represent a long-lived population, which expands rapidly in lymph nodes following subsequent Ag encounter (23, 27). In contrast, effector cells are both $CCR7^-$ and CD45RA and act immediately following Ag exposure, but have limited proliferative capacity (13, 23). A third subset, terminally differentiated memory cells are $CD45RA^+$ and $CCR7^-$ and the most differentiated subpopulation, based on short telomere length and function (26, 28). Naive or non-Ag-experienced T cells characteristically express both CD45RA and CCR7 (13, 26, 28). Combination of markers other than CCR7 and CD45RA may also differentiate subsets of Ag-experienced cells. Fritsch et al. (26) recently proposed the phenotypic classification of $CD4^+$ T cell populations based on expression of CD27 and CCR7. Central memory T cells were defined as $CD27^+$ and $CCR7^+$, effectors as $CD27^+$ and $CCR7^-$, and terminally differentiated T cells as $CD27^-$ and $CCR7^-$. Our aim was to evaluate expression of all of these markers among Ag-experienced T cells induced by BCG. Although several investigators have characterized mycobacteria-specific immune responses by four-color flow cytometry (29–31), these studies could only measure two cytokines or phenotypic markers at a time and thus most likely underestimated the complexity of the response.

Our hypothesis was that BCG vaccination of newborns would induce both $CD4^+$ and $CD8^+$ T cells capable of producing multiple cytokines and that a central memory phenotype of specific cells would be dominant 10 wk after vaccination. We used an intracellular cytokine assay with multiparameter flow cytometry to comprehensively characterize these variables. To achieve our goals, we established clinical structures and optimized techniques (32) to overcome hurdles common to investigation of immunity in infants.

Materials and Methods

Study participants and blood collection

Healthy 10-wk-old infants, routinely vaccinated intradermally with BCG (Statens Serum Institut, Copenhagen, Denmark) at birth, were enrolled in the Cape Town region of South Africa. This area has a very high TB disease incidence in children 5-year old and younger, exceeding 2% per year in certain areas (32). Infants born to HIV-positive mothers, infants known to be HIV positive, infants with suspected or confirmed TB disease, infants with possible exposure to TB disease and infants with any other active or chronic illnesses at the time of enrollment were excluded. Human participation was according to the U.S. Department of Health and Human Services and good clinical practice guidelines. This included protocol approval by the University of Cape Town Research Ethics Committee and written informed consent. Sodium heparinized blood was collected from each infant. Two different cohorts were enrolled to preserve blood volume collected for assessment with two different flow cytometric protocols (see below): 29 infants to assess cytokine expression of T cells and 27 infants to assess the phenotype of IFN-γ- and IL-2-expressing T cells.

Ags and Abs

BCG was reconstituted from the vaccine vial (SSI) at 1.8×10^6 organisms/ml as previously described (32). The positive control streptococcal enterotoxin B (SEB; Sigma-Aldrich) was used at 10 μg/ml. The costimulatory Abs anti-CD28 and anti-CD49d (both from BD Biosciences) were each used at 1 μg/ml. Cytokine profiles of BCG-specific T cells were examined using the following conjugated Abs: anti-CD3 Amcyan (SK7) and anti-IL-2 Alexa 610-PE (5344.111), both custom-conjugated at BD Biosciences; and anti-CD4 Alexa Fluor 700 (RPAT4), anti-CD8 Cy5.5-PerCP (SK1), anti-IL-4 FITC (MP4-25D2), anti-IFN-γ PE (25723.11), anti-IL-10 allophycocyanin (JES3-19F1), and anti-TNF-α Cy7-PE (MAb11), all obtained from BD Biosciences. A separate protocol was used for assessing T cell phenotypes using the following conjugated Abs: anti-CD3 PacBlu (UCHT1), anti-CD4 Cy5.5PerCP (SK3), anti-CD8 Cy5.5PerCP (SK1), anti-CD45RA Cy7PE (L48), anti-CD27 PE (MT271), anti-IFN-γ AlexaFluor 700 (B27) and anti-IL-2 FITC (5344.111), all obtained from BD Biosciences, and anti-CCR7 allophycocyanin (150503), obtained from R&D Systems.

Whole blood intracellular cytokine detection assay

To determine cell-associated cytokine production, 1 ml of heparinized whole blood was incubated with BCG and anti-CD28 and anti-CD49d as described before (32). Blood incubated with SEB and costimulants, or with costimulatory Abs alone (UNS), served as positive and negative controls, respectively. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added during the last 5 h of incubation to capture cytokines intracellularly. After a total

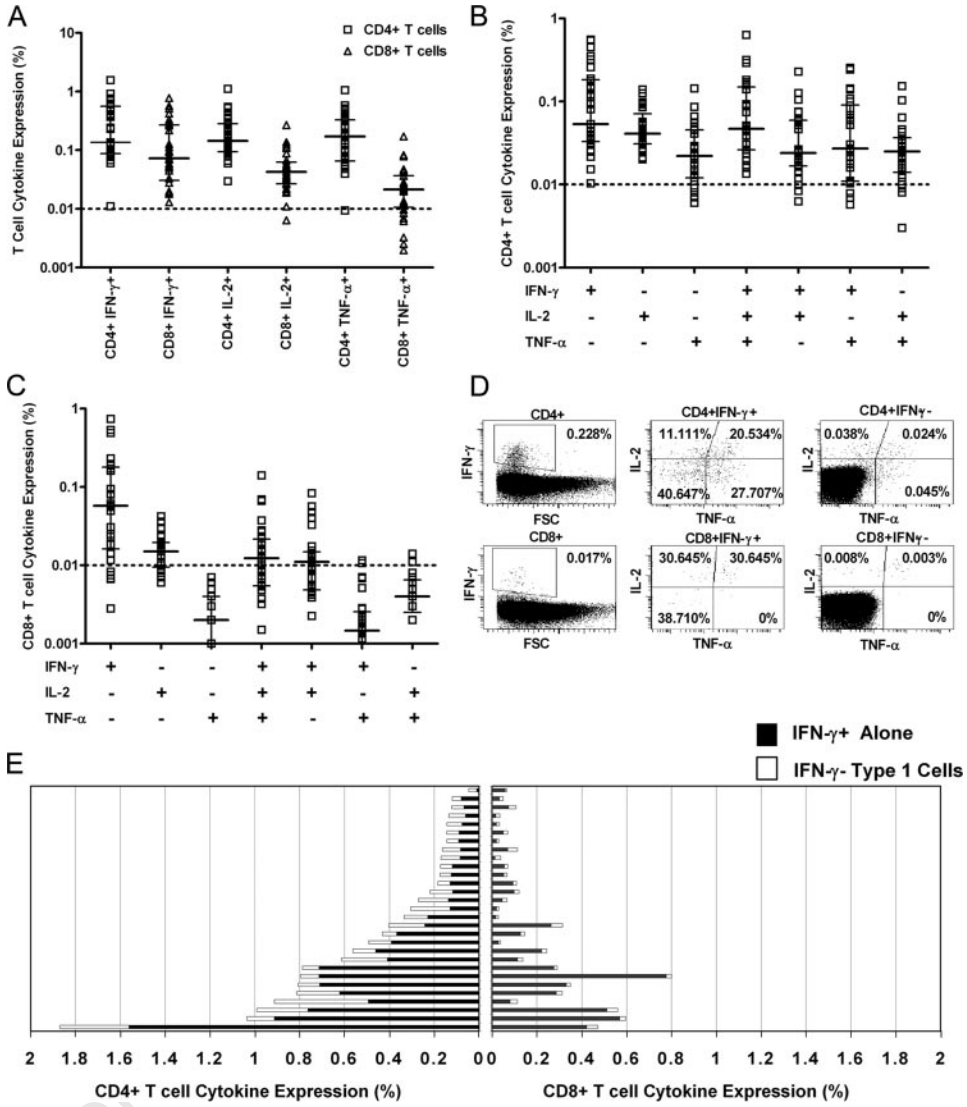

FIGURE 2. Cytokine profiles of BCG-specific T cells in 10-wk-old infants vaccinated at birth. *A,* Frequency of $CD4^+$ and of $CD8^+$ T cells expressing individual type 1 cytokines following incubation of blood with BCG for 12 h in 29 infants. Responses above 0.01% were considered positive. The horizontal line indicates the median and the whiskers indicate the interquartile range. *B,* Frequency of BCG-specific $CD4^+$ T cells expressing different combinations of type 1 cytokines. *C,* Frequency of BCG-specific $CD8^+$ T cells expressing different combinations of type 1 cytokines. *D,* Representative staining of intracellular type 1 cytokines in BCG-specific $CD4^+$ T cells and $CD8^+$ T cells from a single 10-wk-old infant. Cutoff gates for cytokine expression were determined using unstimulated T cells from whole blood incubated with costimulants only. IL-2/TNF-α subset gating was based on patterns of cytokine expression in SEB-stimulated whole blood. *E,* Comparison of frequency of $CD4^+$ and of $CD8^+$ T cells expressing IFN-γ (dark bars) with frequency of T cells expressing IL-2 and/or TNF-α without IFN-γ (light bars) in 29 BCG-vaccinated infants.

incubation of 12 h, RBC were lysed and white blood cells were fixed with FACS Lysing Solution (BD Biosciences), followed by cryopreservation.

