THE EFFECT OF BIRTH WEIGHT AND GESTATIONAL AGE ON BCG-INDUCED IMMUNE RESPONSES IN INFANTS FOLLOWING BCG VACCINATION

By

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Declaration

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Abbreviations

Antibody	Ab
Antigen	Ag
Antigen presenting cells	APC
Acute phase protein	APP
Bacillus Calmette-Guérin	BCG
Bromodeoxyuridine	BrdU
Carboxyfluorescein diacetate succinimidyl ester	CFDA-SE
Cell Preparation Tubes	CPT
Cytomegalovirus	CMV
Cytotoxic T-lymphocyte	CTL
Dendritic cell	DC
Diphtheria, tetanus, acellular pertussis	DTap
Deoxyribonucleic acid	DNA
Dimethylsulfoxide	DMSO
Endoplasmic reticulum	ER
Enzyme-linked immunosorbent assay	ELISA
Enzyme-linked immunospot assay	ELISpot
Extended Programme on Immunisation	EPI
Extensively drug-resistant	XDR

Fas-ligand	Fas-L
Forward scatter	FSC
Forward scatter area	FSC-A
Gestational age	GA
Haemophilus influenzae type b	Hib
Hepatitis B surface antigen	HBsAg
High-resolution computed tomography	CT
Human immunodeficiency virus	HIV
Immunoglobins	Ig
Inactivated polio virus	IPV
Interferon	IFN
Intellectual quotient	IQ
Interleukin	IL
Intracellular cytokine staining	ICS
Intra-uterine growth restriction	IUGR
Large for gestational age	LGA
Low birth weight	LBW
Membrane attack complex	MAC
Major histocompatibility complex	MHC
Mice cytomegalovirus	MCMV
Mycobacterium tuberculosis	M.tb

Multidrug-resistant	MDR
Natural killer cell	NK cell
Nitric oxide	NO
Normal birth weight	NBW
Oral polio virus	OPV
Oregon Green (2', 7'-difluorofluorescein)	OG
Pathogen-associated molecular patterns	PAMPS
Pattern recognition receptor	PRR
Peripheral blood mononuclear cells	PBMC
Phorbol 12-myristate 13-acetate and Ionomycin	PMA/I
Photomultiplier tubes	PMT
Polysaccharide	PS
Positron emission tomography	PET
Pneumococcal	Pn
Pre-term	PT
Purified protein derivative	PPD
Reactive oxygen species	ROS
Regulatory T cell	Treg
Retinoic acid receptor related orphan receptor γt	RORγt
Randomized control trial	RCT
Ribonucleic acid	RNA

Room temperature	RT
South African Tuberculosis Vaccine Initiative	SATVI
Side scatter	SSC
Side scatter	SSC-A
Signal transducer and activator of transcription	STAT
Small for gestational age	SGA
Staphylococcal enteretoxin B	SEB
Stimulation index	SI
T box expressed in T cells	Tbet
T-helper	Th
Transforming growth factor	TGF
Tumor necrosis factor	TNF
Toll-like receptors	TLRs
Tuberculosis	TB
University of Cape Town	UCT
Whole blood intracellular cytokine	WB-ICC
World Health Organisation	WHO

Overall summary

Bacillus Calmette-Guérin (BCG), the only currently licensed tuberculosis (TB) vaccine, provides variable efficacy. Despite the use of BCG, TB remains a global health problem. BCG is administered at birth; however, more than 15% of infants are born preterm [PT (<37 weeks gestation)], or have low birth weights [LBW (<2,500g)], with >90% of these born in developing countries, where the majority of TB cases are found. It is not known how birth weight at the time of vaccination may affect the BCG-induced immune response and subsequent protection against TB. We hypothesised that BCG-vaccinated PT and LBW infants would have a qualitatively and quantitatevely less optimal immune response, compared to term or normal birth weight (NBW) infants.

We designed a study to determine the effects of birth weight and maturity on BCG induced T cell responses, thought to be important mediators in protection against TB. We enrolled healthy infants BCG vaccinated at birth and stratified by birth weight, gestational age (GA), size for GA and gender. At 10 weeks of age, a whole blood intracellular cytokine assay was performed and complemented by assessment of the proliferative potential of BCG-specific T cells from a 6-day Ki67 proliferation assay. Multiparameter flow cytometry was used to measure BCG-specific T cell responses in these two assay systems. This is the first detailed analysis of the functional capacity of BCG-induced T cell responses in PT and LBW infants.

We found that LBW infants had a lower proportion of polyfunctional BCG-specific CD4+ T cells and corresponding higher proportions of single cytokine-producing CD4+ T cells expressing IFN-γ only, compared to NBW infants. No differences were found in the proliferative potential of BCG-specific T cells. Our results suggest that birth weight at time of vaccination may affect the quality of the BCG-induced immune response, and warrant further investigation in larger cohorts. These results may ultimately have important implications for neonatal BCG vaccination practices worldwide.

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and the boxes represent the interquartile range. The Mann Whitney test was used to calculate statistical differences between individual groups. (B) The percentage of vaccinated infants with a detectable BCG-specific CD4+ T cell response above background. Out of 52 LBW and 53 NBW infants, CD4+ T cells from 50 LBW (96%) and 53 NBW (100%) infants responded to BCG. There was no statistical difference between the percentages of infants responding to BCG (Fisher's exact test).

Figure 3.3. Correlation between the birth weight of 105 BCG vaccinated infants and the frequency of BCG-specific CD4+ T cells expressing either: (A) IL-2, (B) IFN-γ, (C) TNF-α or (D) IL-17. Statistical significance was calculated using the Spearman test.

Figure 3.4. Correlation between the gestational ages of 105 BCG vaccinated infants and frequency of BCG-specific CD4+ T cells expressing either: (A) IL-2, (B) IFN-γ, (C) TNF-α or (D) IL-17. Statistical significance was calculated using the Spearman test.

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a slice each for T cells expressing a combination of 3, 2 or 1 cytokine only (B). The Mann Whitney test was used to calculate statistical significance between the individual groups.

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Figure 3.8. Correlation between the birth weight of 105 BCG vaccinated infants and the frequency of BCG-specific CD8+ T cells expressing either IL-2 (A), IFN-

γ (B), TNF-α (C), or IL-17 (D). Statistical significance was calculated using the Spearman test.

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Figure 4.1. Flow cytometry gating strategy and analysis of lymphocyte viability in the Ki67 and Oregon Green (OG) proliferation assays. Cryopreserved PBMC were thawed and cultured with BCG, SEB or medium (UNS; unstimulated) in either a 6-day Ki67 proliferation assay (without OG), or a 6-day OG-based proliferation assay (see *Materials and Methods*, 2.2.5). (A) Gating is first performed on singlets then on live lymphocytes by gating on ViViD^{low} CD3+ cells, from which total CD3+ T cells are differentiated into CD3+CD8+ and CD3+CD8- T cell subsets. This is followed by gating on CD8+ and CD8- OG^{low} (B) or CD8+ and CD8- Ki67+ (C) proliferating cells. CD4+ T cells were defined as CD3+CD8- lymphocytes. Representative data are shown from a BCG stimulated sample. (D) Frequency of viable CD3+ T cells gated on ViViD^{low} CD3+ live cells from Ki67 (n=7) and OG (n=14) proliferation assays. The horizontal line represents the median; the Mann Whitney test was used for comparisons between two groups.

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Ki67+ live cells were differentiated into T cells expressing combinations of IL-2, IFN-γ or TNF-α. CD4+ T cells were defined as CD3+CD8- lymphocytes. (A) Representative dot plots of cytokine expressing cells gated on live CD4+ proliferating cells in one LBW and NBW infant are shown. Percentages shown on the plots are prior to subtraction of cytokine production in the PMA/I negative control sample. The percentage of BCG-specific CD4+ T cells expressing cytokines in LBW (n=18) and NBW (n=18) infants is shown in (B). The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). The horizontal line represents the median and the boxes represent the interquartile range. Data shown in (B) and (C) are after background subtraction of the PMA/I negative control sample (see *Materials and Methods*, 2.2.8). Statistical analyses were determined by the Mann Whitney test.

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Figure 4.7. Cytokine production capacity of BCG-specific proliferating CD8+ T cells. Cryopreserved PBMC were cultured for 6 days with BCG, SEB or medium

(UNS; unstimulated), restimulated for 5 hrs on day 6 PMA/I and analyzed by flow cytometry (see *Materials and Methods*, 2.2.5). Cells gated on proliferating CD3+CD8+ Ki67+ live cells were differentiated into T cells expressing combinations of IL-2, IFN-γ or TNF-α. (A) Representative dot plots of cytokine expressing cells gated on live CD3+CD8+ proliferating cells in one LBW and NBW infant are shown. Percentages shown on the plots are prior to subtraction of cytokine production in the PMA/I negative control sample. (B) Frequency of BCG-specific CD8+ T cells that are proliferating and expressing cytokines in LBW and NBW infants. The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). The horizontal line represents the median and the boxes represent the interquartile range. There was no statistical difference as determined by the Mann Whitney test.

Figure 4.8. The frequency (A) and proportion (B) of BCG-specific CD8+ T cells that are proliferating and expressing combinations of IL-2, IFN-γ or TNF-α cytokines in LBW and NBW infants. Data shown are after background subtraction of the PMA/I negative control sample, and only infants who met criteria for a positive response and minimum numbers of viable CD3+ cells are shown (LBW, n=16 and NBW, n=16) (see *Materials and Methods*, 2.2.8). The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). The horizontal line represents the median and the boxes represent the interquartile range. Each pie is divided into 4 slices that represent the median proportion of the total BCG-specific CD8+ T cell response, a slice each for T cells that are not expressing any cytokines, or a combination of 3, 2 or 1 cytokine only (B). Statistical analyses were determined by the Mann Whitney test.

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CHAPTER ONE

Literature Review

1.1 Overview

Immunity to infection involves the ability of the host to recognise and promptly act to clear invading pathogens. The relevant arm of the immune system acts to prevent infection of either intracellular or extracellular pathogens. In the following sections, characteristics of both innate and adaptive immunity will be described in more detail. After gaining this foundation in immunology, the concept of inducing and manipulating the immune system through vaccination to protect against infection will be introduced. Focus will then be on tuberculosis (TB) disease, the TB vaccine and reasons why, despite its widespread use, TB has become an epidemic. Lastly, an overview of the neonatal immune system and early life vaccines will be presented, illustrating how host factors such as birth weight and maturity may potentially modulate vaccine-induced immune responses.

1.2 The innate immune system

The innate immune system is the first line of defence against invading pathogens. It is rapid, non-specific and innate in the sense that most responses are not affected or enhanced by prior exposure, and innate immune cells are present prior to encounter with pathogen. Innate responses can be divided into 2 phases. The first, simplest and immediate form of innate immunity is preventing the pathogen from entering the host. The skin is the major line of defence and acts as a barrier when intact. Most pathogens fail to survive on the surface of the skin due to the low pH of sweat and sebaceous secretions. In addition, most bodily secretions have antimicrobial properties, such as saliva and tears containing lysozyme, acidic gastric juice, and milk containing lactoperoxidase. Mucus secretions prevent the pathogen from adhering to epithelial cells and mechanical action such as ciliary movement and coughing expel pathogens. Another mode of action is that of microbial antagonism, commensal organisms flourishing in the host suppress pathogens by producing antimicrobial compounds or by outcompeting pathogens for essential resources. However, if

these external barriers fail to prevent infection then the second phase of innate immunity comes into play. (Janeway 2005; Rabson, Roitt et al. 2005; Chaplin 2010)

A pathogen that enters the host may, in some cases, be directly cleared by complement. Complement is a system of plasma proteins present at all times that defend against infection, in particular against extracellular bacteria. In the classical pathway the complement system is activated when antibodies bound to the surface of a pathogen bind the complement component C1. A sequence of cleavage reactions ultimately leads to a C3/C5 convertase enzyme made up of the complement components C4b,2b which cleave the most abundant complement component C3 to C3b. C3b then binds to the surface of bacteria to form a complex that favours its uptake and destruction by phagocytic cells in a process known as opsonisation. There is also an enzymatic molecular cascade leading to the formation of a membrane attack complex (MAC) made up of the terminal complement components C6-C9. The MAC can insert directly into the lipid bilayer of target cells creating pores leading to direct lysis of the pathogen. In the alternative complement pathway, activation is not dependent on Ab but on bound C3b from the classical pathway. Bound C3b binds factor B which in turn is cleaved by protease factor D into Ba and Bb. The C3b,Bb complex of the alternative pathway forms the C3/C5 convertase enzyme which is different but homologous to the C4b,2b complex of the classical pathway. The C3/C5 convertase enzyme of the alternative pathway enhances the effects of the classical pathway by addition of C3b onto the surface of bacteria. Most bacteria are resistant to complement and can activate the phagocytic cells such as macrophages, which in turn activate acute phase protein (APP) production in the liver. These proteins are non-specific and bind to a wide range of microorganisms. Binding of APP to the surface of bacteria enhances complement binding and subsequent destruction by macrophages in the liver (Janeway 2005; Rabson, Roitt et al. 2005; Chaplin 2010).

Natural killer (NK) cells and phagocytic cells such as dendritic cells (DCs) macrophages, monocytes and neutrophils are the major cell types involved in innate immunity. Receptors on NK cells recognise virally infected or tumor cells lacking major histocompatibility complex (MHC) class I molecules. These cells are killed either by apoptosis (programmed cell death) through release of cytotoxic granules or the FAS/FasL (Fas-ligand) pathway (Rabson, Roitt et al. 2005). Phagocytic cells on the other hand have pattern recognition receptors (PRRs) that recognise and adhere to pathogen-associated molecular patterns (PAMPS) on the surface of pathogens. These PAMPs are not found in the host and binding to PRRs activates phagocytic cells to ingest and destroy the pathogen. Phagocytic cells produce reactive oxygen species (ROS) and nitric oxide (NO) that are cytotoxic to both bacteria and viruses. They also produce the cytokine interleukin (IL)-12 important in initiating the differentiation of naïve CD4 T cells to become Th1 cells. Phagocytic cells then bind peptides from ingested pathogens or antigens (Ags) in the context of MHC class I and class II molecules. When these phagocytic cells migrate from infection sites to draining lymphoid organs, the bound peptides are presented to cognate T cells thus activating adaptive immunity. A family of transmembrane proteins known as toll-like receptors (TLRs) found on phagocytic cells but primarily on DCs can also recognise PAMPs and appear to control the type of defensive mechanism initiated. Unlike general phagocytic cells, DCs are professional Ag presenting cells (APCs) that can prime naïve T cells. Activation of TLRs triggers DCs in particular to express proinflammatory cytokine such as tumor necrosis factor (TNF) and interferon γ (IFN-γ), which may ultimately lead to the activation of adaptive immunity. In addition DCs are important producers of chemokines that attract T cells (Janeway 2005; Rabson, Roitt et al. 2005; Chaplin 2010).

For years it was widely believed that the innate immune system responded in a similar way each time it encountered a pathogen without developing immunological memory, a feature attributed to adaptive immunity. However, recent studies have suggested that NK cells develop immunological

memory (O'Leary, Goodarzi et al. 2006; Sun, Beilke et al. 2009), as shown for example in the model of mice cytomegalovirus (MCMV) infection where NK cells are known to be protective. NK cells from cytomegalovirus (CMV) challenged mice adoptively transferred to naïve mice expanded and cleared infection more rapidly upon secondary CMV challenge (Sun, Beilke et al. 2009).

1.3 The adaptive immune system

Many pathogens have evolved mechanisms to evade detection by innate immunity, such as modulation of surface molecules to prevent binding of complement and release of antiphagocytic surface molecules and exotoxins (Rabson, Roitt et al. 2005). The solution to this was the host evolution and development of the adaptive arm of the immune response, which can be divided into humoral (antibody) and cell-mediated responses. Immunological memory, the ability to mount a more efficient and rapid response upon secondary encounter with Ag/pathogen, is the hallmark of adaptive immunity (Janeway 2005; Chaplin 2010).