Cell staining and flow cytometric analysis

To detect intracellular cytokines, cryopreserved cells were thawed, washed in PBS, permeabilized with Perm/Wash solution (BD Biosciences), and incubated at 4°C with fluorescence-conjugated Abs for 1 h. To assess T cell memory phenotypes, a two-step staining method, resulting in optimal staining, was used: cells were thawed, washed in PBS, and permeabilized with Perm/Wash solution and then incubated with surface marker Abs for 1 h at 4°C, followed by an additional hour with Abs specific for intracellular cytokines. Flow cytometric acquisition was completed on a LSRII flow cytometer (BD Biosciences) configured for 3 lasers and 12 detectors. All of the cells in the tube were acquired. Analysis was performed using FACSDiva software (BD Biosciences). Although automated compensation with mouse IgG κ beads was applied, compensation settings were assessed manually after acquisition and adjusted if necessary. Multiparameter panel development (data not shown) included evaluation of appropriate staining controls of Ab and fluorochrome interactions and of spectral overlap using control blood samples (33, 34).

Cutoffs to determine positive cytokine expression in $CD4^+$ and $CD8^+$ T cells from blood incubated with BCG were set using cells from blood incubated with costimulatory Abs alone (negative control) (Fig. 1*A*). Angled cutoff lines were necessary for some fluorochromes, because of data spread at higher fluorescence intensities, following instrument compensation (e.g., Fig. 1). SEB was an excellent positive control for induction of all cytokines, except IL-4. IL-4-expressing HICK-2 cells (BD Biosciences), processed per the manufacturer's protocol, were used as positive control for IL-4. Where distinction between positive and negative surface marker populations was not clear, isotype-matched control Ab staining was used to set cutoffs for phenotypic markers.

Plasma cytokine detection

Plasma was collected from the stimulated whole blood after 7 h and cryopreserved. Later, thawed plasma was used to measure levels of IL-2, IL-4, IL-10, and IFN-γ with multiplex beads according to the manufacturer's instructions (Bio-Rad) and read on a luminometer (Luminex). The range of detection for all cytokines was 1.95–32,000 pg/ml. The optimal plasma dilution for our assay, determined in pilot experiments, was 1/4. Background cytokine levels measured in plasma harvested from unstimulated blood were subtracted from BCG-stimulated blood.

Statistical considerations

Negative control (background) values for cytokine expression were not subtracted from BCG-induced responses, because the median backgrounds for all $CD4^+$ T cell subsets was 0.001% (range, 0.000–0.01%) and for $CD8^+$ T cell subsets was 0.000% (range, 0.000–0.01%). We used an empiric cutoff value of 0.01% as positive: given that a median of 508,509 $CD4^+$ T cells and 188,498 $CD8^+$ T cells was collected, this cutoff was predicted to be >90% different from background, at an α of 0.05 (35). IL-4 and IL-10 expression was reported with backgrounds (see below). Nonparametric tests were used to compare differences in cytokine expression and phenotypic profiles between $CD4^+$ and $CD8^+$ T cells. Associations between cellular expression of cytokines and plasma levels of these were assessed by the nonparametric Spearman test. Data were considered statistically significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism 4 software.

Table I. *Association between type 1 cytokine levels in plasma and frequencies of CD4$^+$ or CD8$^+$ T cells expressing these cytokines after incubation of whole blood with BCG*[a]

	CD4$^+$ T Cells Expressing Cytokine		CD8$^+$ T Cells Expressing Cytokine	
	r	*p*	*r*	*p*
Plasma IFN-γ	0.6793	<0.0001	0.6305	0.0004
Plasma IL-2	0.6441	0.0003	0.6013	0.0009
Plasma TNF-α	0.4034	0.01	0.3187	0.1052

[a] A Spearman test was used to assess correlation in 29 infants.

Results

BCG-specific CD4$^+$ and CD8$^+$ T cell type 1 cytokine production

Intracellular expression of three type 1 cytokines thought to be critical for protective immunity against mycobacteria, IFN-γ, IL-2 and TNF-α (36, 37), was evaluated by incubating blood from 29 BCG-vaccinated infants with BCG for 12 h (Fig. 1). The median frequencies of CD4$^+$ T cells expressing either IFN-γ or IL-2 or TNF-α were similar (Fig. 2*A*). Lower frequencies of CD8$^+$ T cells expressed IFN-γ, IL-2 ($p < 0.05$), or TNF-α ($p < 0.0001$), compared with CD4$^+$ T cells (Fig. 2*A*). There was a strong positive correlation between the frequencies of IFN-γ- or IL-2- or TNF-α-expressing CD4$^+$ and CD8$^+$ T cells ($r = 0.770$, $r = 0.879$, $r = 0.760$, respectively, all $p < 0.0001$, Spearman test).

Analysis of simultaneous expression of IFN-γ, IL-2, and TNF-α on a single cell level revealed seven distinct type 1 cytokine-expressing CD4$^+$ T cell populations (Fig. 2*B*). Among CD8$^+$ T cells, the dominant population expressed IFN-γ only; three other populations were discernable (Fig. 2*C*). Importantly, a substantial proportion of CD4$^+$ T cells expressing IL-2 and/or TNF-α did not coexpress IFN-γ (Fig. 2*D*). Similarly, among CD8$^+$ T cells, a proportion of IL-2-expressing T cells also did not coexpress IFN-γ (Fig. 2*D*). Total type 1 T cell responses were dominated by IFN-γ expression; however, measuring IFN-γ alone did not detect all type 1 cytokine-expressing T cells (Fig. 2*E*).

All three type 1 cytokines could also be detected in plasma (data not shown). There was a significant correlation between plasma levels of IFN-γ, IL-2, and TNF-α and frequencies of CD4$^+$ T cells producing these cytokines (Table I). Plasma IFN-γ and IL-2 also correlated with frequencies of CD8$^+$ T cells producing IFN-γ and IL-2, respectively (Table I).

Taken together, we concluded that BCG vaccination of newborns induces multiple type 1 T cell subsets defined by expression of distinct cytokine combinations.

BCG-specific IL-10 and type 2 cytokine production

The frequency of T cells expressing IL-10 or IL-4 following incubation of whole blood with BCG was low (Fig. 3). Few donors had responses above 0.01%, our cutoff for a positive response, but IL-10 and IL-4 production was consistently above the background expression levels found in blood not incubated with BCG (Fig. 3, *B* and *D*). CD4$^+$ T cell expression of both cytokines was slightly higher than that of CD8$^+$ T cells (CD4$^+$ T cell expression shown in Fig. 3; CD8$^+$ T cells: median, 0.004%; range, 0.001–0.024%, expressed IL-10 and median, 0.005%; range, 0.000–0.011%, expressed IL-4). IL-10 and IL-4 were never coexpressed by cells making type 1 cytokines (Fig. 3, *A* and *C*). Intracellular IL-4 could be readily detected in HICK-2 cytokine-expressing cells, which served as positive control (Fig. 3*C*). IL-4 and IL-10 were detected at low levels in plasma of whole blood incubated with BCG (Fig. 4).

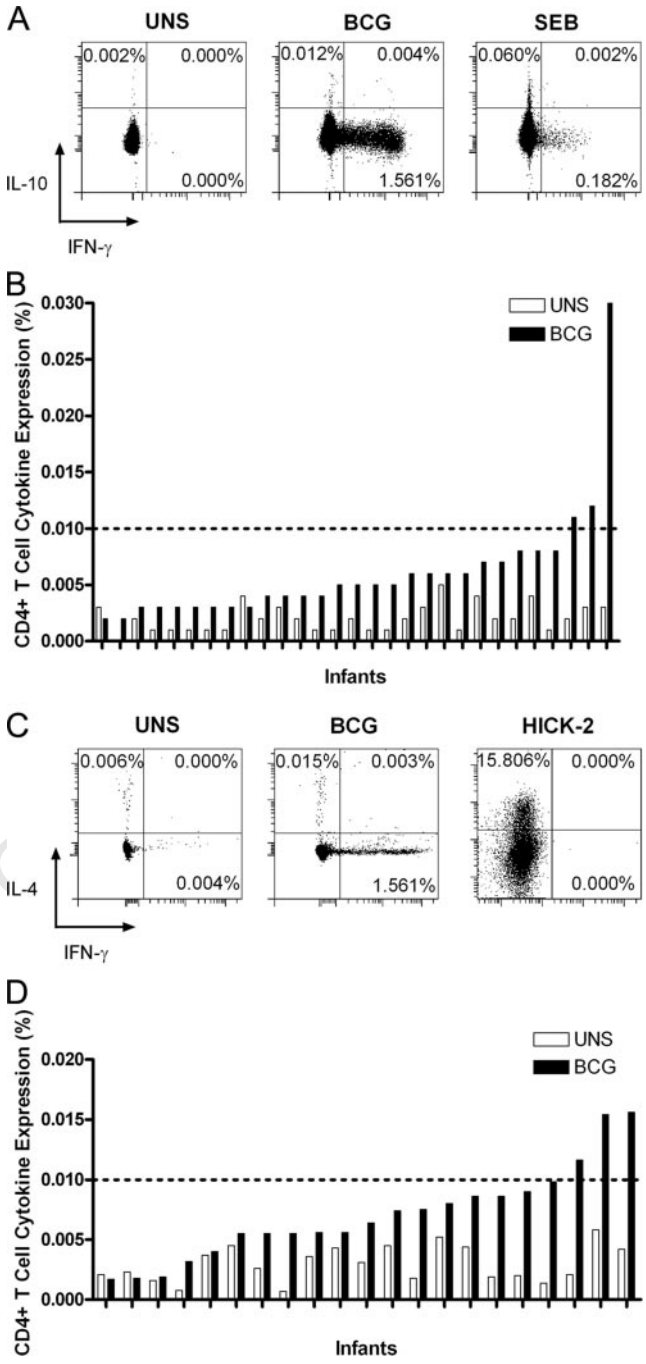

FIGURE 3. Expression of IL-10 (*A*) and IL-4 (*C*) in CD4$^+$ T cells from whole blood from a single, vaccinated infant incubated with costimulants (UNS), BCG, or SEB. Frequency of CD4$^+$ T cells expressing IL-10 (*B*) and IL-4 (*D*) following incubation of blood with costimulants only (light bars) and BCG (dark bars) for 12 h in 29 infants.

We concluded that BCG vaccination of newborns induces low levels of IL-4 and IL-10 expression.

Phenotypic profiles of specific type 1 T cell subsets

Studies in other infectious disease models have shown that distinct populations of Ag-experienced T cells may be associated with long-lived protection (38). We therefore examined the phenotypic

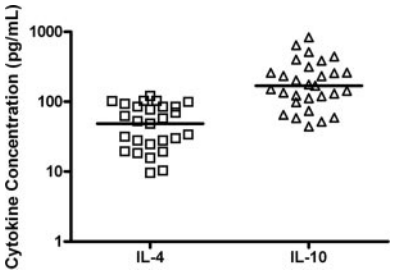

FIGURE 4. Levels of IL-4 and IL-10 in plasma from whole blood incubated with BCG for 7 h. The horizontal line represents the median. Background cytokine levels were subtracted.

profiles of BCG-induced T cells. Specific $CD4^+$ T cells were defined as either IFN-γ- or IL-2-expressing (Fig. 5, *A–D*); frequencies of cells expressing other cytokines were too low to reliably delineate the phenotype. Five major and distinct Ag-experienced $CD4^+$ T cells subsets could be discerned, based on expression of CD45RA, CCR7, and CD27 (Fig. 6, *A–C*). By far the most common phenotype of both IFN-γ- and IL-2-expressing $CD4^+$ T cells was $CD45RA^-CCR7^-CD27^+$ (Fig. 6*A*), a phenotype that has been reported to be characteristic of effector T cells (13). The second most common phenotype among IFN-γ-expressing $CD4^+$ T cells was $CD45RA^-CCR7^-CD27^-$, also characteristic of effector T cells (Fig. 6*A*). Among IL-2-expressing $CD4^+$ T cells, the latter population was significantly less frequent (Fig. 6*A*), while central memory phenotypes were more common: IL-2^+ cells were more likely to be $CD45RA^-CCR7^+CD27^+$, $CD45RA^+CCR7^-CD27^+$, or $CD45RA^+CCR7^+CD27^+$, compared with IFN-γ-expressing $CD4^+$ T cells (Fig. 6*A*). $CD4^+$ T cells that expressed both IFN-γ and IL-2 were predominantly effector memory cells ($CD45RA^-CCR7^-CD27^+$ or $CD45RA^-CCR7^+CD27^+$; Fig. 6*B*). As a comparison, the expression of phenotypic markers among cytokine-negative $CD4^+$ T cells are depicted in Fig. 6*C*.

Phenotypes of BCG-specific $CD8^+$ T cells could reliably be detected only for IFN-γ-producing cells, because the frequencies of IL-2-producing $CD8^+$ T cells were too low. $CD8^+$IFN-γ^+ T cells also displayed a predominant $CD45RA^-CCR7^-CD27^+$ effector phenotype (Fig. 6*D*). Unlike $CD4^+$IFN-γ^+ T cells, a central memory population ($CD45RA^+CCR7^+CD27^+$) was the second

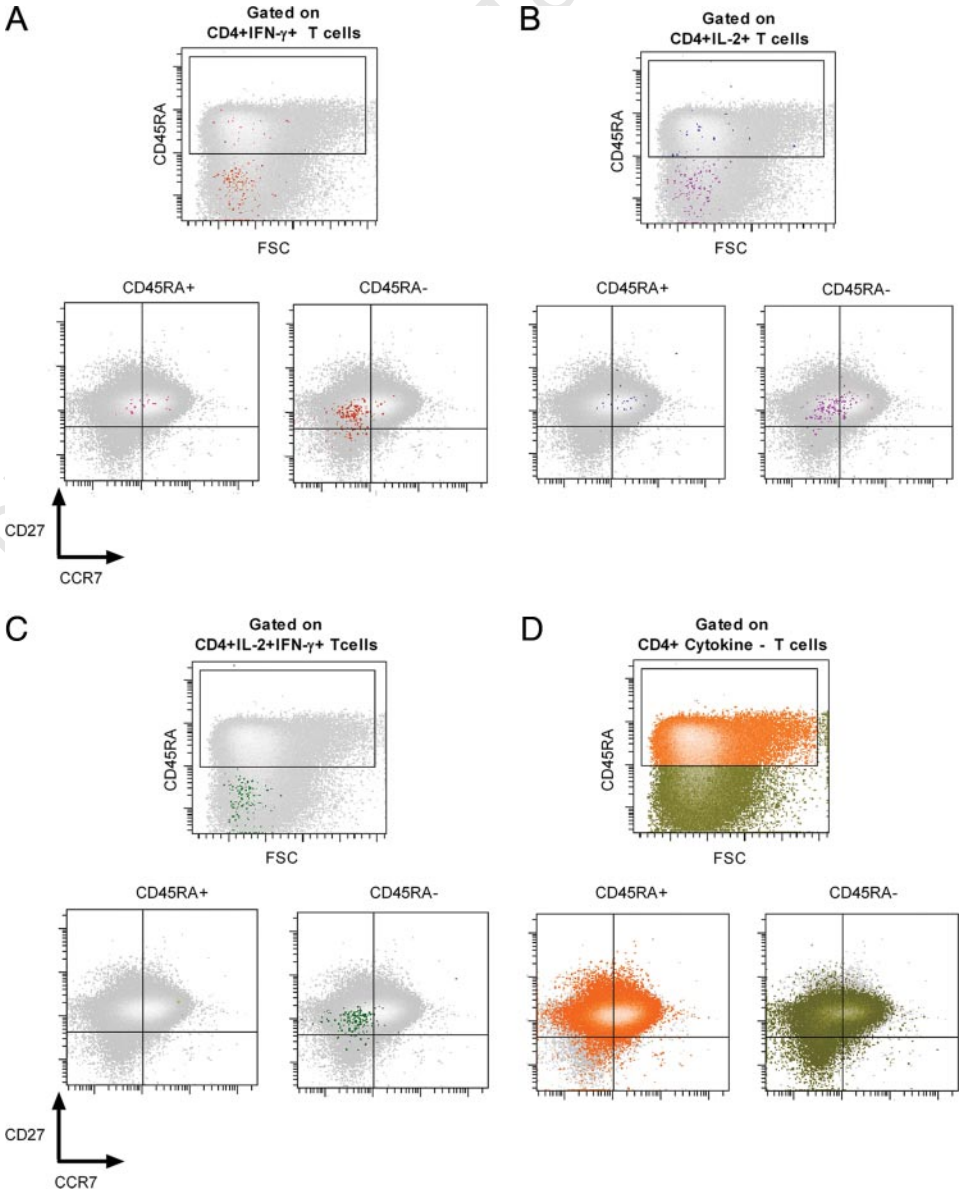

FIGURE 5. Phenotype of BCG-specific $CD4^+$ T cells in 10-wk-old infants vaccinated at birth. Ag-specific $CD4^+$ T cells were identified by expression of intracellular IFN-γ (*A*), IL-2 (*B*), or both cytokines (*C*), and the expression of CD45RA, CCR7, and CD27 was determined in each case. The plots illustrate the distribution of cytokine-expressing cells (in color; foreground) in relation to the entire $CD4^+$ T cell population (gray; background, also shown in *D*).