1.3.1 Humoral response

The main effector cells of the humoral response of adaptive immunity are B-lymphocytes. Millions of B-lymphocytes are produced in the bone marrow, each making only one type of antibody (Ab) with specificity for only one Ag. Antibodies, also known as immunoglobins (Ig) are encoded by multiple gene segments for heavy chains, κ light chains and λ light chains. Ab production defines B cells. The basic Ig structural unit is composed of 2 heavy chains and 2 light chains. The heavy and light chains consist of variable amino acid terminal regions designated as V_H, V_κ and V_λ respectively. These have sub-regions that are hypervariable forming the Ag binding domain of each Ab. Hypervariable regions allow Ab to specifically recognise a wide array of different Ag. The carboxyl

terminal end of each heavy and light chain is constant with 2 heavy chains forming the Fc domain. The Fc domain can bind to cell-surface Fc receptors and is important in activating the complement system. Human Igs are divided into 5 major classes, IgG, IgA, IgM, IgD and IgE, based on the heavy chain constant region. Class switching within germinal centres from IgM and IgD to other classes can occur in an active process of gene mutation that result in changes within the Ag binding domain. Cytokines can partly promote class switching, for example IL-4 and IL-13 to IgE and IL-10 and TGF-β to IgA. Mutations that result in increased Ab affinity for a particular Ag results in the B cell producing that Ab gaining a proliferative advantage within a B cell pool when that particular Ag is encountered again. This is called affinity maturation and that B cell proliferates to dominate the pool of responding cells (Rabson, Roitt et al. 2005; Bonilla and Oettgen 2010; Chaplin 2010).

Antibodies (Abs) expressed on the surface of each B-lymphocytes act as receptors for pathogen antigens (Ag). In this primary response, Ab that correctly binds to Ag causes clonal proliferation of that particular B cell and differentiation into Ab producing plasma and/or memory B cells. The primary response is slow and leads to proliferation of short lived, low affinity Ab with other isotypes of higher affinity appearing only later. Memory B cells are long-lived B cells that require fewer cycles to clonally expand into effectors. During secondary encounter to the same Ag (secondary response), memory B cells are activated resulting in a more rapid response and abundant Ab production of higher affinity (affinity maturation) compared to the primary response. As each B cell makes only one type of specific Ab, plasma cells will produce Ab of one type and specificity. Thus Ag induces clonal selection of plasma cells producing Ab specific to that particular Ag. Once Ab binds Ag it induces the complement system, and enhances phagocytosis (opsonisation) leading to clearance of the Ag. Circulating Abs can also bind and neutralise antiphagocytic surface molecules and exotoxins. This is important to neutralise pathogens that evade complement system and to neutralise toxins that may otherwise damage tissue. (Bonilla and Oettgen 2010; Chaplin 2010).

1.3.2 Cell-mediated response

The humoral response cannot directly target intracellular pathogens. Thus the host developed cell-mediated adaptive immunity to counter intracellular infection. Unlike B cells produced in the bone marrow, the main effectors of cell-mediated adaptive immunity are produced in the thymus gland and are thus called T cells. The T cell receptor (TCR) of naïve T cells, which is analogous to B cell Ag receptors, recognizes Ag presented by major histocompatibility complex (MHC) molecules expressed on APCs. The major class of T cells express the αβTCR and differentiate into CD4 (helper) and CD8 (cytotoxic) T cell subsets (Bonilla and Oettgen 2010; Chaplin 2010). The T cell receptor (TCR) of naïve T cells, which is analogous to B cell Ag receptors, recognizes Ag presented by major histocompatibility complex (MHC) molecules expressed on APCs. MHC molecules are also known as the human leukocyte-associated (HLA) antigens and can be divided into class I and class II MHC molecules. Most conventional T cells express the αβ TCR but a small population, about 10% expresses the γδ TCR. Unlike conventional T cells, γδ T cells can directly recognise non-peptide antigens, such as microbial metabolites and phospholigands, and are enriched in mucosae and epithelial surfaces (Haas, Pereira et al. 1993; Sim, Olsson et al. 1995).

Class I MHC molecules bind endogenous 9-11 amino acid long peptide fragments synthesised from intracellular proteins encoded by the host genome or derived from infecting pathogens. The TCR of cytotoxic CD8 T cells interacts with peptides presented by class I MHC molecules, enhancing CD8 T cell activation. The 3 major types of human class I MHC molecules found on almost all nucleated cells are HLA-A, -B, and -C. In contrast, class II MHC molecules bind exogenous Ags ingested by endocytosis or phagocytosis and degraded to linear fragments by proteolysis within the APC. The TCR of CD4 T helper cells binds peptides presented by class II MHC molecules, enhancing CD4 T cell activation. The main types of cells expressing class II MHC molecules are DCs, B cells and macrophages. Stimulation of epithelial and capillary endothelial cells induces

class II MHC molecules allowing these cells to present peptides at inflammatory sites. Unlike constitutive expression of class I MHC molecules, class II MHC molecules can be induced by activation of innate immunity. The 3 major types of class II MHC molecules in humans are HLA-DR, HLA-DQ and HLA-DP. Cross-presentation of Ag can also occur whereby exogenous Ag is taken up and presented by class I MHC molecules. This is important in overcoming some pathogens such as viruses that can suppress Ag presentation via the classical endogenous pathway (Rabson, Roitt et al. 2005; Bonilla and Oettgen 2010; Chaplin 2010).

CD4 T cells generally support humoral responses and cell-mediated immune responses such as delayed type hypersensitivity responses, among others. Ag specific T-helper cells are activated after recognising Ag presented by APCs. They then secrete cytokines that cause Ag-specific B cells to clonally expand to produce high-affinity Abs. Cytotoxic CD8 T cells have direct effector functions and can kill infected cells and tumours in a contact dependant process. Target cells are killed either by apoptosis through CD8 T cell release of cytotoxic molecules, such as perforin, granzymes and granulysin, or via a Fas/FasL pathway (Bonilla and Oettgen 2010).

Two signals are required for activation resulting in clonal expansion and differentiation of naïve T cells. Naïve T cells are first triggered when the TCR engages with peptide Ag presented by MHC molecules as described above. The second co-stimulatory signal is mediated via the binding of co-stimulatory molecules such as CD28 on the T cell and CD80 (B7.1) or CD86 (B7.2) on the APC. Naïve T cells that receive only the first signal produce only small amounts of IL-2, do not proliferate or differentiate and become anergic to Ag. Sustained proliferation and differentiation of activated T cells is driven by the cytokine IL-2 produced by the activated T cells themselves (Bouneaud, Garcia et al. 2005). Naïve CD8 T cells are already predestined to become cytotoxic CD8 T cells. On the other hand naïve CD4 T cells activated by APC differentiate into T-helper type (Th)-1, Th2, Th17, Th9 or regulatory T cell (Treg) lineages depending on

activation of various transcription factors and also on cytokines in the microenvironment of the naïve cell (Fig. 1.1) (Janeway 2005; Rabson, Roitt et al. 2005; Chaplin 2010).

1.3.3 T cell effector subsets

Innate immune cells can produce the cytokines IL-12 and IFN-γ that bind cell surface receptors inducing pathways for the signal transducer and activator of transcription (STAT)-4 and T box expressed in T cells (T-bet). STAT4 and T-bet bind the IFN-γ promoter driving naïve CD4 T cells to differentiate into Th1 cells. In mice STAT1 may also be an important mediator for TH1 differentiation as shown in a recent study (Ma, Huang et al. 2010). In general these cells support the cell-mediated immune response (Chaplin 2010). Th1 cells express mainly IL-2, IFN-γ and TNF-α and are important for protection against intracellular pathogens. The binding of IL-4 to cell-surface receptors activates STAT6, which initiates differentiation into Th2 cells expressing the transcription factor GATA3 and the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13. Th2 cells in general support the humoral response and are important in protection against extracellular pathogens (Romagnani 1997; Janeway 2005; Rabson, Roitt et al. 2005; Zhu, Yamane et al. 2006; Romagnani 2008; Chaplin 2010).

In humans, the expression of the cytokines IL-1β and IL-6 or IL-1β or IL-23 alone induces the initial commitment of naïve T cells to become Th17 cells. In contrast, murine Th17 cells are produced in response to the simultaneous production of IL-6 and TGF-β. IL-23 expression is necessary for the maintenance of this cell lineage. Th17 cells express the transcription factor RORγt (retinoic acid receptor related orphan receptor γt) and T-bet. Th17 cells mediate protection against extracellular bacteria and fungi by recruiting neutrophils. They express the cytokines IL-22 and mainly IL-17, which has protective effects against bacteria (Curtis and Way 2009; Romagnani, Maggi et al. 2009; Damsker, Hansen et al. 2010). However, Th17 cells may also be pathogenic and have been

implicated in several human diseases. Over-expression of IL-17 is associated with a pro-inflammatory response and high levels of IL-17 have been found for example in patients with rheumatoid arthritis and Crohn's disease (Kirkham, Lassere et al. 2006; Seiderer, Elben et al. 2008). Though unclear, Th17 cells also seem to play a significant role in patients with multiple sclerosis. In patients suffering from opticospinal multiple sclerosis, large amounts of IL-17 have been found in the cerebrospinal fluid (Ishizu, Osoegawa et al. 2005). In addition Th17 cells have also been shown to be able to cross the blood brain barrier resulting in inflammation in the central nervous system parenchyma (Kebir, Kreymborg et al. 2007).

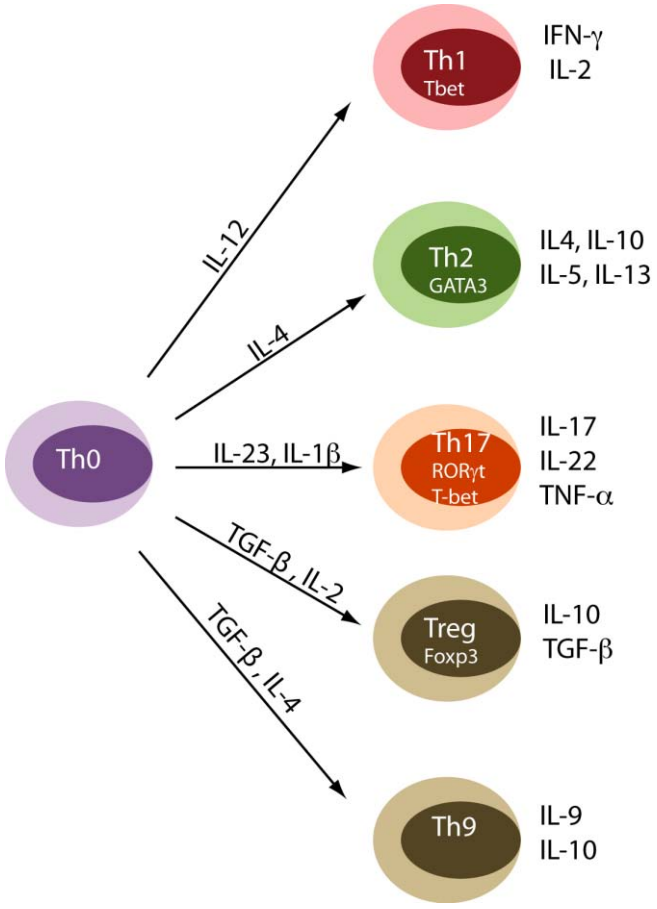

Figure 1.1. Naïve CD4 T cell differentiation into different T-cell effector subsets. Differentiation depends on the type of APC and cytokines in the microenvironment of the cell (arrows). The different T cell subsets express specific transcription factors (shown within the cell) and certain signature cytokines (Adapted from Bonilla and Oettegen 2010).

Another distinct lineage of T cells is that of regulatory T cells (Tregs). The precise signals that polarize naïve T cells to become Tregs are poorly understood, but IL-2 and TGF-β cytokines aid in the maintenance of Tregs. These cells are able to suppress various immune functions (Fig. 1.2), maintaining immune homeostasis and preventing autoimmune and inflammatory diseases (Sojka, Huang et al. 2008). Tregs express the transcription factor FOXP3, the IL-2 receptor α chain (CD25), and the immunosuppressive cytokines IL-10 and TGF-β. Tregs may also play a role in suppressing vaccine induced protective immune responses (Bonilla and Oettgen 2010; Chaplin 2010). IL-4 and TGF-β can also induce Th9 cell differentiation, another recently described lineage of T cells that protect against helminth infections (Ma, Tangye et al. 2010). Th9 cells express large quantities of IL-9, a cytokine previously attributed to the Th2 cell lineage only (Soroosh and Doherty 2009). The specific transcription factor expressed by these cells is yet to be defined but this emphasises that these cells are different from Th2 cells that express GATA3 (Ma, Tangye et al. 2010).

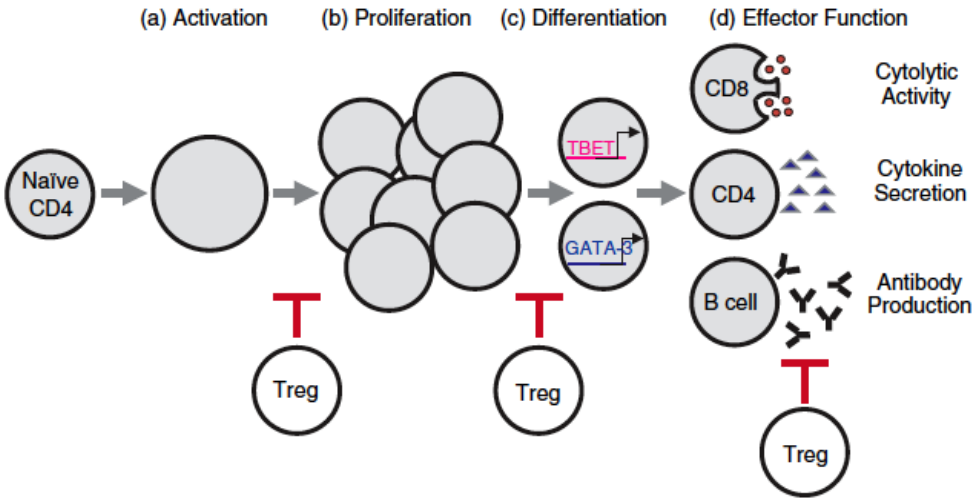

Figure 1.2. Regulatory T cells and the different immune functions they modulate (Sojka, Huang et al. 2008)

In short, the immune system is able to respond appropriately to extracellular and intracellular pathogens. The innate immune mechanisms provide the first line of defence against pathogens. The innate response is non-specific and immediate and may stimulate the induction of the adaptive immune system to clear pathogens that evade innate immunity. The adaptive immune system is a specific form of acquired immunity induced by exposure to the pathogen (Janeway 2005; Calder 2007; Chaplin 2010). Table 1.1 summarizes some important differences between innate and adaptive immunity. Priming of naïve cells during the initial encounter with the pathogen results in clonal expansion of memory cells that can respond more effectively and rapidly upon secondary exposure to the pathogen. This is the hallmark of adaptive immunity that has been used as the basis of prophylactic vaccination strategies against pathogens (Janeway 2005).

	Innate immunity	Acquired immunity
Physiochemical barriers	Skin	Cutaneous and mucosal immune systems
	Mucosal membranes Lysozyme Stomach acid Commensal bacteria in gut	Antibodies in mucosal secretions
Circulating molecules	Complement	Antibodies
Cells	Granulocytes Monocytes/macrophages NK cells	B lymphocytes T lymphocytes
Soluble mediators	Macrophage-derived cytokines	Lymphocytes-derived cytokines

Table 1.1. Differences between innate and adaptive immunity (Calder 2007).

1.4 Immunisation

An estimated four million infants die annually from infections that are highly preventable by vaccination (Bonhoeffer, Siegrist et al. 2006). Immunisation is one of the most important interventions in reducing morbidity and ultimately eradication of these diseases. The basic principles of immunisation are to stimulate the immune system with immunogenic Ags to induce memory cells, and upon exposure to the actual pathogen, memory cells will rapidly and effectively respond to provide protection. Individuals are vaccinated with harmless agents that can either be live attenuated (weakened) vaccine, a killed vaccine, an inactivated toxoid derived from the organism, or protein Ags derived from the organism (Baley and Leonard 2005; Rabson, Roitt et al. 2005). The World Health Organisation Extended Programme on Immunisation, (WHO EPI) started in 1974, recommends that all children be vaccinated against the following important childhood infections for which vaccines exist: tuberculosis (TB), polio, diptheria, whooping cough, tetanus, Hepatits B, measles and *Haemophilus influenzae* type b (Hib) (Baley and Leonard 2005). Additional vaccines are added to the schedule depending on whether a particular vaccine preventable disease is endemic to that country. Since its inception millions of lives have been saved from vaccine preventable diseases.