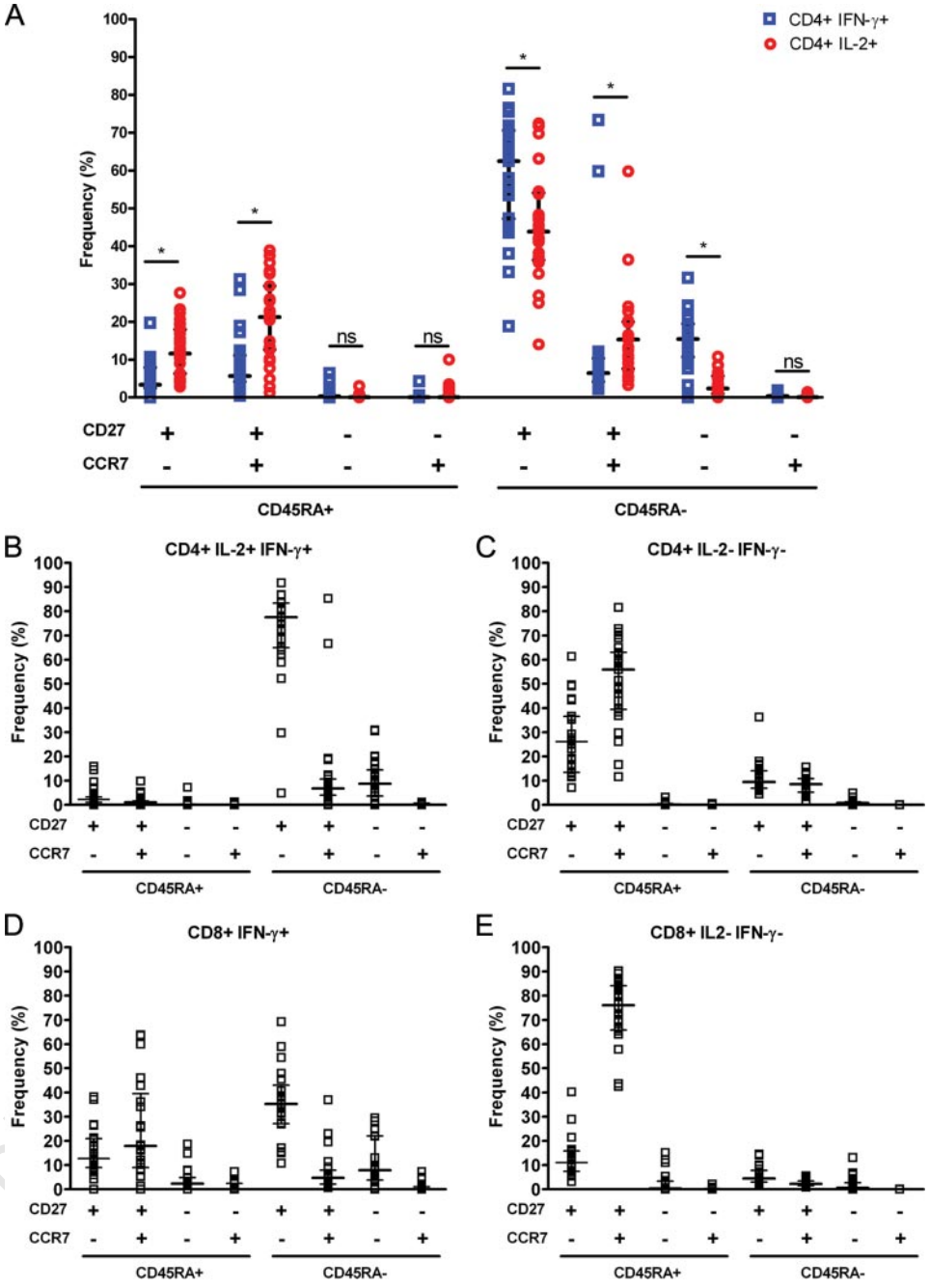

FIGURE 6. Frequency of different subsets of BCG-specific $CD4^+$ T cells, based on expression of CD45RA, CCR7, and CD27 among IFN-γ^+ and IL-2^+ (*A*) and IFN-γ^+IL-2^+ (*B*), expressing $CD4^+$ T cells. *C,* Frequency of these cells among cytokine-negative, i.e., mostly mycobacteria-nonspecific, $CD4^+$ T cells. *D,* Frequency of different subsets of BCG-specific $CD8^+$ T cells among IFN-γ^+ expressing cells. *E,* Frequency of these cells among mycobacteria-nonspecific $CD8^+$ T cells. The horizontal line represents the median and the whiskers the interquartile range. $*$, $p < 0.05$; ns, $p \geq 0.05$.

most common. The expression of phenotypic markers among cytokine-negative $CD8^+$ T cells are depicted in Fig. 6*E*.

We concluded that the majority of specific T cells induced by BCG vaccination of newborns has an effector phenotype and that IL-2 expression is more likely to be associated with a central memory phenotype.

Discussion

We showed that BCG vaccination of human newborns induces a diverse set of T cells, delineated by distinct cytokine production and phenotypic profiles. BCG-specific T cells produced mainly type 1 cytokines, as has been demonstrated before (14–16, 20, 22). However, we showed that not only IFN-γ is produced. A considerable number of IFN-γ-negative $CD4^+$ T cells were present, expressing the other type 1 cytokines IL-2 and TNF-α. Similarly, many $CD8^+$ T cells produced IL-2 in the absence of IFN-γ. The most commonly used measure of mycobacteria-induced immunity today is IFN-γ production, be this to diagnose latent infection via IFN-γ release assays (39, 40) or to describe human immune responses to novel TB vaccines (41). Recent experimental data suggest that measuring IFN-γ may not correlate with vaccination-induced protection against TB (42–44). This strongly supports measurement of all three cytokines on a cellular level to delineate a mycobacteria-specific type 1 response. This is further supported by our observation that diversity in the T cell cytokine response was predominant in our study population, e.g., in some infants cells that produced one cytokine dominated, whereas in others cells that produced three cytokines dominated. Measuring a single component of the immune response may underestimate the magnitude and complexity of BCG-induced immunity.

A BCG-induced $CD8^+$ T cell response was readily detectable, as type 1 cytokine-producing cells. Murine studies suggest that

$CD8^+$ T cells play an important role in control of *M. tuberculosis* infection and contribute substantially to total IFN-γ production (4). Although a BCG-induced $CD8^+$ T cell response has been described before (14, 45), we now show that the response is characterized by both IFN-γ-and IL-2-producing subsets. An interesting observation was that $CD8^+$ T cells produced very little TNF-α. Smith et al. (45) detected similar frequencies of $CD8^+$ T cells expressing TNF-α and IFN-γ following incubation of PBMC with BCG for 6 days. The contrasting low frequency of TNF-α expression observed in our study could be due to differences in assays, since we measured cytokine expression 12 h after incubation of whole blood with BCG. In longer term assays such as those performed by Smith et al. (45), TNF-α production may be derived from newly differentiated effector T cells, whereas our short-term assay measures cytokine-producing potential directly ex vivo. Our results also contrast with data from HIV-infected adults, whose HIV-specific $CD8^+$ T cells readily express TNF-α in short-term intracellular cytokine assays (46, 47), implying that BCG-specific $CD8^+$ T cells in newborns express little TNF-α.

The memory phenotype of BCG-induced T cells has not been reported. We identified five phenotypically distinct subsets within BCG-specific type 1 T cells, based on expression of CD45RA, CCR7, and CD27. The predominant phenotype of both IFN-γ- and IL-2-expressing $CD4^+$ and $CD8^+$ T cells was $CD45RA^-CCR7^-CD27^+$. $CCR7^-$ effector cells are characteristic of persistent activation of T cells seen in chronic viral infections where Ag is not cleared (48–50). Similarly, increased numbers of effector T cells are observed in children with active TB (51). It is possible that *M. bovis* BCG had persisted up to 10 wk of age, the time at which blood was collected from infants, resulting in this predominant phenotype.

Distinct differences in phenotypes were observed among $CD4^+$ IFN-γ- and IL-2-expressing cells. IL-2-expressing cells were significantly more likely to have a central memory phenotype, e.g., $CD45RA^-CCR7^+CD27^+$, compared with IFN-γ-expressing cells. IL-2-expressing T cells induced by BCG vaccination therefore follows patterns similar to those previously described for purified human central memory populations, which are likely to express IL-2 (13). However, our results, from 10 wk after vaccination with BCG, contrast with results obtained 8 wk after vaccination with tetanus toxoid; specific IL-2-expresing central memory cells were present but in a relatively small population, whereas this population was predominant after tetanus vaccination (52). Tetanus toxoid is not a persistent Ag; we therefore hypothesize that viable *M. bovis* BCG persisted following vaccination, driving differentiation predominantly into IFN-γ-expressing effector cells and preventing differentiation into IL-2-expressing central memory cells. An alternate hypothesis is that continuous exposure to environmental mycobacteria may contribute to chronic immune activation and therefore a predominance of effector T cells. The infants in this study were from a region with high rates of environmental mycobacteria and unpublished data suggest that exposure may occur even within the first 10 wk of life. It has been proposed that exposure to environmental mycobacterial Ags in tropical regions may undermine protective immunity induced by BCG (53). A third hypothesis is that the time point at which we measured the host response was too early for full differentiation into the central memory phenotype. A central memory/IL-2 phenotype has been associated with improved prognosis of chronic human viral infections such as HIV (49) and in experimental settings of chronic intracellular bacterial infection with long-lived protection (38). It remains to be determined whether a central memory phenotype is also associated with successful vaccination against TB.