1.5 Tuberculosis epidemiology

In 1882 Robert Koch identified *Mycobacterium tuberculosis* (Mtb) as the causative agent of human tuberculosis (TB). TB is one of the major causes of mortality globally despite availability of a vaccine and access to curative drug therapy. Data collected from 196 countries and territories and published by the World Health Organisation (WHO) gives a good indication of TB incidence and its trends. In 2008 there was an estimated 9.4 million incident cases of TB, up from 9.1 million cases in 2005 and 11.1 million (range, 9.6-13.3 million) prevalent cases (WHO; Global Tuberculosis control: a short update to the 2009 report). TB

therefore remains a significant public health problem. The WHO Millennium goal is to reverse this incidence of TB disease globally with a target date set for the year 2015. However the African and European regions are behind track in reaching their set targets. For example, in 2005 the annual TB incidence in the Western Cape region of South Africa was the highest in the world, with an estimated 1037 cases per 100,000 population (Cowley, Govender et al. 2008). Based on the most recent data of all TB incidence cases, South Africa has the fifth highest rate in the world, behind India, China, Indonesia and Nigeria (WHO TB report 2009). The global distribution of new TB cases is shown in Fig. 1.3.

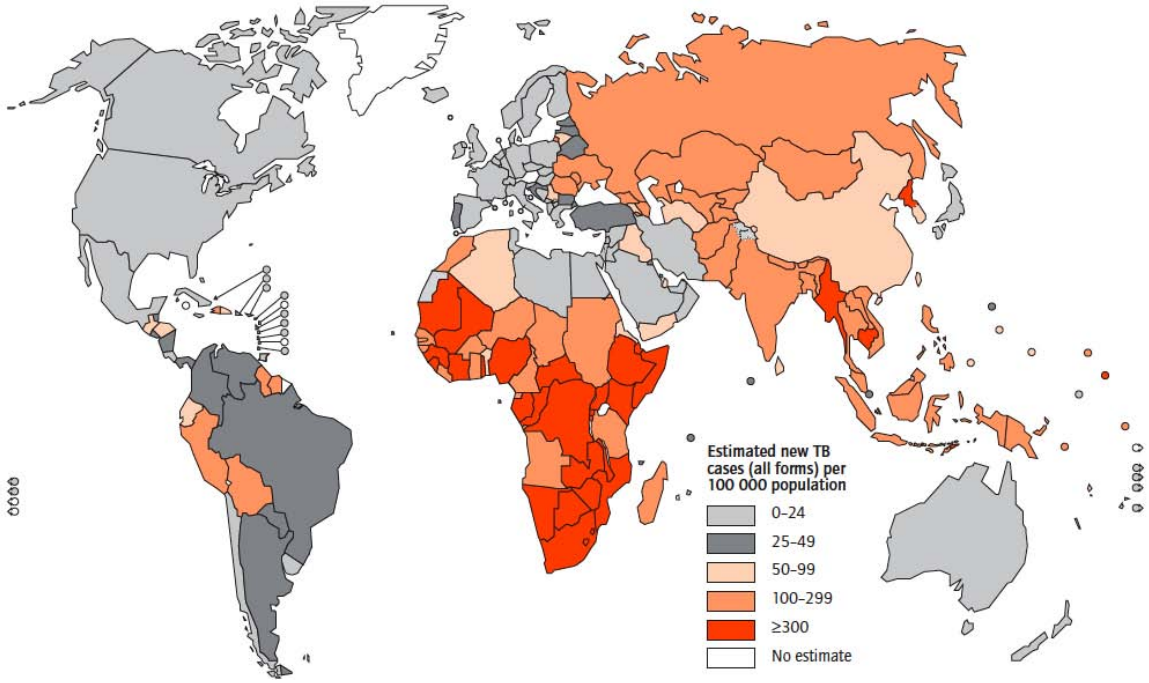

Figure 1.3. Estimated rates of new TB cases worldwide in 2008. (Adapted from the WHO; Global Tuberculosis control: a short update to the 2009 report).

The rise in incidence cases is largely in developing countries and has been attributed mainly to population growth and human immunodeficiency virus (HIV)-associated TB due to HIV co-infection (Corbett, Watt et al. 2003; Dye, Watt et al. 2005). Of all the new TB cases in 2008, 15% were HIV-positive and 78% of

these were found in developing countries in the African region (WHO; Global Tuberculosis control: a short update to the 2009 report). HIV-infected individuals have an estimated 15% annual risk of TB disease, compared to a 10% lifetime risk in HIV negative individuals (Whelan, Pathan et al. 2009).

In addition, multidrug-resistant (MDR) TB is on the rise with an estimated 50 million people infected (Dye, Espinal et al. 2002; Kaufmann 2002). MDR TB is resistant to the two first-line anti-TB drugs, isoniazid and rifampacin. The lengthy course of TB treatment leads to poor compliance and coupled with poor drug access in developing countries makes TB difficult to eradicate (Flynn 2004). Though rare, extensively drug-resistant (XDR) TB poses an even greater challenge to successful eradication of TB. XDR TB is resistant to almost every anti-TB drug, including the first-line and second line drugs, fluoroquinolones and at least one of the injectable anti-TB drugs (Gandhi, Moll et al. 2006). There is thus an urgent need to find ways to better control and eliminate TB. One of the best ways to control this epidemic is through prevention of TB disease by use of an effective vaccine.

1.6 Pathogenesis and the immune response to TB

An overview of the success of TB as a pathogen will help in understanding why it has become a global health problem. TB is contagious and is transmitted mainly via the respiratory route with humans as the natural host (Flynn 2004). Once in the lungs, Mtb replicates within alveolar ducts and within alveolar macrophages that transport the bacilli to draining lymph nodes where they present Ags to CD4 T cells. Mtb also infects macrophages recruited to the site of infection from the bloodstream. These cells are not activated and thus cannot kill Mtb. Neutrophils are the main host cell infected with Mtb in TB patients (Eum, Kong et al. 2010), however Mtb can infect other cell types such as macrophages, DCs and epithelial cells (McDonough and Kress 1995; Abadie, Badell et al. 2005). Binding of Mtb to PRRs and to other receptors such as TLR2 and complement receptor 3

(CR3) facilitates uptake of Mtb into phagocytic cells (Schlesinger 1993; Ernst 1998).

After Mtb infection the pathogen may be immediately cleared in a process that is yet to be defined in detail. However, approximately 5% of Mtb-infected people will fail to clear infection and develop primary tuberculosis within the first two years of infection, and another 5% develop TB later in life, whilst the remaining 90% control infection and establish an asymptomatic, latent infection. If not cleared, chronic Mtb infection leads to the formation of a granuloma. Mtb is able to survive within macrophages by preventing the phagosome-lysosome fusion pathway. A granuloma consists of fibroblasts, both CD4 and CD8 T cell lymphocytes and B cells surrounding macrophages, some infected with Mtb. The granuloma functions to contain the infection in localised sites preventing spread and allowing immune cells to act on infected cells. Caseous necrosis of the granuloma may occur forming cavities within the lung resulting in disseminated TB in other organs such as the liver and spleen, but more commonly Mtb remains in granulomas formed in these other organs.

The metabolic state of Mtb within the granuloma is not well known but it was initially believed to be in a dormant non-replicative state. However, recent animal studies employing use of necropsies and imaging techniques such as high-resolution computed tomography (CT) and positron emission tomography (PET) suggest that Mtb may be metabolically active and replicating within the granuloma. Thus latency may represent a wide range of infection states. This latent phase can last for a lifetime and is characterised by a lack of clinical symptoms and adverse effects as Mtb has developed escape mechanisms and is able to survive within the granuloma. This presents a huge problem, as latently infected individuals are a reservoir of TB and may reactivate to develop TB disease. A reactivation of latent TB infection occurs in up to 10% of individuals, who fail to control infection. Reactivation may be as a result of a genetic predisposition, immunosuppression due to age or HIV infection, or malnutrition. This is mostly seen in adults and the disease presents itself as highly infectious

pulmonary TB. However, in immunocompromised individuals where granuloma formation may be compromised, Mtb infection is not well contained and can become a systemic disease spreading to other organs, as seen in HIV-infected individuals (Flynn 2004; North and Jung 2004; Barry, Boshoff et al. 2009).

1.6.1 T cell responses to Mtb

In most individuals an adaptive immune response is triggered to control infection. Mtb is an intracellular pathogen that usually resides in the vacuole of infected macrophages, and thus an effector T cell response rather than an antibody-mediated response is required to clear infection. Following infection and uptake of Mtb by phagocytic cells such as macrophages and dendritic cells, IL-12 is released promoting a Th1 response. Infected macrophages can present Mtb Ags to T cells via the MHC Class II pathway resulting in a CD4 T cell response. CD4 T cells produce various cytokines but mainly produce IFN-γ in response to Mtb infection (Flynn 2004).

As mentioned earlier, Mtb resides in intracellular vacuoles and thus Mtb Ags are mainly presented to CD4 T cells. However some studies have shown that Ag presentation may occur via the MHC class I pathway, thus activating CD8 T cells. During the innate immune response APCs may cross-present Ag to CD8 T cells (Schaible, Winau et al. 2003; Morel, Badell et al. 2008). CD8 T cells may be cytotoxic, targeting Mtb and clearing infection. Cells may be lysed by the action of perforin, which forms pores in the cell membrane of infected cells. Direct killing of Mtb is through release of granulysin, which gains access to intracellular Mtb through pores first created by perforin (Stenger, Hanson et al. 1998). Cell death may lead to the killing of Mtb but may also release bacteria that can further infect activated macrophages (Flynn 2004).

CD4 and CD8 T cells express IFN-γ and TNF-α resulting in the activation of macrophages to become mycobacteriocidal (Flynn and Chan 2001). Individuals with mutations for IL-12p40 receptor, IFN-γ receptor or genes for IFN-

γ production are more susceptible to Mtb, highlighting the importance of these cytokines (Lammas, De Heer et al. 2002). Studies have shown that even though IFN-γ is a requirement for a protective response, it is not a good immune correlate of protection against development of TB disease (Flynn 2004; Kagina, Abel et al. 2010). The importance of TNF-α is highlighted in patients who become more susceptible to severe forms of disseminated TB after undergoing anti-TNF-α treatment for rheumatoid arthritis or Crohn's disease (Keane, Gershon et al. 2001). TNF-α also plays an important role in macrophage activation by acting synergistically with IFN-γ to maintain the granuloma (Cooper 2009).

1.7 Diagnosis

A pathological delayed-type hypersensitivity response is characteristic of an immune response to mycobacteria. This hypersentivity response can be initiated by the classic tuberculin skin test (TST/ Mantoux skin test). In this test, purified protein derivative (PPD) from Mtb is injected intradermally in the forearm. If the person responds an induration of the skin occurs and interpretations of results are usually done 48-72hrs after start of the test. Indurations of 10mm or more in diameter are considered as a positive test result indicative of mycobacterial infection (Gallant, Cobat et al. ; Huebner, Schein et al. 1993). This indicates prior exposure to mycobacteria but not necessarily disease. A subset of TB exposed people do not respond to the PPD test indicating that their innate immune system prevented infection but the mechanism by which this happens is largely unknown (Flynn 2004). However, this test is of little use in diagnosing active TB disease due to high rates of false positives (cross-reactive) and false negatives. Exposure to environmental mycobacteria and prior bacillus Calmette-Guérin (BCG) vaccination in individuals receiving the PPD test can lead to false positives due to cross-reactive immune responses to shared Ag in PPD and other mycobacteria. Active TB is thus commonly diagnosed by standard tests based on history of TB exposure, radiological tests and microbiological confirmation of Mtb in smear and

culture tests from sputum or other disease sites (Chan, Heifets et al. 2000; Negrete-Esqueda and Vargas-Origel 2007).

1.8 BCG vaccine

The most widely used and the oldest of the vaccines to date is the BCG (Bacillus Calmette and Guérin) vaccine, the only licensed TB vaccine. In their search for a TB vaccine, Albert Calmette and Camille Guérin started working on the *Mycobacterium Bovis* strain from tuberculosis mastitis infected cows at a time when there was minimal understanding of the bacterium and the host immune system. This strain was serially passaged 231 times from 1906 to 1919 in medium containing glycerol, potato slices and beef bile. During this period the strain lost its virulence, first to calves and then to guinea pigs, and the first attenuated BCG vaccine was tested in humans in 1921 by Weil Halle (Fine 2000).

More than 4 billion people have received BCG since the start of its routine use in the 1960s. In the past the vaccine has been administered via different routes but the WHO currently recommends the intradermal route (Bricks 2004). The WHO also recommends that newborns in highly TB endemic countries be BCG vaccinated at birth unless they are symptomatically infected with human immunodeficiency virus (HIV) (Baley and Leonard 2005). BCG is also contradicted in persons with leukaemia, persons undergoing immunosuppressive therapy and pregnant mothers (Hussey, Hawkridge et al. 2007). The WHO does not recommend BCG vaccination after 1 year of age because protection is variable and uncertain. More than 4 billion people have received BCG since the start of its routine use in the 1960s. According to recent WHO-UNICEF estimates, in 2009 there was an estimated 88% global BCG immunisation coverage, whilst in South Africa this was estimated at 94% of the target population. Despite its extensive coverage TB still remains the leading cause of death from a curable infectious disease worldwide.

The original strain of BCG maintained by serial passage at the Pasteur Institute was distributed to manufacturers worldwide. Different laboratories maintained their own strains under different sub-culturing conditions. However the original BCG strain at the Pasteur institute was lost and as it was never cloned this has resulted in many different BCG strains from manufacturers around the world. Genotypic analyses show undoubtedly that these strains differ from each other and from the original BCG strain. Currently the major producers of BCG vaccines are Pasteur-Merieux-Connaught, the Danish Statens Serum institute, Evans Medava and the Japan BCG laboratory in Tokyo (Fine 2000; Hussey, Hawkridge et al. 2007).

1.8.1 BCG vaccine efficacy

The BCG vaccine protects infants against the more severe forms of TB such as TB meningitis and miliary TB, but protection in adults and infants against pulmonary TB is variable, resulting in the controversy surrounding this vaccine (Fine 1995; Hussey, Hawkridge et al. 2007). A meta-analysis of published reports has shown variable efficacy rates against pulmonary TB ranging from 0-80% (Trunz, Fine et al. 2006; Hussey, Hawkridge et al. 2007; Negrete-Esqueda and Vargas-Origel 2007). Years later a BCG induced memory response can be detected in vaccinated individuals. However, the response wanes with time and some studies have indicated that an immune response wanes after 20 years but may persist for as long as 60 years (al-Kassimi, al-Hajjaj et al. 1995; Aronson, Santosham et al. 2004).

Some possible reasons for variable efficacy rates are environmental mycobacteria, helminth infections and host genetics (Hussey, Hawkridge et al. 2007). These may modulate the immune response to BCG and interfere with BCG-induced protection. Exposure to other environmental mycobacteria post-BCG vaccination may lead to cross-reactive immunity that blocks BCG-induced protective immune responses (Brandt, Feino Cunha et al. 2002; Flaherty,

Vesosky et al. 2006). Environmental mycobacteria are thought to also mask BCG induced immunity. In masking, antimycobacterial immunity induced by environmental mycobacteria cannot be improved upon by subsequent vaccinations (Buddle, Wards et al. 2002). This may be the underlying reason why BCG is less effective in tropical regions where environmental mycobacteria are generally more abundant (Fine 2000). Failure of BCG to provide adequate protection in environments where chronic infections are prevalent has led to the hypothesis that chronic helminth infections may affect BCG efficacy (Hori, Watanabe et al. 1993). Helminth infection may skew the immune system towards a Th2 type response (Kullberg, Pearce et al. 1992) instead of a Th1 response required for protection against Mtb. The environment alone cannot explain the variable efficacy of BCG. Host genetics pay a role as BCG induced protection varies between different populations (Colditz, Brewer et al. 1994; Fine, Carneiro et al. 1999).

Thus much work is underway to understand BCG, TB and to produce new TB vaccines. Although knowledge in this field is on the rise little is actually known about the host factors affecting BCG induced immunity to prevent TB. To advance in the development of a more efficacious vaccine against TB, it is important to characterise host factors that may modulate the immune response induced by BCG vaccination at birth, as infants are potentially the target population for new improved vaccines against TB.

Other potentially important factors that may influence BCG induced immunity are birth weight, gestational age (GA) and size for GA at time of vaccination. Firstly, a better understanding of these host factors and lessons that can be learnt from other early life responses and vaccines is needed before investigating them in the context of BCG vaccination.

1.9 Infant birth weight and size for gestational age

The term low birth weight (LBW) refers to infants born weighing less than 2,500g. Infants weighing between 1,000 and 1,499g are classified as very LBW (VLBW) and extremely LBW if they weigh between 500 and 999g. Premature or pre-term (PT) LBW infants are infants born ≤37 weeks gestation, and term LBW infants are born between 38 and 41 weeks of gestation. LBW infants born after 41 weeks are classified as post term. Size for gestational age is based on foetal body weight percentiles and standard growth curves of infants. Small for gestational age (SGA) infants have a birth weight less than the lower 10^{th} percentile of the standard growth curves. Appropriate for gestational age (AGA) infants have birth weights between the 10^{th} and 90^{th} percentile, and large for gestational age (LGA) infants have a birth weight above the 90^{th} percentile. Intra-uterine growth restriction (IUGR) is commonly a result of poor maternal nutrition but can be any process limiting intra-uterine growth resulting in LBW. LBW and IUGR do not always represent the same situation, as some SGA infants are healthy whilst some AGA infants show signs of IUGR (Valero De Bernabe, Soriano et al. 2004; Raqib, Alam et al. 2007).

LBW is an important public health indicator. There are increased rates of morbidity and mortality in LBW infants compared to NBW infants. Consequences of prematurity, LBW and IUGR on development can also extend to later years in life. Depending on the specific cause, these infants may have increased rehospitalisation rates, lower intellectual quotients (IQ), impaired neurological functions and may develop hypertension, diabetes and coronary heart disease as adults (Ballow, Cates et al. 1986; Valero De Bernabe, Soriano et al. 2004). The reasons for LBW are complex but the primary cause is premature birth. The risk factors that may contribute to LBW are extensively reviewed by Bernabe *et al* (Valero De Bernabe, Soriano et al. 2004). Table 1.2 below summarises some of these risk factors and their outcomes.

Risk Factors	Outcome
Smoking (Tobacco is most common)	Higher incidence of PT and LBW infants.
Alcohol consumption	Foetal alcohol syndrome (FAS) may result depending on alcohol dosage and is related to an increase in LBW
Maternal nutrition	Moderately related to IUGR leading to LBW
Marital status and maternal stress	LBW infants are usually born to single mothers or to couples where the father was absent during pregnancy.
Educational status	Decreased level of educational status is possibly linked to increase in LBW incidence
Socio-economic level (strongly related to all the risk factors above)	The lower the socio-economic level the higher the incidence of LBW.
Ethnicity	LBW incidence is higher in black women than white women.
Genetic	40% of LBW is hereditary (60% environmental)
Maternal Age	LBW incidence is increased at the extremes of female reproductive life (15-19yrs and 35-40yrs)
Multiple births	Decreased availability of nutrients to each foetus. May result in IUGR and lead to LBW

Table 1.2. Risk factors and outcomes pertaining to low birth weight (Valero De Bernabe, Soriano et al. 2004).

2.0 Birth weight, maturity and the neonatal immune response

The innate and adaptive immunity of neonates is fundamentally the same as that of adults, as described in the previous sections. However, some functional differences in the human neonatal immune system have been observed. These differences have resulted in the classification of the neonatal immune system as immature, compared to adults.

As discussed earlier, APCs induce the adaptive immune response. Neonatal APCs are functional but express low levels of CD80 and CD86, co-stimulatory molecules required for the clonal expansion and differentiation of naïve T cells. Thus neonatal DCs have a reduced capacity to promote Th1

responses (Goriely, Vincart et al. 2001; Velilla, Rugeles et al. 2006). Even though at the mRNA level TLR expression in adults and neonates is similar, impairment of TLR1, TLR2, TLR4 and TLR6 immune function have been described. For example, expression of the proinflammatory cytokines TNF-α and IL-12 in response to TLR4 ligands, such as bacterial lipopolysaccharide (LPS), is low in neonates compared to adults. TLR4 protein expression has been found to be lower in PT infants compared to term infants. Furthermore, neonatal monocytes exhibit a lower capacity to phagocytose *E.coli* compared to adult monocytes; this impairment is even more pronounced in PT infants born <30 weeks gestation (Velilla, Rugeles et al. 2006). PT LBW infants are particularly prone to pneumococcal infections, whilst LBW infants are at a greater risk of pertussis infections (Bonhoeffer, Siegrist et al. 2006). Taken together, these findings may explain the increased susceptibility of PT infants to infections that are otherwise not as harmful in full term infants and adults (Velilla, Rugeles et al. 2006).

Expression of immunosuppressive cytokines by Tregs helps to maintain immune homeostasis. Tregs are found in high numbers within the uterine lining and play an important role in maintaining pregnancy by suppressing inflammatory responses. Tregs also develop spontaneously in the human foetus and in newborn infants. Unlike adult cells, Tregs found in cord blood have a naïve phenotype with high levels of CD45RA expression. At birth the frequency of Tregs is higher in PT infants compared to full term infants. This suggests that infant Tregs may be detrimental for Ag specific immune responses, and particularly more so in PT infants. Expression of trophoblast derived IL-10 is another immunoregulatory mechanism to maintain pregnancy. IL-10 inhibits Th1 responses that would otherwise harm the foetus. A characteristic of this immunoregulation is the impaired CD4 T cell expression of IFN-γ and IL-2 and responses that are skewed towards a Th2 response in neonates compared to adults. Decreased expression of IFN-γ is due to hypermethylation of certain sites of the promoter region of the IFN-γ gene (Melvin, McGurn et al. 1995; Velilla, Rugeles et al. 2006).

A study by Gasparoni *et al* evaluating immune development showed not only that neonatal responses are impaired but that there is also a gradual maturation from birth to adulthood. In this study, IL-2, IL-4, IL-10 and IFN-γ expression was compared among very pre-term infants (20-29 weeks GA), PT infants (30-37 weeks GA), term infants (38-42 weeks GA), children (3-10 years old) and adults. The percentage of naïve T cells expressing CD45RA was higher in newborns compared to all other groups. IL-2, IL-10 and IFN-γ expression by T cells was found to be negatively associated with GA. The activity of NK cells was impaired in neonates and was positively associated with GA (Gasparoni, Ciardelli et al. 2003).

There is also evidence that gradual maturation of the neonatal immune system is directly correlated with vaccine-induced protection. Antibodies directed against most bacterial polysaccharides (PSs) are only produced in infants 2 years or older, leaving younger infants susceptible to invasive disease caused by organisms such as *Haemophilus influenza* type B (Hib). Vaccines given in the first 6 months of life, with the exception of BCG, require several doses to achieve protective responses. A single dose of either oral polio virus (OPV) or diphtheria-tetanus toxoid at birth induces very low levels of neutralising Ab (Siegrist 2001). A gradual increase in responses with age has been reported, for example the Hib conjugate vaccine is most immunogenic when administered as a single dose at 15-23 months of age, compared to 3 doses in the first 6 months of life. In addition, Hib induces higher serum Ab levels at 4-6 months than at 2-3 months of age (Siegrist 2001). A similar gradual increase has also been observed with measles vaccination, with lower Ab responses at 6 months compared to 9, 12 and 15 months of age (Gans, Arvin et al. 1998; Bonhoeffer, Siegrist et al. 2006).

The following vaccines are all recommended by the American Academy of Pediatrics to be administered at the full dosage to healthy PT LBW infants and to term NBW infants at 2 months of age: Hib conjugate; diphtheria, tetanus, acellular pertussis (DTap); inactivated polio virus (IPV) and Pneumococcal (Pn) conjugate. These vaccines induce significantly higher neutralising Abs in infants

when administered at this age than at birth (Baley and Leonard 2005). The hepatitis B vaccine has a different regime for PT LBW infants compared to term infants. Birth weight affects the immunogenicity of this vaccine, as infants weighing less than 2,000g are less likely to mount sufficiently high levels of neutralising Ab. It is recommended that the hepatitis B vaccine be delayed if the mother is hepatitis B surface Ag (HBsAg) negative and the infant weighs less than 2,000g. These infants should only be vaccinated once they reach a birth weight of 2,000g, or when they are 2 months of age (Baley and Leonard 2005).

LBW as a result of nutritional deficiencies may also affect the immune system as growth of lymphoid organs is sensitive to nutrition and is positively associated with age (Langley-Evans and Carrington 2006). Anti-typhoid Ab responses in a group of Filipino adolescents was positively associated with birth weight (McDade, Beck et al. 2001). Studies in the Gambia have shown that birth in the rainy hungry season (July-December) resulting in LBW, compared to birth in the harvest season (January-June), is associated with increased mortality from infectious disease later in life (Moore, Cole et al. 1997; Moore, Cole et al. 1999). The hungry season is marked by increased seasonal infections and shortage of food when food supplies from the previous harvest season run out (Moore, Fulford et al. 2004). These infants were found to have lower expression of CD154, a ligand that enhances the killing capacity of human macrophages and monocytes and which may play a role in protection against Mtb (Miles, van der Sande et al. 2008).

Lastly, the neonate humoral response is also relatively immature at birth compared to adults. In the first year of life Ab responses are weak and short lived. An infant's immune system largely depends on passively transferred maternal antibodies (Abs) for protection. Maternal immunoglobulin (Ig)-G Abs are mostly transferred across the placenta during the third trimester of pregnancy. During breast feeding IgG and IgA Abs are transferred to the neonate. Maternal Abs confer passive immunity for up to 18 months against bacterial and viral

pathogens that the mother was immune to prior to giving birth or to breastfeeding (Baley and Leonard 2005; Janeway 2005).

The evidence presented here indicates that some neonatal immune responses are immature, or different, compared to adult responses. There is also evidence of a gradual maturation of the immune system in neonates to adults. Functional impairment of the neonatal immune system may explain the variable Th1 responses to vaccines and increased susceptibility to intracellular pathogens usually exhibited by neonates.

3.0 Current study: Aims and objectives

Neonatal immunological immaturities may be more pronounced in the least mature group of PT LBW infants. Evidence of neonatal immune immaturity provides rationale for performing a study assessing the effects of birth weight and gestational age on BCG-induced cellular immunity.

Aim 1:

To determine the effect of birth weight and gestational age on the phenotype and magnitude of the BCG induced T cell response

Hypothesis: BCG-vaccinated PT and LBW infants would have a lower frequency of BCG-specific T cells, compared to term NBW infants.

Aim 2:

To determine the effect of birth weight and gestational age on the proliferative potential of BCG-specific T cells following BCG vaccination

Hypothesis: BCG vaccination at birth induces antigen-specific T cells with a greater proliferative and cytokine producing capacity in NBW infants compared with LBW infants.

CHAPTER TWO

Methodology

2.1 Overview

An adaptive immune response mediated by lymphocytes is critical in the clearance of various pathogens. Many novel vaccines aim to induce an antigen-specific T cell response, and measuring this cellular immune response is important in assessing vaccine immunogenicity and efficacy. Several methods, many of them based on flow cytometry, can be used to study the frequency and phenotype of antigen-specific T cells.

Antigen-specific T cells may be indirectly detected by measuring secreted cytokines at the population level, by an enzyme-linked immunosorbent assay (ELISA). An enzyme-linked Immunospot (ELISpot) assay is more sensitive and can be used to detect secreted cytokines at the single cell level. T cell effector functions, such as cytotoxicity, may be detected *in vitro* by a chromium release assay. Target cells labelled with ^{51}Cr and pulsed with antigen are incubated with cytotoxic T-lymphocytes (CTLs). CTLs specifically kill target cells and thus cell-mediated cytotoxicity can be measured by the release of radioactive ^{51}Cr into the culture supernatant (Kern, LiPira et al. 2005).

Alternatively, T cells may be directly analysed by staining them with fluorescently labelled major histocompatibility complex (MHC) tetramers. These specific MHC tetramers have a high avidity to target T cell receptors, ensuring a high signal when analysed by flow cytometry (Thiel, Scheffold et al. 2004). Antigen-specific T cells may also be directly detected by their *in vitro* ability to proliferate, or synthesise deoxyribonucleic acid (DNA) following activation as described later (2.1.2).

The advent of intracellular cytokine staining (ICS) to detect cytokine expression at the single cell level (Suni, Picker et al. 1998) has greatly advanced the field of cellular immunology. T cells are stimulated with antigen to induce cytokine production, and then fixed, permeabilised and stained with fluorescently-conjugated monoclonal antibodies (Abs) (Kern, LiPira et al. 2005). ICS assays were first used to detect responses in peripheral blood mononuclear cell (PBMC)

cultures and later modified for use in whole blood cultures in field studies (Suni, Picker et al. 1998). This modified whole blood intracellular cytokine (WB-ICC) assay is described in more detail below (2.1.1).

2.1.1 Whole blood intracellular cytokine (WB-ICC) assay and flow cytometry

The whole blood intracellular cytokine (WB-ICC) assay is one method that can be used to measure the frequency of cytokine-producing, antigen-specific T cells in adults and infants (Fuhrmann, Streitz et al. 2008). The advantages outlined below made this the main assay of choice in this study to measure BCG-specific T cell responses.

The WB-ICC assay has been optimised and shown to be a sensitive and specific assay with reproducible results (Hanekom, Hughes et al. 2004). This assay does not require carbon dioxide and requires only a small volume of blood, which is immediately stimulated with antigen after venipuncture (Hanekom 2005). It has been optimised to cater for studies where blood collection is done offsite, typically at a rural clinic far from the laboratory where the immunological assays are done. Blood collected at the rural clinic is incubated with antigen and transported in a portable incubator to the laboratory where incubation is continued in a programmable waterbath and the assay completed with the fixation and cryopreservation of white cells (Hanekom, Hughes et al. 2004; Thiel, Scheffold et al. 2004).

To initiate the short-term stimulation with antigen, blood is collected in sodium heparanised syringes and added to polypropylene tubes containing an antigen of choice in the presence of co-stimulatory Abs such as anti-CD28 and anti-CD49d. Co-stimulants are added to enhance the specific stimulatory capacity of effector T cells and maximize the detection of cells producing cytokines such as IFNγ. Whole blood may be incubated in a short term assay (<20 hrs), or in longer term assays (up to 7 days) after first diluting it in RPMI media. A Golgi transport inhibitor, such as Brefeldin A or monensin, is added during the last several hours of incubation to block secretion of cytokines by

preventing protein transport from the endoplasmic reticulum (ER) to the Golgi complex. After incubation, cells are harvested, red blood cells lysed, the white cells fixed and cells or ribonucleic acid (RNA) cryopreserved for later analysis. Plasma from the incubated whole blood may also be collected prior to addition of Brefeldin A and stored for later analysis of soluble factors secreted during the initial stimulation period.

After antigen stimulation of whole blood, intracellular cytokine staining of lymphocyte populations may be performed. Most of the detectable intracellular cytokines are a result of addition of a protein transport inhibitor that blocks cytokine secretion and allows for detection of cytokines within the cytoplasm of cells. Thus stimulated, fixed cells that have been cryopreserved are thawed, and permeabilised to allow detection of intracellular cytokines. Cytokines of interest found within the permeabilised cells are detected by staining with fluorescently-conjugated monoclonal Abs (Soares, Scriba et al. 2008).

Lastly, stained cells are analysed by flow cytometry (Janeway 2005), a technique to detect fluorescently labelled particles as they flow in a fluid system through a laser beam. Particles such as fluorescently labelled cells are forced through a nozzle in a single fluid stream of droplets that flows perpendicular to the laser beam. When they intercept the laser beam they will deflect and scatter the incident laser light whilst the fluorochrome is excited, thus emitting fluorescence. The physical properties of each particle determine the extent of light scatter. Light scattered in the forward direction, forward scatter (FSC) gives information on the size and surface area of the particle. Light scattered at an angle of 90° to the incidence laser beam is called side scatter (SSC) and gives information on the granularity of the particle. Scattered light and fluorescence signals given off by the fluorescently stained particles are detected by sensitive photomultiplier tubes (PMTs). Finally, signals are converted to information that can be analysed by a computer (Janeway 2005). Multiparameter flow cytometry allows measurement of several fluorochromes simultaneously on a single cell basis. In this study, the use of a three-laser LSR II flow cytometer enabled the acquisition and detection of cells co-stained with different fluorochrome-

conjugated monoclonal Abs (Fuhrmann, Streitz et al. 2008). After acquisition, data analysis enables the delineation of different cell types, cell subsets and intracellular cytokines expressed.