We found that a significant amount of specific $CD4^+$ and $CD8^+$ T cells had a phenotype traditionally regarded as naive, i.e., $CD45RA^+$ and $CCR7^+$. In a recent study of children with TB, Caccamo et al. (54) also described this population, identified as specific by MHC class I pentamers of Ag-85A. We propose that this $CD45RA^+CCR7^+$ population reflects early differentiation into Ag-specific cells, before loosing CD45RA expression.

We could detect very low intracellular expression of the type 2 cytokine IL-4 above background values. Expression of type 2 cytokines has been associated with a suboptimal immune response to mycobacteria (55). For example, Ordway et al. (56) showed that long-term control of latent *M. tuberculosis* infection in humans appeared to be associated with optimal type 1 cytokine production and absence of detectable type 2 cytokine production. They showed that high percentages of IL-4-expressing $CD8^+$ and $\gamma\delta$ T cells soon after *M. tuberculosis* infection were associated with ultimate development of TB disease. The IL-4-expressing cells were detected after incubation of PBMC for 6 days, which contrasted with our 12-h assay; longer term assays may be required to detect these cells in the setting of BCG vaccination of the newborn. Intracellular expression of IL-10 could also be detected in our cohort, was low, and, like IL-4 expression, was never coexpressed with any type 1 cytokine. We propose that the IL-10-expressing T cells were induced regulatory T cells, which are expected to be present at low frequencies. Regulatory T cells control conventional effector immune responses, are induced by infections (57) and likely also by vaccination with BCG.

Our results clearly demonstrate the advantages of complex multiparameter flow cytometric analysis for deciphering a vaccination-induced immune response. However, this technology is not readily available. It is therefore important to note, from our findings, that when detection of IFN-γ or IL-2 is the aim and flow cytometry is not available, plasma levels may serve as surrogates of T cell cytokine production. We showed that plasma levels of type 1 cytokines correlated strongly with intracellular expression despite the fact that non-T cells also have the ability to make these cytokines (58, 59). When only four-color flow cytometry is available, inclusion of all three type 1 cytokines in one-color channel for detecting the total type 1 response may be the most useful. This proposal is supported by observations in HIV infection, where the presence of "polyfunctional" $CD8^+$ T cell populations, i.e., HIV-specific $CD8^+$ T cells that coexpress multiple cytokines, was associated with better clinical outcome (46). The observation in a mouse model of *Leishmania major* infection that polyfunctional T cell induction is also associated with the best outcome (60) suggests that measurement of these cells may also be important following BCG vaccination, since the mechanisms of immune protection are very similar for *Leishmania* and mycobacteria. Regardless, measurement of all three type 1 cytokines in one color would still not delineate the complexity of cytokine expression profiles. This may be important, since other studies of HIV infection showed that IL-2 expression, rather than IFN-γ or dual expression, correlated with the best clinical outcome (49). We therefore propose that delineation of multiple cytokine-expressing subsets, individually, will be important to investigate protective immunity against TB, either following natural infection or following vaccination.

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Disclosures

The authors have no financial conflict of interest.

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Research paper

Novel application of Ki67 to quantify antigen-specific *in vitro* lymphoproliferation

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ABSTRACT

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

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

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

1. Introduction

Proliferation and clonal expansion of antigen-specific T cells are critical functions for mediating protective immunity and immunological memory (Rosenberg et al., 1997; Combadiere et al., 2004). Previously, the most widely used method for detection of antigen-specific T cell proliferation has involved incorporation of ^{3}H-thymidine into DNA of dividing cells (Payan et al., 1983; Marchant et al., 1999). This technique has largely been replaced by flow cytometric assays of proliferation. Examples include fluorescent dye dilution assays, using CFSE or its derivative, Oregon Green (OG) (Magg and Albert 2007; Wallace et al., 2008; MacMillan et al., 2009), and assays that detect the DNA intercalating agent, 5-bromo-2′-deoxyuridine (BrdU), detected by fluorochrome-conjugated antibody staining (Dolbeare et al., 1983; Houck and Loken 1985; Rosato et al., 2001). The advantages of these assays are that they allow co-staining with other markers, enabling delineation of cellular sub-populations according to phenotype and functional characteristics, such as cytokine production (Lyons, 2000; Bachmann et al., 2005; Precopio et al., 2007).

Abbreviations: PPD, purified protein derivative; TT, tetanus toxoid; OG, Oregon Green.

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

In this study, we show that Ki67 expression in T cells is a specific and quantitative indicator of proliferation, and that results are comparable to those when proliferation is measured by other methods. We also show that measurement of Ki67 may be applied to longitudinal monitoring of vaccine-specific T cell responses. Overall, the Ki67 assay offers a reliable, versatile and simple method for detection of antigen-specific T cell proliferation.

2. Materials and methods

2.1. Study subjects

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

2.2. Whole blood BrdU incorporation assay

Whole blood (125 μL diluted 1:10 in warm RPMI 1640) was incubated with antigens for 6 days at 37 °C with 5% CO_2. Antigens were used at the following final concentrations: 1×10^5 cfu/mL Danish BCG (Danish strain 1331; Statens Serum Institut), 1 μg/mL TB10.4 protein (kindly provided by Tom Ottenhoff, Leiden University, Leiden, Netherlands), 2 μg/mL *M. tuberculosis* purified protein derivative (PPD, Statens Serum Institut) and 0.16 IU TT (Tetavax, Sanofi Pasteur). On day 6 (day 3 for PHA), 10 μmol/L BrdU (Sigma-Aldrich) was added for the last 5 h of culture. When intracellular cytokine expression was assessed, 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 1.5 μg/mL ionomycin (Sigma-Aldrich) and 1.5 μg/mL Brefeldin A (Sigma-Aldrich) were also added during the last 5 h of culture. Control antigens included 1 μg/mL phytohaemagglutinin (PHA; positive control, Sigma-Aldrich), medium only (unstim., negative control) or, for intracellular cytokine assays, medium with PMA and ionomycin (unstim-PI). On day 6, cells were harvested with 2 mM EDTA (Sigma-Aldrich) and red blood cells lysed. White cells were stained with a viability dye (LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Invitrogen), fixed in BD FACS Lysing Solution (BD Biosciences) according to manufacturer's instructions and cryopreserved until analysis.

2.3. PBMC isolation and the OG assay

PBMC were isolated by density gradient centrifugation and immediately stained with 10 μg/mL of CellTrace Oregon Green 488 (Molecular Probes, Invitrogen) per 1×10^7 cells and rested overnight at 37 °C, 5% CO_2. Cells were either incubated with medium or 1×10^5 cfu/mL Danish BCG, 0.5 μg/mL PPD, 1 μg/mL TB10.4 protein or 0.05 μg/mL staphylococcal enterotoxin B (SEB, positive control, Sigma-Aldrich), for 6 days at 37 °C with 5% CO_2. On day 6 for some assays, PBMC were restimulated with 50 ng/mL PMA, 250 ng/mL ionomycin and 10 μg/mL Brefeldin A for a further 5 h. Finally, PBMC were stained with LIVE/DEAD Fixable Violet Dead Cell Stain, fixed with BD FACS Lysing Solution (BD Biosciences) and cryopreserved until analysis.

2.4. Antibodies and flow cytometry

The following monoclonal antibodies were used for phenotypic and/or intracellular cytokine staining: CD3-QDot 605 (UCHT1), CD4-PerCP (SK3), CD8-PerCP-Cy5.5 (SK1), Ki67-PE (B56), IFN-γ-Alexa Fluor 700 (B27), TNF-α-PE-Cy7 (MAb11), IL-2-APC (MQ1-17H12), and anti-BrdU-FITC (B44). All antibodies were from BD Biosciences except for CD3-QDot 605, which was from Invitrogen. Samples were acquired on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA).

2.5. Data analysis

Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. Single-stained or unstained mouse κ beads were used to calculate compensations for every run. In some experiments CD4+ T cells were gated as CD3+ CD8− lymphocytes, because PMA and ionomycin stimulation strongly down-regulates CD4 expression on T cells. Data were analysed with FlowJo software v.8.8.6 (Treestar Inc.), Pestle v 1.6.2 and Spice v 4.3.2 software (provided by M. Roederer, National Institutes of Health, Bethesda, MD). Statistical analyses were calculated using GraphPad Prism v 4.0.