Several factors must first be considered when developing an optimum polychromatic Ab panel for flow cytometry so that results are reliable and reproducible. Firstly, the fluorochrome-Ab combination should be carefully chosen based on expression levels of the target protein to allow optimum detection. Proteins of interest that are expressed at low levels should ideally be labeled with the brightest fluorochromes in that Ab panel. The amount of Ab should be titrated to determine the Ab volume that gives the best signal to noise ratio. Unwanted spectral overlap of one fluorochrome into the detector of another fluorochrome is another problem to consider as it can complicate data analysis. Such fluorescence spill-over can be reduced or eliminated by titrating antibodies and carefully choosing fluorochromes with different excitation spectra. Spectral overlap can be assessed by investigating the frequency of the labeled protein with and without each single fluorochrome in that Ab panel in "fluorescence minus one" experiments. In addition with the use of appropriate compensation controls and analysis software, spectral overlap may be compensated for using flow cytometry analysis software such as FlowJo. The number of fluorochromes in a panel is also important, as spectral overlap and measurement errors worsen as the number of fluorochromes increase, even when the panel is properly compensated. Testing the desired Ab panel with different permutations of fluorochrome-Ab combinations can be time consuming and expensive. A common method of optimising a polychromatic Ab panel is to first optimise a smaller subset of the desired panel and then to systematically add and optimise additional fluorochrome-Abs until the panel is complete (Baumgarth and Roederer 2000; Roederer 2001; McLaughlin, Baumgarth et al. 2008).

2.1.2 Lymphocyte proliferation assay and flow cytometry

Another method to measure adaptive immune responses in vitro is by the ability of lymphocytes to proliferate and expand in response to antigen stimulation. Lymphocytes can be isolated from peripheral blood by density gradient centrifugation of anticoagulated blood over a layer of Ficoll. The resulting cell population, consisting of mainly lymphocytes and monocytes, are called peripheral blood mononuclear cells (PBMCs) (Gulati 2009).

PBMCs, either cryopreserved or freshly isolated, can be incubated with specific antigens in either short or long-term assays. Proliferation of antigen specific cells is usually detected following 6-7 days of antigen stimulation *in vitro*, and may be measured by the uptake of radioactive nucleotides, such as tritiated thymidine (^{3}H), or nucleotide analogues, such as bromodeoxyuridine (BrdU), into the DNA of replicating cells (Weston and Parish 1990; Fulcher and Wong 1999). The amount of these incorporated DNA precursors is proportional to the amount of proliferation.

Another method to monitor lymphocyte proliferation is based on the combined use of cell trace dyes and flow cytometry. In this assay system, PBMC are stained with a cell trace dye such as carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) or Oregon Green (OG: 2', 7'-difluorofluorescein), and labelled cells incubated with antigen. Cell trace dyes bind cellular proteins and when these stained cells proliferate, the cell trace dye is partitioned equally between daughter cells and the fluorescence intensity decreases by 50% with each cell division. The advantage of using cell trace dyes is that the number of cell divisions and the precursor cell frequency of antigen-specific cells can be quantified by flow cytometry. Another advantage is that cells may be co-stained with phenotypic markers and intracellular cytokines followed by multiparameter flow cytometric analysis, thereby allowing detailed characterization of populations of antigen-specific T cells with proliferative capacity (Lyons and Parish 1994; Lyons 2000; Wallace, Tario et al. 2008).

Intracellular detection of the human nuclear protein Ki-67 presents another flow cytometry-based method of assessing antigen-specific proliferation (Shedlock, Talbott et al. 2010). Most circulating lymphocytes isolated from peripheral blood are in the resting phase until stimulated with antigen where upon they actively divide (Gulati 2009). Ki67 can be used as a marker of proliferation *in vitro* as it is expressed in the nuclei of actively dividing cells during the G_1, G_2, S and mitosis phase, but not in resting cells (G_0) (Jeurink, Vissers et al. 2008). Proliferating cells can then be detected by intracellular staining of Ki-67 using fluorescently-conjugated monoclonal Abs.

The cytokine production capacity of antigen-specific proliferating T cells can be further assessed following restimulation of cells with specific antigens or mitogens, such as phorbol 12-myristate 13-acetate (PMA) and ionomycin, for the final 4-6 hours of the incubation period. Cells may also be stained with viability dyes enabling the differentiation of live cells from dead cells by flow cytometry. Furthermore, cell culture supernatants can be harvested at the end of the 6-day incubation period and stored for later analysis of soluble molecules that have been secreted and accumulate in the media over the culture period.

In summary, various ways to measure antigen-specific responses have been developed, however the resources available for the current study have influenced the choice of assays in our experimental system. South Africa is a developing country and participant recruitment was done at an established clinical site in a rural area. The WB-ICC assay was chosen as the most practical assay in our setting as outlined before, and the use of Ki67 as a sensitive marker of antigen-specific T cell proliferation was explored.

2.2 Materials and methods

2.2.1 Study participants

Healthy infants not requiring hospitalization, vaccinated intradermally or percutaneously within 24h of birth with Japanese BCG vaccine (strain 172, Japanese BCG laboratory) were recruited from Worcester in the Western Cape region of South Africa. Participants were selected from a larger randomised control trial (RCT; n=11,670) investigating the incidence of TB over 2 years in infants vaccinated at birth intradermally or percutaneously with Japanese BCG (Hawkridge, Hatherill et al. 2008). For this study, enrolled infants were stratified by birth weight into one of the following groups based on World Health Organisation (WHO) definitions: (i) low birth weight (< 2,500g) and (ii) normal birth weight (≥ 2,500g). The gestational age, as calculated from the mothers last menstrual period, was recorded for all infants. Maturity was noted and standard definitions used to classify infants as pre-term (PT) if born at ≤ 37 weeks gestation, and as term if born between 38 and 41 weeks gestation. Size for gestational age was based on fetal body weight percentiles and standard growth charts of infants aged 28-43 weeks (Naeye and Dixon 1978). Infants with a birth weight below the 10^{th} percentile were classified as small for gestational age (SGA). Appropriate for gestational age (AGA) infants had birth weights between the 10^{th} and 90^{th} percentile. Large for gestational age (LGA) infants had a birth weight above the 90^{th} percentile. Infants presenting with any chronic or acute illness at 10 weeks of age were excluded. At 10 weeks of age, at a routine childhood immunization visit, approximately 10mls of heparinised blood was collected by a dedicated qualified nurse by standard venipuncture technique. Two millilitres of blood were immediately processed for use in a whole blood intracellular cytokine (WB-ICC) assay described later. Approximately 8ml of blood was collected in Vacutainer Cell Preparation Tubes (CPT, Becton Dickinson). Written, informed consent was obtained from the parents or legal

guardian. The study protocol was approved by the Human Research Ethics Committee of the University of Cape Town.

2.2.2 Peripheral blood mononuclear cell (PBMC) collection

Blood was collected in Vacutainer Cell Preparation Tubes (CPT, Becton Dickinson) and peripheral blood mononuclear cells (PBMCs) isolated by density gradient centrifugation over a layer of Ficoll (HISTOPAQUE®-1077: Sigma-Aldrich). Multiple aliquots of PBMCs at 10 million per cryovial were cryopreserved in 10% dimethylsulfoxide (DMSO), 40% fetal calf serum (FCS) and 50% RPMI, and stored in liquid nitrogen until use.

2.2.3 Antigens

Viable *Mycobacterium bovis* Bacillus Calmette-Guérin [(BCG), Danish strain 1331, Statens Serum Institute)] was incubated with: (i) heparinised whole blood at a final concentration of 1.6×10^6 CFU/ml of blood, or (ii) 2×10^5 PBMCs at a final concentration of 2×10^5 CFU/ml. Staphylococcal enteretoxin B (SEB; Sigma-Aldrich), included as a positive control, was incubated with: (i) heparinised whole blood and (ii) 2×10^5 PBMCs at a final concentration of 10μg/ml and 0.05μg/ml, respectively. On the 6^{th} day of the proliferation assay, PBMCs were restimulated with 50ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 250ng/ml of ionomycin (I; Sigma-Aldrich).

2.2.4 Whole blood intracellular cytokine (WB-ICC) assay

The co-stimulatory Abs anti-CD28 and anti-CD49d (0.5μg/ml each, BD Biosciences) were included in all conditions of the WB-ICC assay. One ml of peripheral blood was incubated with BCG, whilst 500μl blood incubated with SEB served as a positive control. As a negative control, 500μl blood was incubated with costimulatory Abs alone. The WB-ICC assay was performed as described

previously (Hanekom, Hughes et al. 2004). Briefly, blood was incubated with antigens at 37°C in a portable incubator and transferred 100km by car from Worcester to the South African Tuberculosis Vaccine Initiative (SATVI) research laboratory, where the incubation was continued in a 37°C programmable waterbath. After 7h, Brefeldin A (10µg/ml; Sigma-Aldrich) was added and the incubation continued for an additional 5h. Cells were harvested after a total incubation time of 12h. EDTA was added (2mM, Sigma Aldrich), red blood cells lysed and white blood cells fixed by diluting the blood 1:10 with FACS Lysing solution (BD Biosciences). After a 10min incubation in FACS Lysing solution at room temperature (RT), the fixed white blood cells were centrifuged at 1400rpm for 7min at RT. Cells were resuspended in cryo-solution by first resuspending them in 250µl RPMI followed by addition of 250µl 20% DMSO (E Merck) in FCS. The fixed white blood cells were cryopreserved and stored in liquid nitrogen until use.

2.2.5 Six day PBMC proliferation assay

Cryovials containing infant PBMCs stored in liquid nitrogen were thawed quickly in a 37°C waterbath. The contents of each vial were transferred dropwise into a 15ml conical tube containing 10ml 12.5% heat inactivated human AB serum in RPMI (complete medium) and 2.5µg/ml DNase (Sigma-Aldrich). Tubes were centrifuged at 400g for 10min, supernatant decanted and cells resuspended gently with 1ml of complete medium. After resuspension, cells were counted with a Coulter machine (Beckman Coulter A^c.T diff^{TM} Analyser) and the concentration adjusted to 1 x 10^6/ml with complete media containing 2mM L-glutamine (Bio Whittaker). Using a Gilson p200 pipette, 2 x 10^5 cells per well were transferred to wells of a 96-well, round-bottomed plate (NUNC). Cells were plated in triplicate wells for each stimulation condition.

Cells were rested overnight at 37ºC, 5% CO_2 in a humidified incubator before addition of antigen. After resting, 2 x 10^5 cells per well were either left

unstimulated (negative control) or stimulated with BCG and SEB as described earlier (*2.2.3*). Cells were then incubated for 6 days in a 96-well, round-bottomed plate (NUNC) at 37ºC, 5% CO_2 in a humidified incubator.

A dye dilution method where cells were labeled with Oregon Green (OG: 2', 7'-difluorofluorescein; Molecular Probes) was also used in some experiments to investigate antigen-specific proliferation. For the OG proliferation assays, cryopreserved PBMCs were thawed and washed as outlined above, followed by another wash in PBS, supernatant decanted and cells resuspended gently in 1ml PBS. Cells were counted with a Coulter machine (Beckman Coulter A^c.T diff^{TM} Analyser) and adjusted to no more than 1 x 10^7/ml viable cells in PBS. Prior to resting, an equal volume of 10μg/ml working solution of OG in PBS was added to each 15ml conical tube containing the adjusted number of resuspended viable cells. Tubes were mixed gently, left to stand at RT for 3min, mixed vigorously by vortexing for 10sec, followed by 4min incubation at RT. An equal volume of PBS was added followed by gentle mixing and 4min incubation at RT. Each tube was filled to the top with PBS and centrifuged at 400g for 5min. The supernatant was decanted and cells resuspended in 1ml of complete medium containing 2mM L-glutamine (Bio Whittaker). Cells were rested overnight in a 96-well plate in a 37ºC, 5% CO_2 humidified incubator, antigen added the following day and cells incubated for 6 days as described above.

On the 6th day of all proliferation assays, cells were restimulated with PMA and Ionomycin (PMA/I); Brefeldin A (10μg/ml) was added and cells incubated for 5h at 37ºC, 5% CO_2 in a humidified incubator. Negative controls consisted of unstimulated PBMCs, and unstimulated PBMCs from day 1 of the assay restimulated on day 6 with PMA/I (PMA/I negative control). After 5h, 2mM EDTA was added and cells incubated for 15min at RT. Cells were centrifuged at 2200rpm and washed in PBS, centrifuged, and supernatant removed. Cells were stained for 30min at RT with violet viability dye (ViViD; LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Molecular Probes, Invitrogen). After staining, cells were washed in PBS and centrifuged at 2200rpm, supernatant removed and cells fixed

by the addition of FACS Lysing solution for 10min at RT. Stimulated, fixed cells were cryopreserved and stored in 96-well plates (NUNC) at -80ºC until use.

2.2.6 Antibodies and staining of cryopreserved whole blood and PBMCs

Fixed, stimulated cells from the WB-ICC assay were placed in a 37°C waterbath for quick thawing. Once thawed, samples were transferred by pipette to Falcon 2054 FACS tubes containing 3mls of PBS. FACS tubes were centrifuged at 2000rpm for 5min, supernatant decanted and cells resuspended gently in 1ml of 1X Perm/Wash buffer (BD Biosciences). Cells were left at RT in Perm/Wash buffer for 10min to permeabilise them. After 10min incubation, FACS tubes were centrifuged at 2000rpm for 5min at RT. Supernatant was decanted and cells stained at 4°C for 1h in the dark with the following fluorochrome-conjugated monoclonal Abs: anti-CD3 Pacific Blue (UCHT1), anti-CD4 Qdot 605 (S3.5), anti-CD8 Cy5.5-PerCP (SK1), anti-IL2 FITC (5344.111), anti-IFNγ Alexa Fluor 700 (B27), anti-TNFα Cy7-PE (MAb11) and anti-IL17 Alexa Fluor 647 (eBio64CAP17).

For analysis of 6-day proliferation assay samples, frozen NUNC plates containing fixed stimulated cells were thawed at RT and centrifuged at 2300rpm. Supernatant was removed, cells resuspended in Perm/Wash buffer (BD Biosciences) and incubated at RT for 10min to permeabilise cells. Thereafter cells were transferred to FACS tubes and centrifuged at 2000rpm for 5min and supernatant decanted. Cells were stained at 4°C for 1h in the dark with the following fluorochrome-conjugated monoclonal Abs: anti-CD3 FITC (Leu-1), anti-CD8 Cy5.5-PerCP (SK1), anti-IL2 APC, anti-IFNγ Alexa Fluor 700 (B27), anti-TNFα Cy7-PE (MAb11) and anti-Ki67 PE (eBio64CAP17). In the OG-based proliferation assay, anti-CD3 Qdot 605 (UCHT1) was used in place of anti-CD3 FITC (Leu-1).

All Abs were obtained from BD Bioscience, except for anti-Ki67 PE and anti-IL17 Alexa Fluor 647 obtained from eBioscience, and anti-CD3 Qdot 605 and anti-CD4 Qdot 605 obtained from Invitrogen.

After 1h incubation FACS tubes containing labelled cells were washed with 2ml Perm/Wash buffer, centrifuged for 5min at 2000rpm, supernatant decanted and cells resuspended in 100μl of PBS. Acquisition was completed on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA).

2.2.7 Flow cytometric analysis

Analysis of flow cytometry data was performed using FlowJo software version 8.8.5 (Treestar, San Carlos, CA). Separate compensation tubes for each fluorochrome-conjugated monoclonal Ab were prepared by first adding one drop each of anti-mouse Ig, κ and negative control (FBS) compensation beads (BD™ CompBeads Set Anti-Mouse Ig, κ) followed by the appropriate monoclonal antibody. Anti-rat Ig, κ and negative control (FBS) compensation beads (BD™ CompBeads Set Anti-Rat Ig, κ) were used with the rat Ab anti-IL2 APC. Anti-CD3 Pacific Blue (UCHT1) was used to compensate for ViViD. In a dye dilution method where cells were stained with OG, anti-IL2 FITC (clone 5344.111) was used to compensate for OG. Compensation beads were stained at 4°C for 1h in the dark to allow labelling.