3. Results

3.1. Ki67 is a specific marker of in vitro lymphoproliferation

Ki67 is expressed by all cells undergoing cycling (Lopez et al., 1991; Scholzen and Gerdes, 2000). We investigated the kinetics of Ki67 expression in T cells cultured over 6 days. Whole blood was either cultured in the absence of antigen (unstimulated), or in the presence of purified protein derivative (PPD) or anti-CD3 and anti-CD28 (αCD3/αCD28). Expression of Ki67 was quantified each day. Ki67 expression

was low in unstimulated CD4+ T cells on day 1 (24 h, median, 0.62%), and by day 6, had decreased to <0.1% of CD4+ T cells (median, 0.08%, Fig. 1A). PPD stimulation resulted in Ki67 expression levels above those in unstimulated cells between days 2 and 4; expression peaked on day 6 (Fig. 1A and B). High expression of Ki67 was observed following polyclonal T cell stimulation with αCD3/αCD28; Ki67 was observed responses were high on day 1 already, peaked on day 3, and declined thereafter (Fig. 1A and C).

Next, we assessed proliferation by Ki67 detection in whole blood from 15 healthy donors, after 6-day culture with no antigen, or with PPD. All donors had undetectable or very low frequencies of Ki67+ CD4+ T cells in unstimulated blood (median, 0.07%). PPD stimulation resulted in higher frequencies of Ki67+ CD4+ T cells in all donors (median, 46.1%, Fig. 1D).

We also determined whether proliferation could be detected by assessing Ki67 expression in PBMC. Again, Ki67 expression identified *in vitro* CD4+ T cell proliferation; frequencies of Ki67+ cells after PPD stimulation consistently exceeded those in unstimulated PBMC, at a median of 21.7% (Fig. 1E).

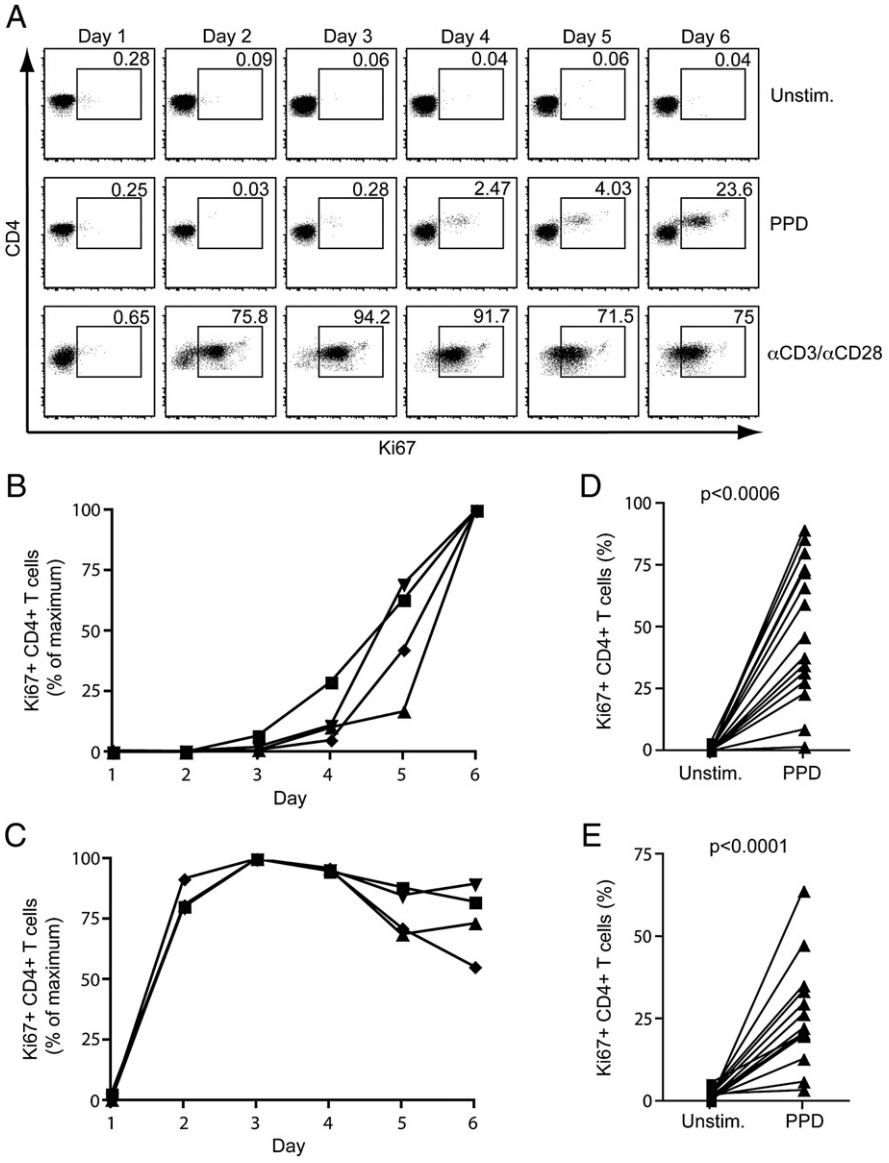

Fig. 1. Ki67 as a specific marker of *in vitro* lymphoproliferation. Whole blood from healthy donors was incubated with the indicated antigens and Ki67 expression quantified on a daily basis over 6 days. (A) Representative example showing the frequencies of Ki67 expression by CD4+ T cells after incubation of whole blood with medium only (unstim.), PPD or αCD3/αCD28 over 6 days. Dotplots were gated on live, CD3+ CD4+ lymphocytes. Ki67+ CD4+ T cell frequencies after (B) PPD stimulation or (C) αCD3/αCD28 stimulation in 4 donors. Data are expressed as a percentage of the maximum response. The frequency of Ki67+ CD4+ T cells is indicated in each plot. (D) Frequencies of Ki67 expressing CD4+ T cells in whole blood from 15 donors after 6-day culture with medium only (unstim.) or PPD. (E) Frequencies of Ki67+ CD4+ T cells in PBMC from 14 donors. Differences were calculated using the Wilcoxon matched pairs test.

These data suggest that in 6-day PBMC or whole blood culture with antigen, Ki67 expression is up-regulated in T cells undergoing *in vitro* proliferation.

3.2. Comparison of Ki67 expression with BrdU and Oregon Green assays

Next, we compared our Ki67-based proliferation assay with more traditional flow cytometric proliferation assays, i.e., those measuring BrdU incorporation and dye dilution of OG (Fig. 2).

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

The OG assay requires uniform labelling of cells prior to long-term culture. In contrast to results from the BrdU assay, the OG and Ki67 assays yielded remarkably similar frequencies of proliferating, specific T cells; Ki67+ and OG^{low} CD4+ T cell frequencies were not different in PPD or TB10.4-stimulated PBMC (Fig. 2D, E and F).

Frequencies of Ki67+ CD4+ T cells correlated strongly with BrdU+ CD4+ T cell frequencies (Fig. 3A and B). Similarly, a strong correlation was found between frequencies of antigen-specific Ki67+ and OG^{low} CD4+ T cells (Fig. 3C and D).

These data show that frequencies of proliferating T cells detected by Ki67 expression agree with frequencies detected with conventional proliferation assays.

3.3. Cytokine expression profiles of proliferating CD4+ T cells

The functional capacity of cells that have expanded during the 6-day culture may be assessed by short-term polyclonal re-stimulation with PMA and ionomycin on day 6. This induces cytokine production, which can be measured by intracellular staining. We compared expression of IFN-γ, IL-2 and TNF-α by Ki67+ CD4+ T cells with expression of these cytokines in BrdU+ or OG^{low} CD4+ T cells. When Ki67 and BrdU assay results were compared, similar expression of IFN-γ and TNF-α was observed in proliferating CD4+ T cells. BrdU+ CD4+ T cells yielded higher proportions of IL-2+ cells than Ki67+ CD4+ T cells but these differences were small (Fig. 4A and B). Similar expression profiles of IFN-γ, IL-2 and TNF-α were observed when comparing Ki67+ and OG dilution (Fig. 4C and D).

3.4. Intra-assay variability of Ki67 proliferation assay

To test the reproducibility of the Ki67 proliferation assay, we performed 5 proliferation assays per donor on whole blood from 3 healthy adult volunteers. Intra-assay coefficient of variation (CV) values for PPD-specific Ki67+ CD4+ T cells were between 2% and 3%, and for Ki67+ CD8+ T cells, which were present at lower frequencies than Ki67+ CD4+ T cells, between 10 and 16%. Even lower CV values were observed for PHA-stimulated blood, which induced the highest frequencies of Ki67+ T cells (Table 1). These results indicate that the Ki67 proliferation assay generates highly reproducible findings.

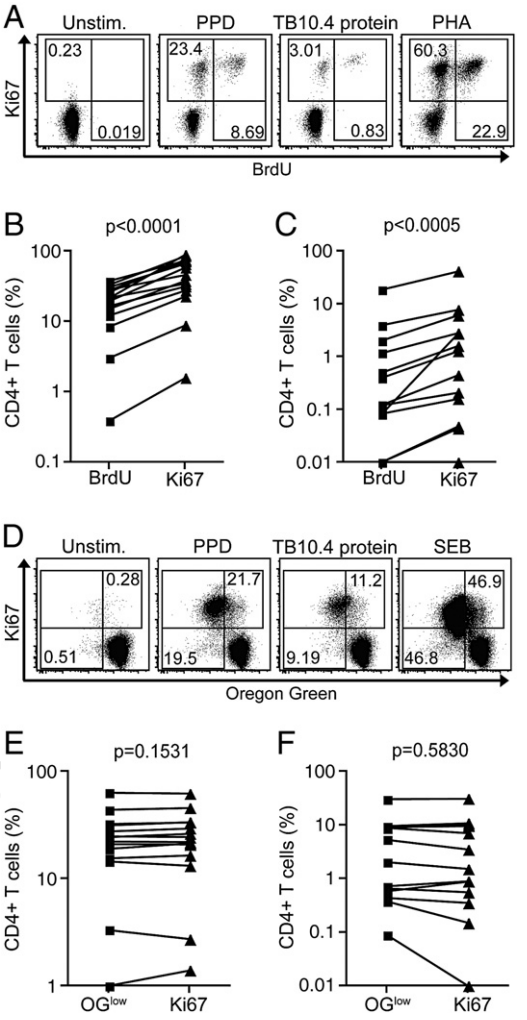

Fig. 2. Comparison of the Ki67 proliferation assay with the BrdU and Oregon Green proliferation assays. (A) Representative dotplots showing Ki67 versus BrdU expression by CD4+ T cells in whole blood. Dotplots are gated on live, CD3+ CD8− lymphocytes. Frequencies of (B) PPD- and (C) TB10.4-specific CD4+ T cell proliferation as detected by Ki67 expression or BrdU incorporation ($n=15$). CD4+ T cells are defined as CD3+ CD8− T cells (see Data analysis). (D) Representative dotplots showing Ki67 and dye dilution of Oregon Green by CD4+ T cells in PBMC. Dotplots are gated on live, CD3+ CD8− lymphocytes. Frequencies of (E) PPD- and (F) TB10.4-specific CD4+ T cell proliferation as detected by Ki67 expression or dye dilution of Oregon Green (OG^{low}) in 14 donors. CD4+ T cells are defined as CD3+ CD8− T cells (see Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

3.5. Measurement of vaccination-induced T cell proliferation

To establish if Ki67 can be used to measure vaccine-specific T cell proliferation, we determined Ki67 expression in T cells before and 11–13 days after tetanus toxoid (TT) re-immunisation of healthy, 18 month old infants. This post-vaccination time point was selected because it coincides with the peak TT-specific CD4+ T cell response in healthy adults (Cellerai et al., 2007).

The frequency of proliferating, Ki67+ CD4+ T cells observed pre-vaccination, following *in vitro* incubation of whole blood with TT, was low (median, 0.15%). After vaccination, TT-specific CD4+ T cell proliferation increased markedly (median, 3.77%,

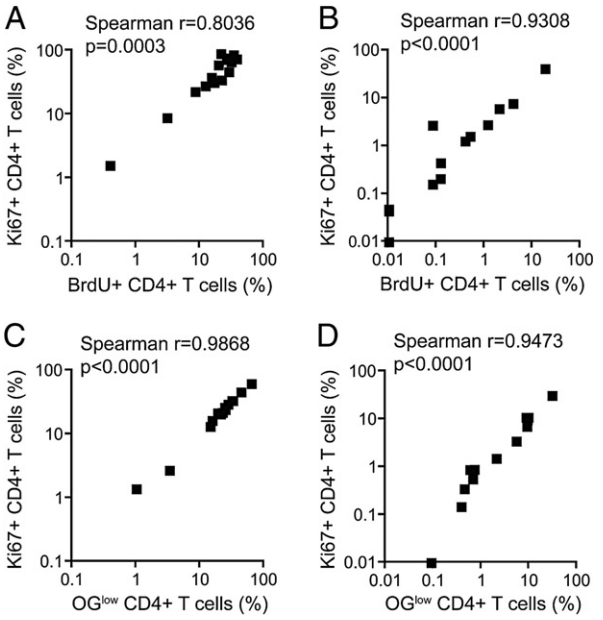

Fig. 3. Correlations between Ki67+ CD4+ T cell expression and BrdU incorporation or dye dilution of Oregon Green (OG^{low}). Whole blood was incubated with (A) PPD or (C) TB10.4 for 6 days ($n=15$). PBMC were incubated with (B) PPD or (D) TB10.4 for 6 days ($n=14$). Correlations were calculated using a Spearman's rank correlation coefficient.

Fig. 5A and B). To control for possible non-specific up-regulation of Ki67 after TT vaccination *in vitro*, we also quantified BCG-specific T cell proliferation pre- and post-vaccination. Frequencies of BCG-specific Ki67+ CD4+ T cells were not different before and after TT vaccination (Fig. 5A, C and D).

It is well established that vaccination-induced T cell proliferation results in increased *in vivo* and, thus, *ex vivo* expression of Ki67 (Cellerai et al., 2007; Miller et al., 2008). To determine whether "background" expression levels of Ki67, reflecting *in vivo* T cell turnover, affected the detection of antigen-specific T cell proliferation *in vitro*, we quantified Ki67 expression directly *ex vivo* in whole blood from toddlers before and 11–13 days after TT vaccination. High *ex vivo* frequencies of Ki67+ CD4+ T cells were readily detected in all toddlers before and after TT vaccination (Fig. 5E and F). Importantly, after 6 days of culture in the absence of antigen, Ki67 expression decreased markedly to background levels (Fig. 5E and F).

These data suggest that *in vivo* T cell turnover does not interfere with the specificity of the Ki67 proliferation assay. This assay is therefore specific for the detection of antigen-specific T cell proliferation *in vitro*.

4. Discussion

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

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

Cellular proliferation in PBMC samples is routinely evaluated by dye dilution methods, using CFSE or derivatives such as OG (Robinson and Amara, 2005). A recent non-human primate study has proposed measurement of *in vitro* proliferation by the combined analysis of Ki67 and side scatter properties of cells (Shedlock et al., 2010). The authors demonstrate a correlation between this assay and the CFSE dilution assay. In this study, we show that the proliferation events detected by loss of OG dye are virtually identical to the Ki67+ events. From this we reasoned that Ki67 expression is an accurate measure of T cell proliferation as only cells that have completed cycling display a decrease in OG fluorescence intensity. Limitations of many protein reactive dye compounds include cellular toxicity (Last'ovicka et al., 2009; Shedlock et al., 2010) and sensitivity to pH and light (Wallace et al., 2008). The Ki67 proliferation assay requires no incubation or washing steps prior to or during the culture, and exposure of cells to toxic compounds is eliminated. Additionally, since labelling of cells is not required before antigen stimulation, detection of Ki67 by flow cytometry can be performed on antigen-stimulated cells after cryopreservation. A limitation of Ki67 as a proliferation marker is its inability to resolve the number of proliferation cycles that cells have undergone, as can be done with dye dilution assays (Parish, 1999; Lyons and Doherty, 2004). Enumeration of cell cycles enables calculation of the original precursor frequency of specific cells, since the number of cells and their respective number of divisions are known (Givan et al., 1999).

Monitoring vaccine-induced T cell proliferative potential is important for determining vaccine take, memory function and long-term persistence of vaccine-specific responses. Previous studies have quantified Ki67 expression directly *ex vivo* as a measure of the vaccine-induced proliferative response (Miller et al., 2008), or in combination with activation markers to identify antigen-specific T cells (Stubbe et al., 2006). To detect increases in the expression of Ki67, these studies relied on low-level Ki67 expression before vaccination in healthy adults. Direct *ex vivo* detection of antigen-specific Ki67 expression may thus be challenging in individuals with high levels of *in vivo* T cell proliferation — such as those resulting from recent vaccinations or infections. We observed high *ex vivo* frequencies of Ki67+ CD4+ T cells in toddlers, suggesting elevated levels of *in vivo* T cell turnover. This turnover is likely to be