Background fluorescence was determined automatically from single stained compensation beads, and compensation levels adjusted manually if necessary. Forward scatter area (FSC-A) versus forward scatter height (FSC-H) was used to exclude doublets and to gate on singlet cells followed by further analysis.

To analyse flow cytometry data from the WB-ICC assay, a lymphocyte gate was made by plotting FSC-A versus side scatter area (SSC-A). Total T cells were gated on by plotting CD3+ events versus IFN-γ, followed by a CD4+ versus CD8+ plot. Total cytokines produced by either CD4+ or CD8+ T cells were

determined by Boolean analysis of all possible combinations (see *Chapter 3,* Fig. 3.1A).

After excluding doublets in data from the 6 day PBMC proliferation assay, live cells were gated on by plotting the viability marker ViViD versus CD3+ events. The proliferation of live cells dim for ViViD and positive for CD3 were then analysed by plotting CD8 versus Ki67 or CD8 versus OG. This was followed by gating on CD8+ Ki67+/CD8+ OG^{low} and CD8- Ki67+/ CD8- OG^{low} proliferating cells. CD4+ T cells were defined as CD3+CD8- lymphocytes. Total cytokines produced by either of these proliferating cell populations was determined by Boolean analysis of all possible combinations. Cytokine expression was expressed as the frequency of grandparent, i.e. the percentage of live cytokine-producing and proliferating T cells within the total CD4+ or CD8+ population

Gates for proliferating and cytokine-producing cells were based on the negative control sample.

2.2.8 Statistical analysis

A response in the WB-ICC data assay was considered positive if it was greater than the median plus 3 times the median absolute deviation (Median + 3MAD) of the negative control samples. Background subtraction of proliferation data was based on the PMA/I negative control. Infants were excluded if the absolute number of viable CD3+ cells acquired was below the 25^{th} percentile of viable CD3+ cells acquired in the negative control, i.e <5,000 cells. Eleven infants failed to meet this cut-off and where excluded from further analysis. A response was considered positive if the number of proliferating cells in the stimulated sample was at least >20 cells above background proliferation in the negative control. Proliferative response was expressed as a stimulation index (SI) by dividing the percentage of Ki67+ T cells in the BCG-stimulated sample by the percentage of Ki67+ T cells in the unstimulated (PMA/I negative control) sample. Data for both assays were excluded if the positive control failed to work. Data were analysed in Pestle v1.6.2 (Dr M. Roederer, VRC/National Institutes of Allergy and Infectious

Diseases/National Institutes of Health, Bethesda, MD) and Spice v 5.0.5013 software (Dr M. Roederer and Joshua Nozzi, National Institutes of Allergy and Infectious Diseases/National Institutes of Health). A nonparametric Spearman test was used to determine associations between cytokine responses and birth weight. The Mann-Whitney test was used for comparisons between 2 groups and the Kruskal-Wallis test for comparisons between 3 groups. A p value $p<0.05$ was considered significant. Statistical analysis was performed using GraphPad prism v5.0a (GraphPad, San Diego, CA, USA).

CHAPTER THREE

The effect of birth weight and gestational age on the cytokine production profile of BCG-specific T cells

3.1 Summary

BCG, the only currently licensed tuberculosis (TB) vaccine, provides variable protection against TB. Immunogenicity studies have indicated a wide range in the frequencies of specific T cells induced by BCG vaccination, and one of the host factors that could contribute to this variability is birth weight. We designed a study to determine if birth weight (BW) and gestational age (GA) affect the magnitude and quality of the BCG-induced immune response. We hypothesised that BCG-vaccinated pre-term [PT (<37 weeks gestation)] and low birth weight [LBW (<2,500g)] infants would have a qualitatively and quantitatevely less optimal immune response, compared to term or normal birth weight (NBW) infants.

105 healthy infants were enrolled from Worcester, South Africa and BCG vaccinated at birth. Infants were stratified by birth weight, GA, size for GA and gender. At 10 weeks of age, a whole blood intracellular cytokine assay and multi-parameter flow cytometry was used to measure the BCG-induced immune response.

Compared with NBW infants, LBW infants had a lower overall frequency of IL-2+ and TNF-a+ BCG-specific CD4 T cells, and a positive correlation was observed between the frequency of BCG-specific IL-2+ CD4+ T cells and birth weight. Moreover, we observed a lower proportion of polyfunctional BCG-specific CD4+ T cells and corresponding higher proportions of single cytokine-producing CD4 T cells expressing IFN-γ only, in LBW compared to NBW infants.

These data suggest that LBW infants may have a reduced capacity to induce BCG-specific polyfunctional CD4+ T cells. The presence of these cells, following vaccination, has been associated with improved outcome in murine models following experimental TB infection. These results suggest that birth weight at time of vaccination may affect the quality of the BCG-induced immune response, and warrant further investigation in larger cohorts, and may ultimately have important implications for infant vaccination practices worldwide.

3.2 Introduction

Mortality due to infectious disease is high, with an estimated 4 million infant deaths annually, which occur most frequently in developing countries within the neonatal period soon after birth (Garcia, Fadel et al. 2000; Bonhoeffer, Siegrist et al. 2006). Immunisation is one of the most important interventions in containing infectious diseases (Baley and Leonard 2005). More than 15% of infants are born pre-term [PT (<37 weeks gestation)], or have low birth weights [LBW (<2,500g)], with >90% of these born in developing countries (Okan, Karagoz et al. 2006). Vaccine administration may be delayed in PT and LBW infants, or may not occur at all, leading to higher morbidity rates compared to term and normal birth weight (NBW [≥2,500g]) infants. Delays are usually due to a belief that factors such as birth weight, current weight, or degree of prematurity should influence the timing of immunisations. Only later in life do vaccination rates for both PT and LBW infants increase to similar rates to that of term and NBW infants (Langkamp, Hoshaw-Woodard et al. 2001; Baley and Leonard 2005; Batra, Eriksen et al. 2009). On the other hand vaccinating PT and LBW infants early in life may induce sub-optimal immune responses due to their developing immune systems (see *Chapter 1*, 2.0). Thus it is important to fully characterise vaccine-induced immune responses in PT and LBW infants to optimise vaccination strategies in order to decrease morbidity rates in this group of infants.

As discussed in Chapter 1 the neonatal immune system may be immature compared to adults, for example neonatal antigen presenting cells are inefficient at promoting Th1 responses and activating naïve T cells. Immaturities may be even more pronounced in PT and LBW infants. There is a gradual maturation of the immune system from birth to adulthood that may affect vaccine induced immune responses (see *Chapter 1,* 2.0). A study by Kagina *et al* on delaying BCG vaccination from birth to 10 weeks highlights the importance of maturational changes in the immune system that comes with age. Healthy term NBW infants were BCG vaccinated at birth or at 10 weeks of age. At 10, 20 and 50 weeks post-vaccination blood was collected and responses measured in a WB-ICC assay. Kagina *et al* observed that infants vaccinated at 10 weeks had greater

frequencies of BCG-specific CD4+ T cells, in particular polyfunctional BCG-specific CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2. These differences persisted and were most significant at 50 weeks post vaccination (Kagina, Abel et al. 2009). Kagina *et al's* results showed an enhanced BCG-specific qualitative response in infants vaccinated at 10 weeks of age, implying that they are immunologically more mature than infants at birth.

Compared to vaccination at birth, PT and LBW infants have been shown to mount a protective immune response following vaccination at 2 months of age, as shown by several studies that have evaluated protective antibody (Ab) titre levels to DTP (diptheria, tetanus, pertussis), hepatitis B, poliovirus and *Haemophilus influenzae* type b (Hib). However, these protective vaccine induced immune responses are frequently lower than those of term infants, and medically unstable PT LBW infants may also have lower Ab titres to these vaccines (Kim, Chung et al. 1997; Patel, Butler et al. 1997; Schloesser, Fischer et al. 1999; Baley and Leonard 2005). Previous studies have indicated that adequate levels of protective Ab titres to serotype 3 polio vaccine may be reduced in PT and LBW infants compared to term infants (D'Angio, Maniscalco et al. 1995). In general the same vaccination guidelines for term NBW infants may be applied to PT and LBW infants, with some exceptions (Esposito, Serra et al. 2009). Hepatitis B recommendations are complex and are based on the mothers' hepatitis B infection status and the infant's birth weight; infants weighing 2,000g are less likely to have protective levels of neutralising Ab compared to infants weighing ≥2,000g (Baley and Leonard 2005). These studies highlight the variations in PT and LBW infant immune responses and show that under certain conditions, birth weight and gestational age (GA) may lead to a suboptimal response to some childhood vaccines.

Tuberculosis (TB) kills 1.7 million people worldwide each year, and South Africa has the fifth highest incidence of the disease in the world. We focused on assessing the immune response to Bacillus Calmette-Guérin (BCG), the only currently licensed TB vaccine, which is administered at birth to all infants in South Africa. Studies in term infants have shown that BCG effectively protects

against severe childhood forms of TB disease (Trunz, Fine et al. 2006), however it is not clear if birth weight and maturity influence BCG immunogenicity (Okan, Karagoz et al. 2006). In TB endemic areas such as South Africa, infants should be vaccinated as soon as possible after birth (Fine, Carneiro et al. 1999). The World Health Organisation (WHO) recommends that PT infants should be vaccinated at the chronological age of 40 weeks, a practise seldom followed. Problems associated with calculating gestational ages (GA) result in BCG being administered when infants reach a birth weight of ≥2,500g, instead of when they reach a chronological age of 40 weeks, as proposed by the WHO (Roth, Jensen et al. 2004).

Previous studies on the immune responses to BCG vaccination in PT and LBW infants have yielded conflicting conclusions. Sedaghatian *et al* suggested that PT infants vaccinated at birth have a lower response to BCG as indicated by poor BCG scar formation, and low responses to tuberculin purified protein derivative (PPD). In this study, 101 PT infants were BCG vaccinated at birth and responses measured 2-4 months later. Overall, 32% did not develop a BCG scar following vaccination, and of the 70 infants that returned for tuberculin skin test measurements, 31% were non-reactive (Sedaghatian and Kardouni 1993). In contrast, in a study of 35 PT infants vaccinated at 2-3 months of age, Okan *et al* found high rates of BCG scarring and positive TST conversion (Okan, Karagoz et al. 2006). However these two studies by Okan *et al* and Sedaghatian *et al* were limited in not having a control group of term infants. In yet another study by Sedaghatian *et al*, PT immune responses to BCG were compared to term infants. BCG scarring was found to be largest in term infants vaccinated at birth, compared to PT infants vaccinated at birth or at 40 weeks of age. When these 3 groups of vaccinated infants were further analysed, BCG scarring and TST measurements were found to be associated with sex and birth weight. Female infants were more likely to have a BCG scar and a positive TST conversion (Sedaghatian, Hashem et al. 1998). Negrete-Esqueda *et al* found similar responses to TST and rates of BCG scarring when 50 PT and 50 term infants BCG vaccinated at birth were directly compared, concluding that PT infants may

be BCG vaccinated at birth (Negrete-Esqueda and Vargas-Origel 2007). Others have also compared subgroups of term infants to determine the effect of intra uterine growth restriction (IUGR). Using TST measurements, 57 term, small for gestational age (SGA) infants were compared to 52 term, appropriate for gestational age (AGA) infants and immunogenicity to BCG was found to be similar between the two groups (Mussi-Pinhata, Goncalves et al. 1993).

Previous studies of BCG-induced immune responses in PT and LBW infants have measured immunity by Elisa, lymphoproliferation assays, TST and the presence of a BCG scar. One disadvantage of these bulk assay approaches is that a more detailed analysis of specific responses on a single cell level is not possible (Thiel, Scheffold et al. 2004). Not only have these few studies either been inconclusive or yielded conflicting results, they have also highlighted the wide variation in infant immune responses to BCG. Differences in methodology may also explain in part the conflicting and variable results obtained in previous studies. In addition, TST reactivity after BCG vaccination is is not generally thought to influence the degree of protection offered by BCG, and up to 25% of infants do not develop a BCG scar following vaccination (al-Kassimi, al-Hajjaj et al. 1995; Negrete-Esqueda and Vargas-Origel 2007).

Recently, the use of intracellular cytokine staining and multiparameter flow cytometry has allowed the detailed study of antigen-specific immune responses on a single cell basis (Suni, Picker et al. 1998), and has been applied to the study of mycobacteria-specific responses (Hanekom, Hughes et al. 2004; Hanekom 2005; Soares, Scriba et al. 2008; Kagina, Abel et al. 2009). These studies on term, NBW infants and adults have shown detectable functional BCG-specific CD4+ and CD8+ T cells of varying frequency that express complex patterns of IFN-γ, IL-2 and TNF-α cytokines (Murray, Mansoor et al. 2006; Soares, Scriba et al. 2008). The T-helper type 1 (Th1) cytokines such as IFN-γ, IL-2 and TNF-α have been used routinely to measure BCG immunogenicity (Marchant, Goetghebuer et al. 1999; Murray, Mansoor et al. 2006; Kagina, Abel

et al. 2010). However, these studies have not addressed the effect of maturity and birth weight on the cytokine profile of BCG-induced T cell responses.

In conclusion, studies to date have not fully addressed BCG induced immune responses in PT and LBW infants. These infants may miss out on the BCG induced protective effects against severe childhood forms of TB. Considering the high PT and LBW rates in developing countries, we investigated the effect of GA and birth weight on BCG immunogenicity. We aimed for the first time to assess the BCG induced T cell responses of medically stable PT and LBW infants on a single cell basis using a whole blood intracellular cytokine staining assay and multiparameter flow cytometry. We hypothesised that BCG vaccinated PT and LBW infants have a lower frequency of BCG-specific T cells compared to term or NBW infants. A better understanding of the effects of birth weight and GA on the BCG induced immune response may ultimately lead to more effective BCG vaccination policies of PT and LBW infants worldwide.

3.3 Results

3.3.1 Participant characteristics

One hundred and five BCG vaccinated, healthy infants were enrolled in the study. Blood was collected at 10 weeks of age from infants who were stratified according to birth weight, gestational age, gender and maturity (see *Materials and Methods,* 2.2.1). Participant characteristics are summarized in Table 3.1.

Characteristic	Low birth weight	Normal birth weight
Subjects (n)	52	53
Male, n (%)	26 (50)	27 (50.94)
Gestational age, weeks (range)	37.2* (32 - 40)	37.5* (32 - 42)
Small for Gestational age, n (%)	39 (75)	5 (9.43)
Large for Gestational age, n (%)	0	14 (26.92)
Delivery mode C-section, n (%)	9 (17.31)	9 (16.98)
Ethnicity, Mixed race, n (%)	50 (96.15)	46 (86.79)
Birth weight, kg (range)	2.17* (1.56- 2.49)	3.06* (2.50 -4.50)

*mean

Table 3.1. Study participant characteristics

3.3.2 Flow cytometric detection of BCG-specific T cell responses in whole blood

To investigate the effect of birth weight on the frequency and profile of BCG-specific T cells in infants 10 weeks post-vaccination, BCG-stimulated cells from a WB-ICC assay (see *Materials and Methods*, 2.2.4) were thawed and analysed by multiparameter flow cytometry. The sequential gating strategy (Fig. 3.1A) was used to delineate different cell types (CD3+/CD3-), cell subsets (CD4+/CD8+) and intracellular cytokines expressed (IL-2, TNF-α, IFN-γ and IL-17). Representative intracellular cytokine staining data for CD4+ T cells from a LBW and NBW infant are shown (Fig. 3.1B). Cytokine gates were based on the unstimulated sample and were kept constant for each infant's corresponding SEB and BCG stimulated condition. All infants responded to SEB stimulation. We observed that both LBW and NBW 10-week old infants BCG vaccinated at birth had a detectable specific immune response to BCG using the WB-ICC assay. Antigen specificity of this assay in detecting BCG-induced responses in 10-week old BCG vaccinated infants has been shown previously in a study by Kagina *et al*, who showed that BCG vaccinated but not unvaccinated infants display BCG-specific T cell responses as measured by the WB-ICC assay (Kagina, Abel et al. 2009).

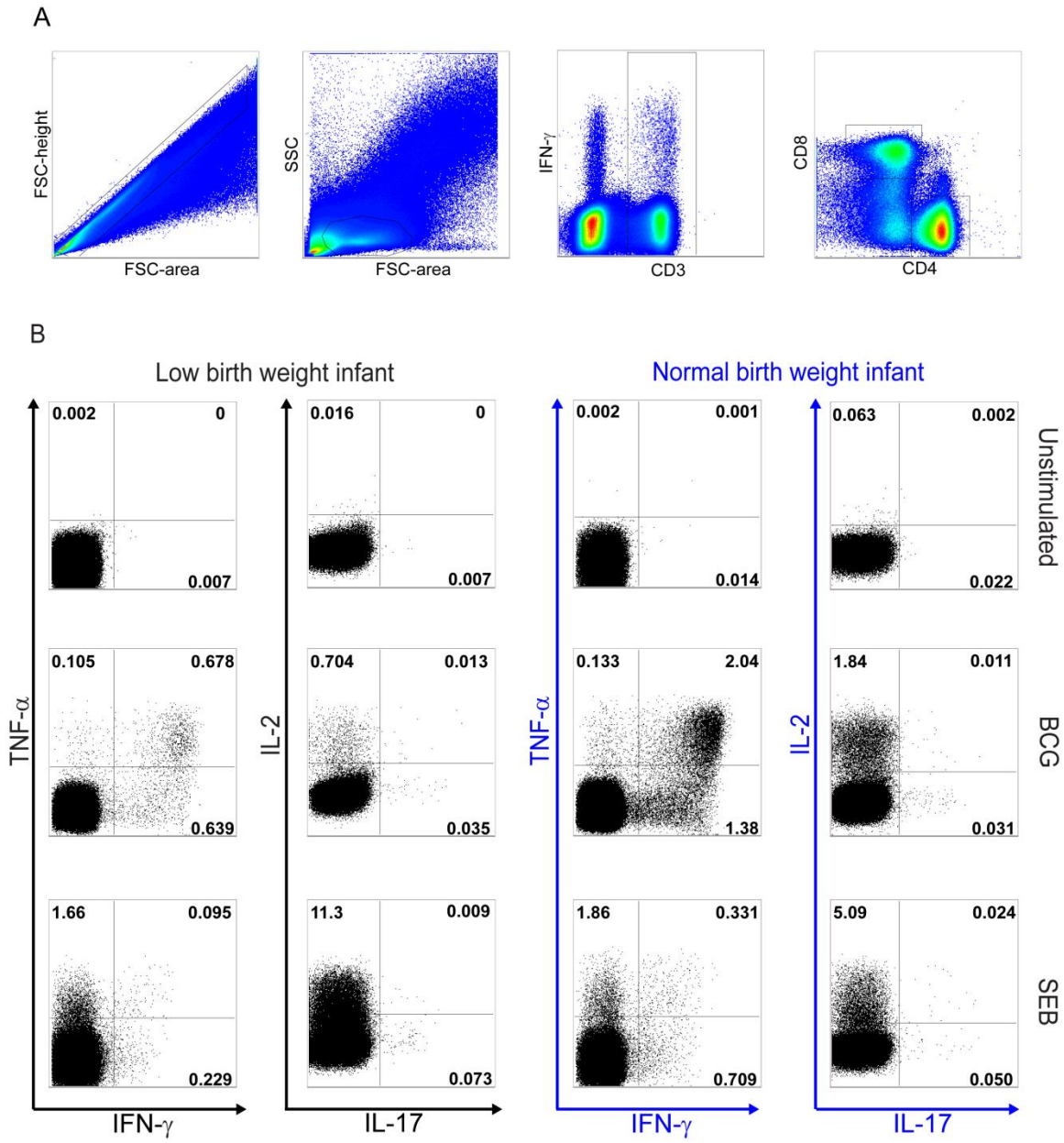

Figure 3.1. Flow cytometry gating strategy and representative data from the whole blood intracellular cytokine staining (WB-ICC) assay. (A) Gating is first performed on singlets then lymphocytes, followed by CD3+ T cells, from which CD4+ and CD8+ T cells are differentiated. In (B) the percentage of CD4+ T cells expressing combinations of IL-2, IFN-γ, TNF-α or IL-17 cytokines are shown for a low birth weight (LBW) and normal birth weight (NBW) infant; percentages shown on the plots are prior to subtraction of cytokine production in the negative control sample. Representative data are shown from a negative control (unstimulated), BCG and SEB stimulated sample.

3.3.3 Frequency of BCG-specific CD4+ T cells expressing Th1 and Th17 cytokines.

Expression of the T-helper type 1 (Th1) cytokines IL-2, IFN-γ and TNF-α has been frequently used to measure BCG immunogenicity (Marchant, Goetghebuer et al. 1999; Murray, Mansoor et al. 2006; Soares, Scriba et al. 2008; Kagina, Abel et al. 2010). There is increasing evidence that Th17 cells play an important role in *M.tb* infection (Chen, Zhang et al.). Recently, the presence of BCG-specific CD4+ Th17 cells expressing IL-17 was shown for the first time in whole blood of infants following BCG vaccination (Kagina, Abel et al. 2010).

We therefore assessed the total frequency of BCG-specific CD4+ T cells producing IL-2, IFN-γ, TNF-α or IL-17, and compared responses between LBW and NBW infants. NBW infants had a higher total frequency of BCG-specific IL-2 and TNFα–producing CD4+ T cells than LBW infants (Fig. 3.2A). No differences between LBW and NBW infants were found in the total BCG-specific CD4+ T cell expression of IFN-γ or IL-17.

To determine whether birth weight influenced the rate of detection of BCG-specific CD4+ T cell responses, we compared the percentage of infants with a positive BCG-specific CD4+ T cell response between LBW and NBW infants. Determination of a positive response is described in *Materials and Methods,* 2.2.1. All NBW infants and 96% of LBW (n=50) infants had a detectable BCG-specific CD4+ T cell response above background (Fig. 3.2B). There was no difference in the frequency of BCG-specific CD4+ T cell responders between NBW and LBW infants. These results indicate that birth weight alone does not affect the ability to detect BCG-specific CD4+ T cell responses ex vivo in our assay system.

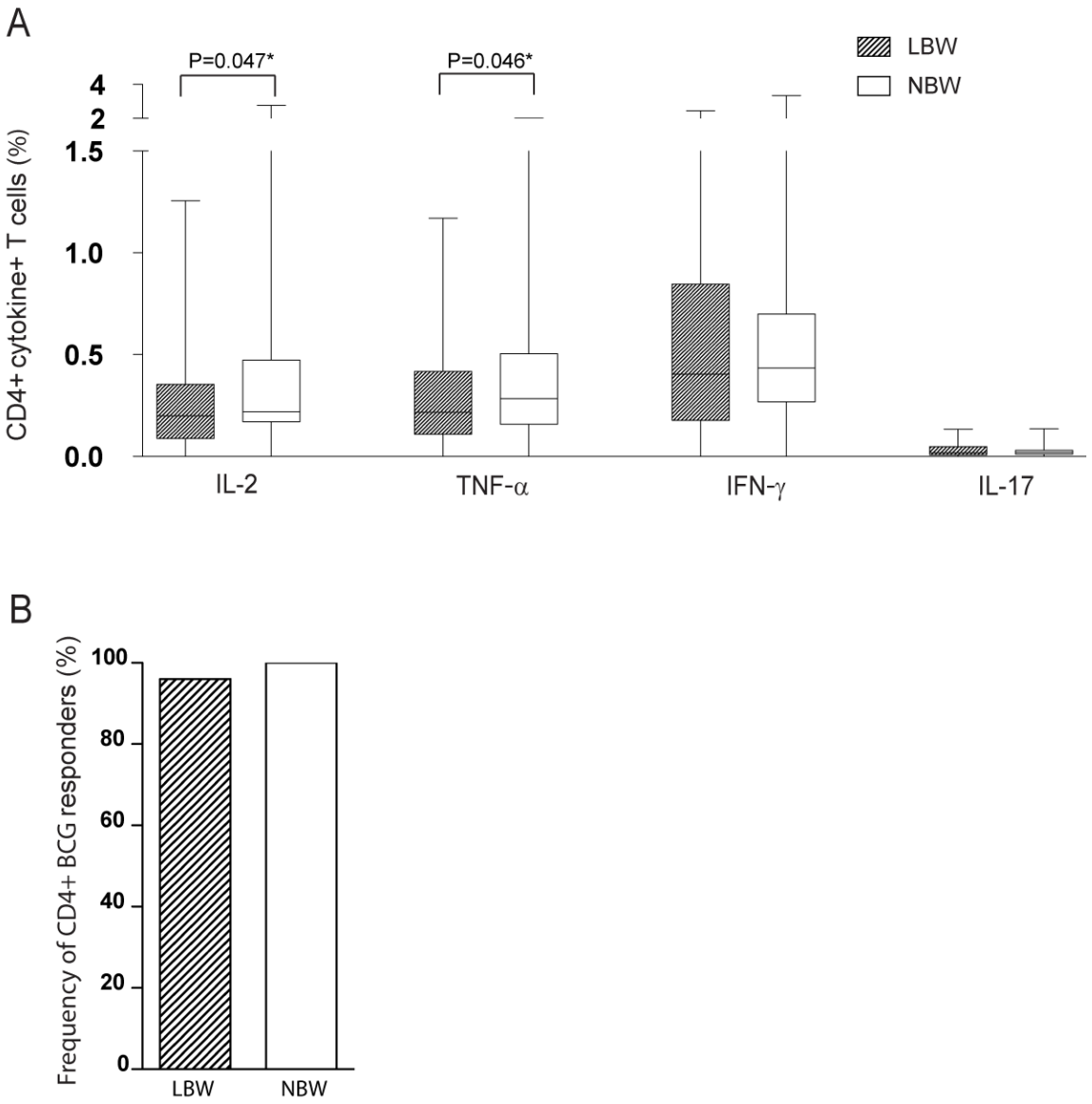

Figure 3.2. (A) Frequency of BCG-specific CD4+ T cells expressing cytokines in LBW (n=52) and NBW (n=53) infants. The horizontal line represents the median and the boxes represent the interquartile range. The Mann Whitney test was used to calculate statistical differences between individual groups. (B) The percentage of vaccinated infants with a detectable BCG-specific CD4+ T cell response above background. Out of 52 LBW and 53 NBW infants, CD4+ T cells from 50 LBW (96%) and 53 NBW (100%) infants responded to BCG. There was no statistical difference between the percentages of infants responding to BCG (Fisher's exact test).

3.3.4 Association between birth weight and gestational age and total frequencies of BCG-specific cytokine+ CD4+ T cells

The analyses described above were done by categorically grouping infants as either LBW or NBW. We next investigated whether there was a direct correlation between BCG-specific responses and birth weight as a continuous variable (Fig. 3.3). We found a positive association between the total frequency of BCG-specific CD4+ T cells expressing IL-2 and birth weight (Fig. 3.3A). No associations were found with birth weight and total frequencies of BCG-specific CD4+ T cells producing IFN-γ, TNF-α or IL-17 (Fig. 3.3B-D).

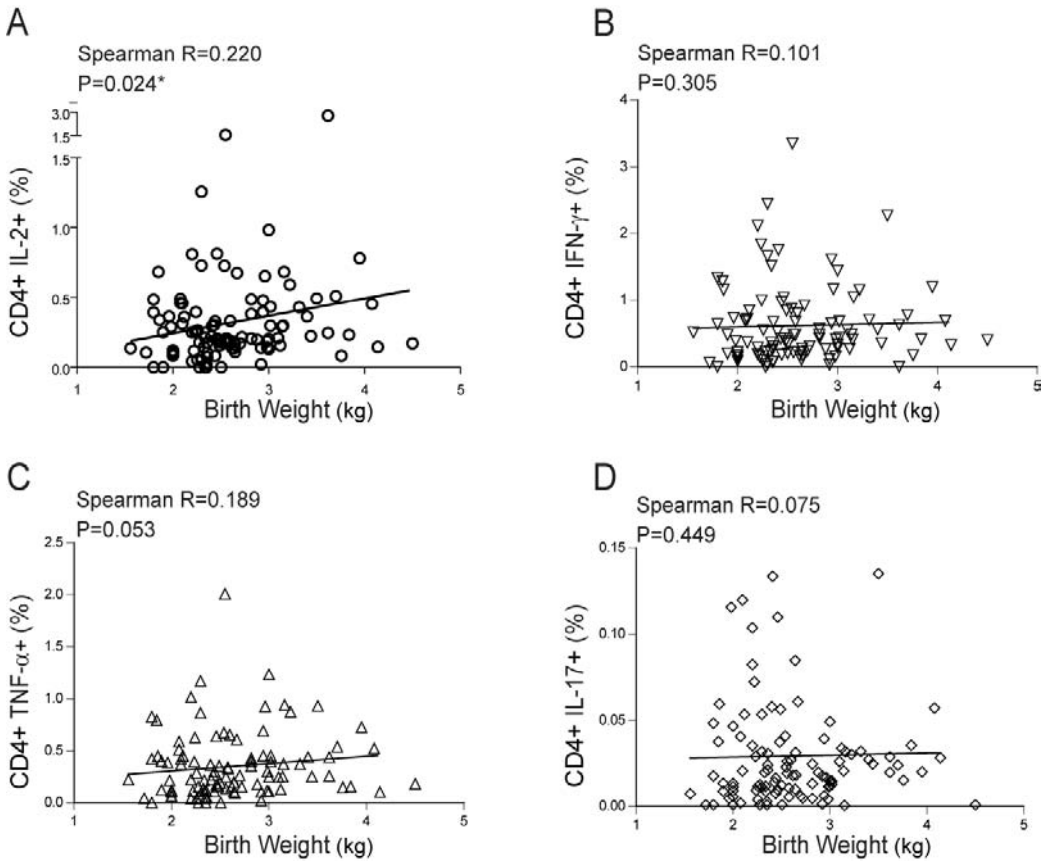

Figure 3.3. Correlation between the birth weight of 105 BCG vaccinated infants and the frequency of BCG-specific CD4+ T cells expressing either: (A) IL-2, (B) IFN-γ, (C) TNF-α or (D) IL-17. Statistical significance was calculated using the Spearman test.

To assess the influence of GA, we investigated the association between the frequency of BCG-specific CD4+ T cell responses and GA (Fig. 3.4). Although there was a positive association between CD4+ T cells expressing IL-17 and GA (Fig. 3.4D), no associations were found between GA and any of the Th1 cytokines measured (Fig. 3.4A-C). These results suggest that birth weight may have a greater influence on BCG immunogenicity than GA.