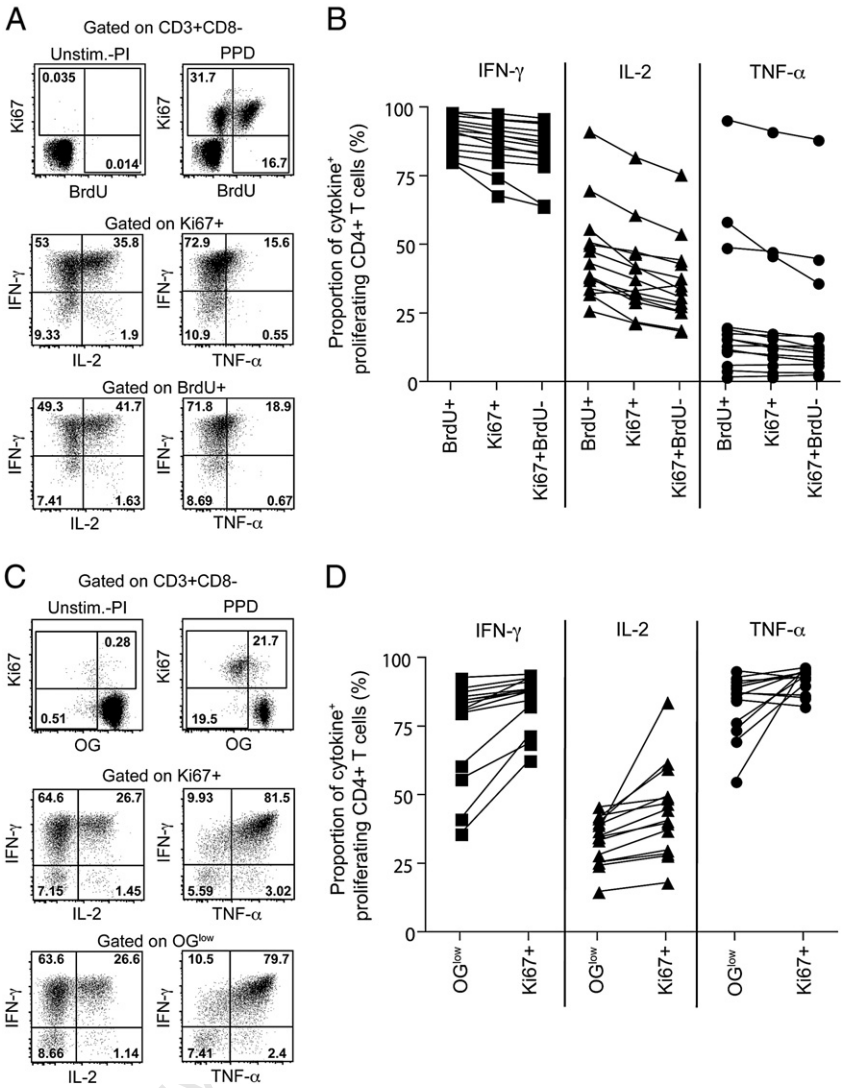

Fig. 4. Cytokine expression profiles of proliferating CD4+ T cells. Whole blood or PBMC were cultured for 6 days with no antigen or PPD. On day 6, cells were restimulated with PMA and ionomycin for 4 h in the presence of Brefeldin A to detect cytokine expression by proliferating T cells. Representative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

driven by routine childhood vaccinations and exposure to infections, common in this age group. Whole blood cultured in the absence of antigen reduced Ki67 expression to barely detectable levels by day 6, presumably due to cells reverting to a quiescent state. Therefore, this 6-day assay proved to be sufficiently specific and sensitive for the identification of rare,

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

Subset	Donor 1		Donor 2		Donor 3	
	Ki67+ CD4+	Ki67+ CD8+	Ki67+ CD4+	Ki67+ CD8+	Ki67+ CD4+	Ki67+ CD8+
PPD stimulation						
Mean	66.2	10.31	79.82	8.3	62.79	3.03
SD	1.88	1.66	1.46	0.86	1.33	0.34
CV	2.84	16.14	1.83	10.42	2.12	11.17
PHA stimulation						
Mean	94.5	91.26	94.7	91.7	76.65	74.54
SD	0.87	1.14	0.81	1.11	3.32	2.4
CV	0.92	1.25	0.86	1.21	4.33	3.21

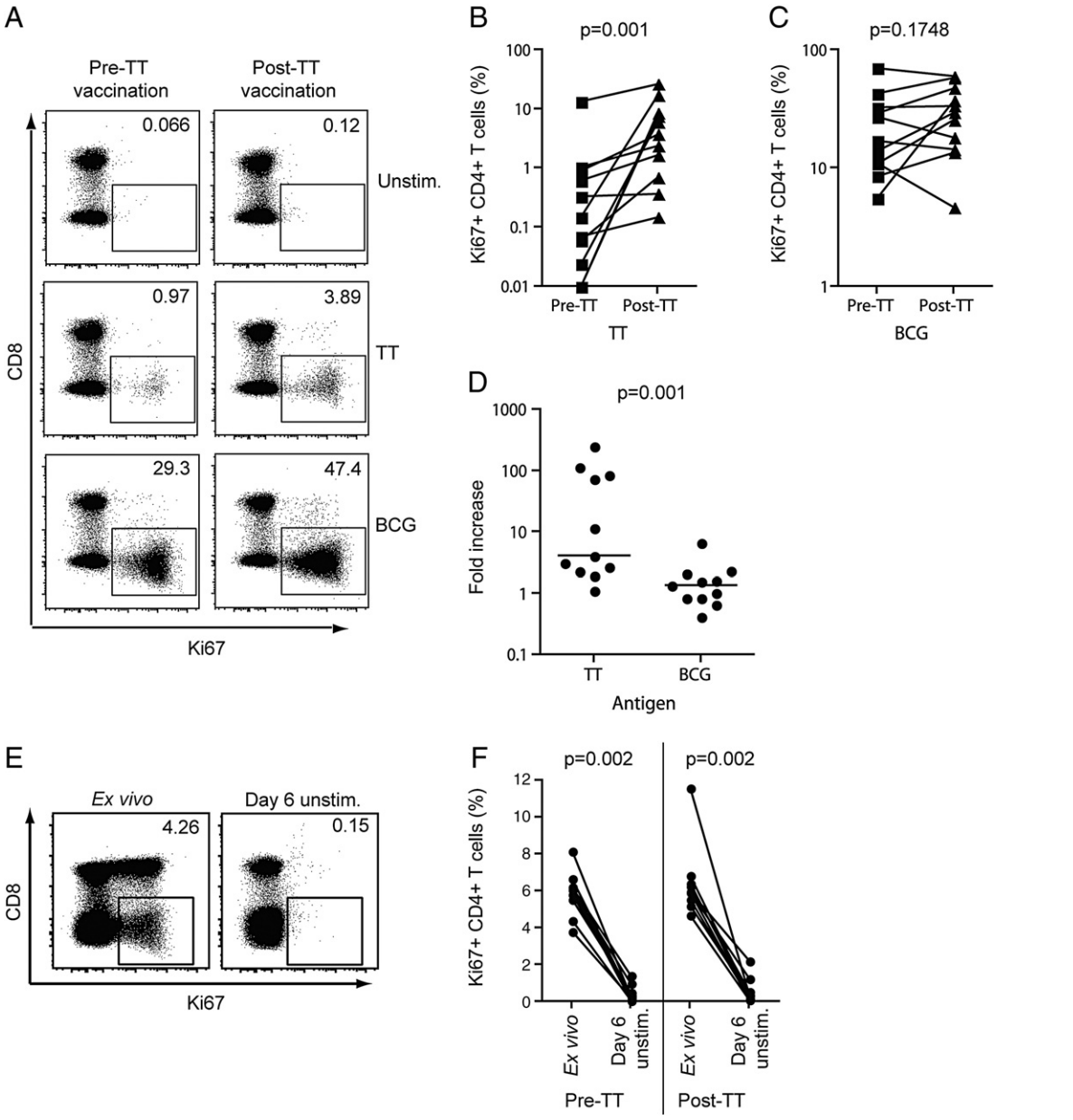

Fig. 5. Monitoring of vaccine-induced T cell proliferation. (A) Dotplots showing Ki67 expression by CD4+ T cells from a representative 18 month old toddler before (pre-TT) and after TT vaccination (post-TT). Dotplots are gated on live, CD3+ lymphocytes. Values in each dotplot represent the frequency of Ki67+ T cells within the CD3+ CD8− T cell population. Frequencies of (B) TT-specific and (C) BCG-specific CD4+ T cells pre- and post-TT vaccination in 11 toddlers. CD4+ T cells are defined as CD3+ CD8− T cells (see Data analysis). (D) Relative increase in TT-specific or BCG-specific CD4+ T cells pre- and post-TT. The lines represent the medians. (E) Dotplots depicting frequencies of Ki67+ CD4+ T cells in whole blood directly *ex vivo* or after culture in the absence of antigen (unstim.) for 6 days. Values in each dotplot represent the frequency of Ki67+ T cells within the CD3+ CD8− T cell population. (F) Frequencies of Ki67+ CD4+ T cells directly *ex vivo* or after culture for 6 days with medium ($n = 11$). CD4+ T cells are defined as CD3+ CD8− T cells (see Data analysis). Differences were calculated using the Wilcoxon matched pairs test.

antigen-specific T cells following vaccination in the context of high *ex vivo* frequencies of Ki67+ T cells.

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

Conflict of interest

The authors declare no financial or commercial conflicts of interest.

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