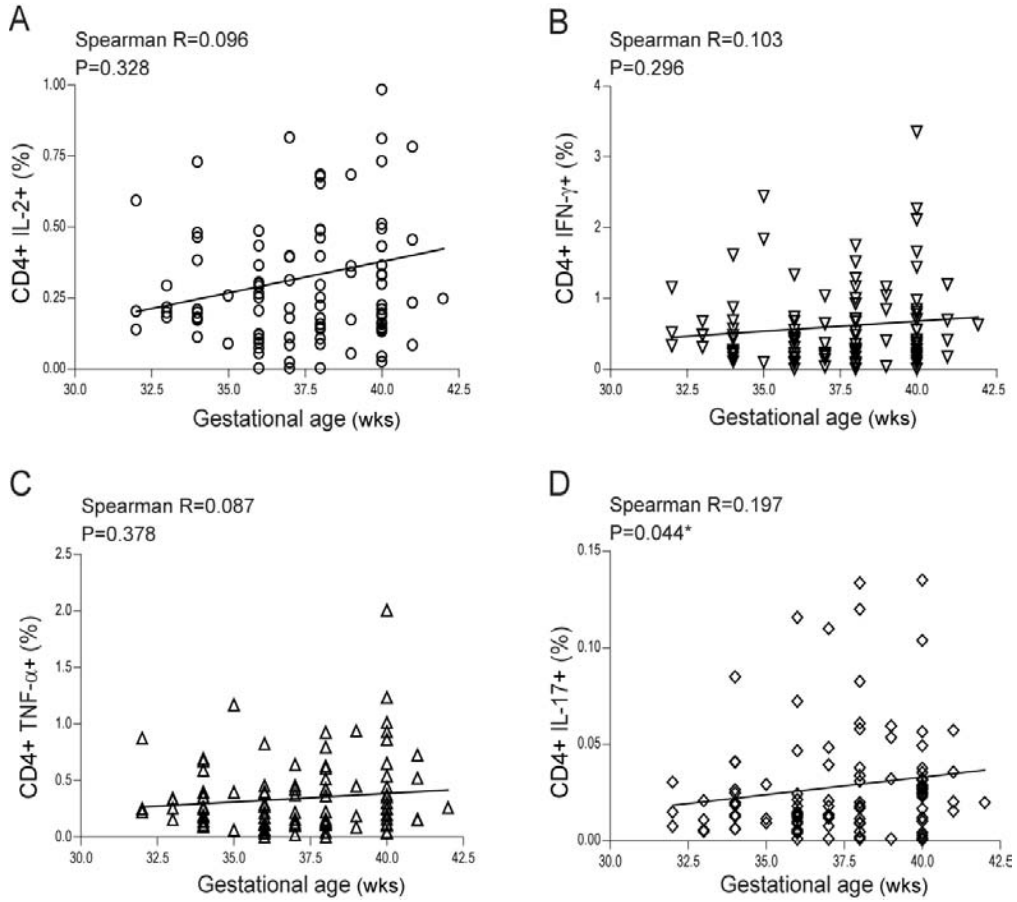

Figure 3.4. Correlation between the gestational ages of 105 BCG vaccinated infants and frequency of BCG-specific CD4+ T cells expressing either: (A) IL-2, (B) IFN-γ, (C) TNF-α or (D) IL-17. Statistical significance was calculated using the Spearman test.

3.3.5 Effect of birth weight on the cytokine profile of BCG-specific CD4+ T cell responses

After evaluating the effects of birth weight on the total frequency of cytokine+ BCG-specific CD4+ T cells, we next assessed the patterns of co-expression of multiple cytokines in individual T cells. We used multiparameter flow cytometry to measure individual BCG-specific CD4+ T cells expressing combinations of IL-2, IFN-γ, TNF-α or IL-17. Boolean analysis of these produced 15 distinct cytokine expressing T cell subsets, providing a detailed analysis of the functional capacity of these BCG-specific T cells.

We first compared the frequency of BCG-specific CD4+ T cells expressing combinations of IL-2, IFN-γ, TNF-α or IL-17 in NBW and LBW infants. Similar frequencies of CD4+ T cells expressing combinations of these cytokine populations were found in the two groups of infants (Fig. 3.5A). Consistent with previous findings, the predominant BCG-specific response in both groups was that of single cytokine-producing CD4+ T cells expressing IFN-γ only and polyfunctional CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 together (Soares, Scriba et al. 2008; Kagina, Abel et al. 2009). We next addressed the quality of the response by assessing the contribution of each subset of cytokine-producing cells to the total BCG-specific CD4+ T cell response. For this analysis, we only analyzed infants with a detectable BCG-specific CD4+ T cell response (see *Materials and Methods,* 2.2.1). Using these criteria, we excluded 2 LBW infants whose BCG-specific CD4+ T cell response was not above background levels. We then compared the proportion of BCG-specific CD4+ T cell subsets expressing 15 different combinations of IL-2, IFN-γ, TNF-α or IL-17 in NBW (n=53) and LBW (n=50) infants (Fig. 3.5B). In both groups of infants the predominant BCG-specific cytokine-producing subsets were CD4+ T cells expressing IFN-γ only and CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 together. However, LBW infants had a lower proportion of polyfunctional BCG-specific CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 together, compared to NBW infants, and correspondingly higher proportions of single cytokine-

producing CD4+ T cells expressing IFN-γ only. Lastly, LBW infants had a lower proportion of BCG-specific CD4+ T cells co-expressing IFN-γ and TNF-α together compared to NBW infants. Notably, when IL-17 was measurable, it was found to be expressed alone (Fig. 3.5). These results suggest that LBW infants may have a reduced capacity to induce polyfunctional CD4+ T cells following BCG vaccination. Although we found differences by grouping infants categorically as either LBW or NBW, there was no direct correlation between the proportion of BCG-specific CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 together or single cytokine-producing BCG-specific CD4+ T cells expressing IFN-γ only and birth weight (data not shown). The same analysis was done by gestational age and no association was found (data not shown).

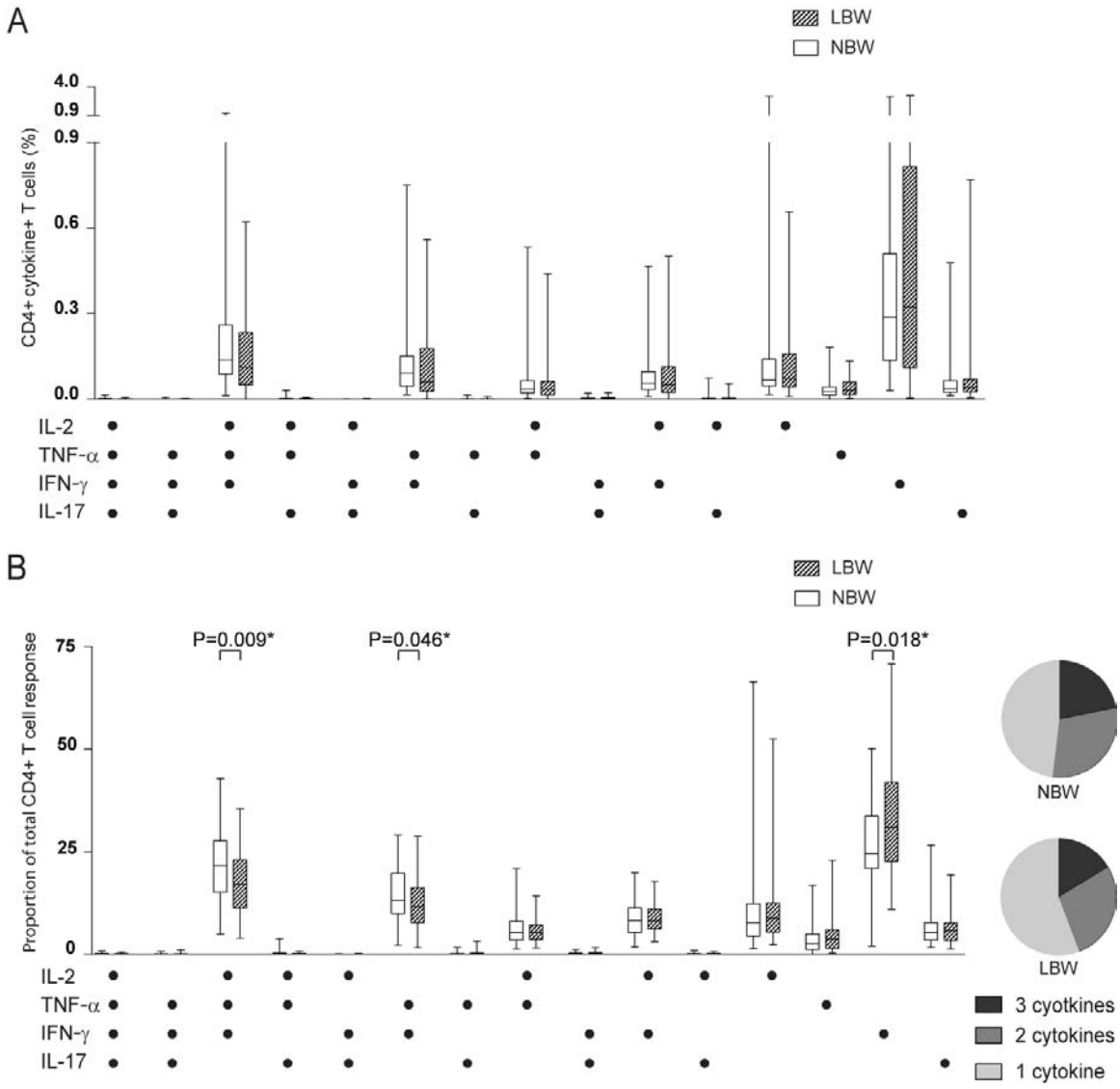

Figure 3.5. The frequency and proportion of BCG-specific CD4+ T cells expressing combinations of IL-2, IFN-γ, TNF-α or IL-17 in LBW and NBW infants. The frequency (A) of CD4+ T cells expressing combinations of cytokines for all infants is shown (LBW, n=52 and NBW, n=53 respectively). Only infants with a detectable BCG-specific CD4+ T cell response above background (LBW, n=50 and NBW, n=53; see *Materials and Methods*, 2.2.8) were included when calculating the proportion of BCG-specific CD4+ T cells expressing each combination of cytokines (B). The horizontal line represents the median and the boxes represent the interquartile range. Each pie is divided into 3 slices that represent the median proportion of the total BCG-specific CD4+ T cell response, a slice each for T cells expressing a combination of 3, 2 or 1 cytokine only (B). The Mann Whitney test was used to calculate statistical significance between the individual groups.

3.3.6 Size for gestational age, maturity and gender do not affect the cytokine profile of BCG-specific CD4+ T cell responses

As we observed a difference in the proportion of specific cytokine-producing subsets contributing to the total BCG-specific CD4+ T cell response between NBW and LBW infants, we performed a similar analysis in infants stratified into 3 groups: size for gestational age, maturity and gender. For this analysis, we only analyzed infants with a detectable BCG-specific CD4+ T cell response (see *Materials and Methods,* 2.2.1). We excluded 2 male infants who were not BCG responders, both of whom were small for gestational age, 1 was term and the other pre-term. We thus evaluated responses in small for gestational age (SGA; n=42), appropriate for gestational age (AGA; n=47), large for gestational age (LGA; n=14), term (n=65), pre-term (n=38), male (n=51) and female (n=52) infant BCG responders. The proportion of polyfunctional BCG-specific CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 together (Fig. 3.6A), co-expressing IFN-γ and TNF-α together (Fig. 3.6B), and single cytokine-producing BCG-specific CD4+ T cells expressing IFN-γ only (Fig. 3.6C), were not different by size for GA, maturity or gender. We also compared the proportion of BCG-specific CD4+ T cells expressing all other combinations of IL-2, IFN-γ, TNF-α or IL-17 and found no differences (data not shown). We concluded that the qualitative response to BCG vaccination is not affected by size for GA, maturity and gender.

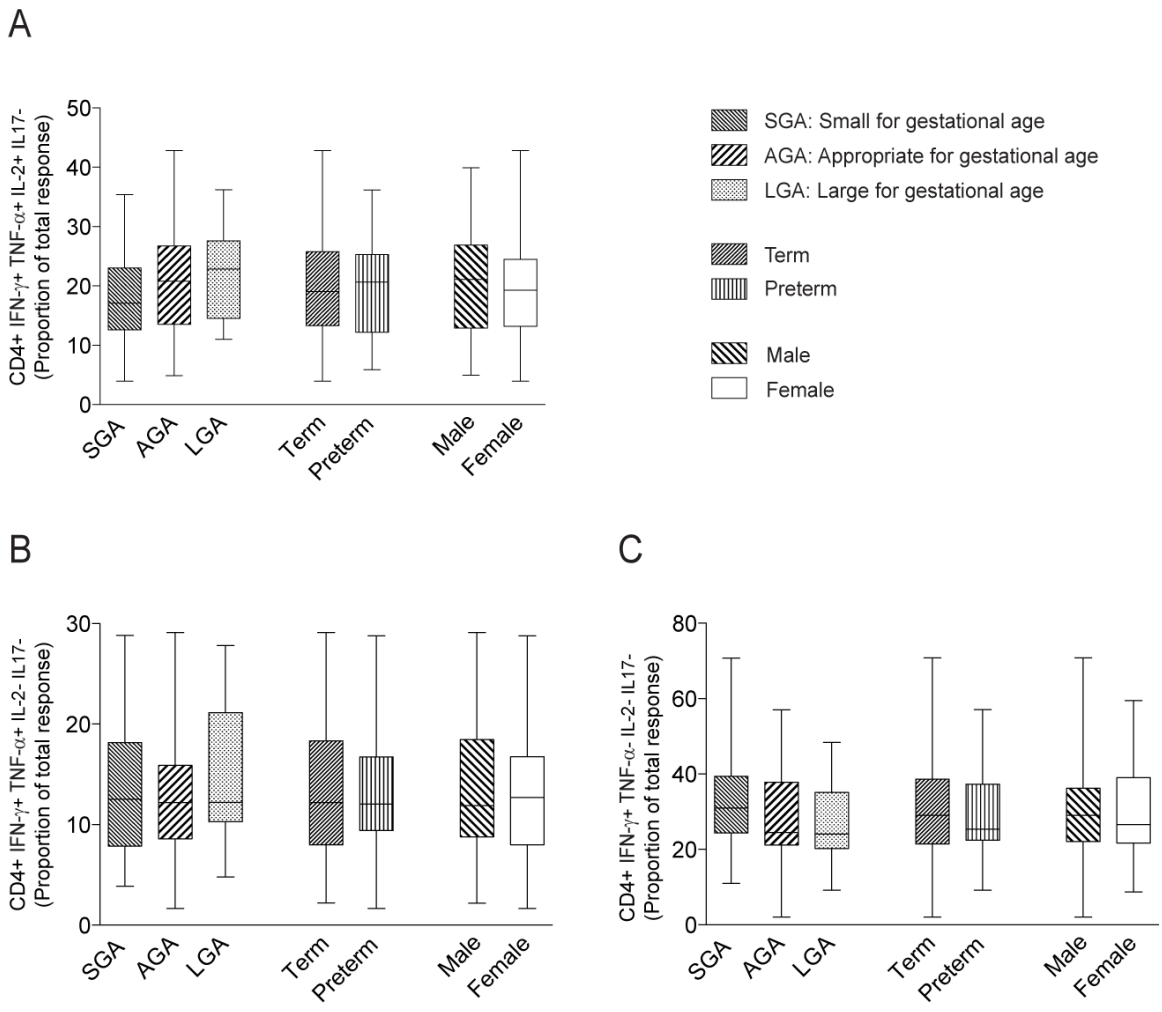

Figure 3.6. Proportions of polyfunctional BCG-specific CD4+ T cells stratified by size for gestational age, term status, and sex. The proportions of BCG-specific CD4+ T cells co-expressing IL-2, IFN-γ and TNF-α cytokines together (A), co-expressing IFN-γ and TNF-α cytokines only (B), or expressing IFN-γ only (C) are shown. Only infants that responded to BCG are shown and were stratified as follows: small for gestational age (SGA; n=42), appropriate for gestational age (AGA; n=47), large for gestational age (LGA; n=14), term (n=65), pre-term (n=38), male (n=51) and female (n=52). The horizontal line represents the median and the boxes represent the interquartile range. The Mann Whitney test was used for comparisons between two groups, and the Kruskal-Wallis test was used for comparisons of three groups.

3.3.7 Frequency of BCG-specific CD8+ T cells.

Following BCG vaccination, BCG-specific CD8+ T cells are induced (Murray, Mansoor et al. 2006; Soares, Scriba et al. 2008). As we observed a difference in the total frequency of TNF-α and IL-2-producing BCG-specific CD4+ T cell responses by birth weight, we next investigated whether the BCG-specific CD8+ T cell response was affected by birth weight.

We assessed the total frequency of BCG-specific CD8+ T cells expressing cytokines in NBW and LBW infants. There was no difference in the total frequencies of BCG-specific CD8+ T cells producing IL-2, IFN-γ, TNF-α or IL-17 between LBW and NBW infants (Fig. 3.7A). We also compared the percent frequency of responders between LBW and NBW infants. After applying a cut-off for a positive BCG-specific CD8+ T cell response (see *Materials and Methods,* 2.2.1), 77% of NBW infants (n=41) and 75% of LBW infants (n=39) had a detectable BCG-specific CD8+ T cell response in our assay system (Fig. 3.7B). We concluded that equal numbers of infants responded to BCG vaccination as measured by our assay system, and that birth weight does not affect the frequency of cytokine+ BCG-specific CD8+ T cells.

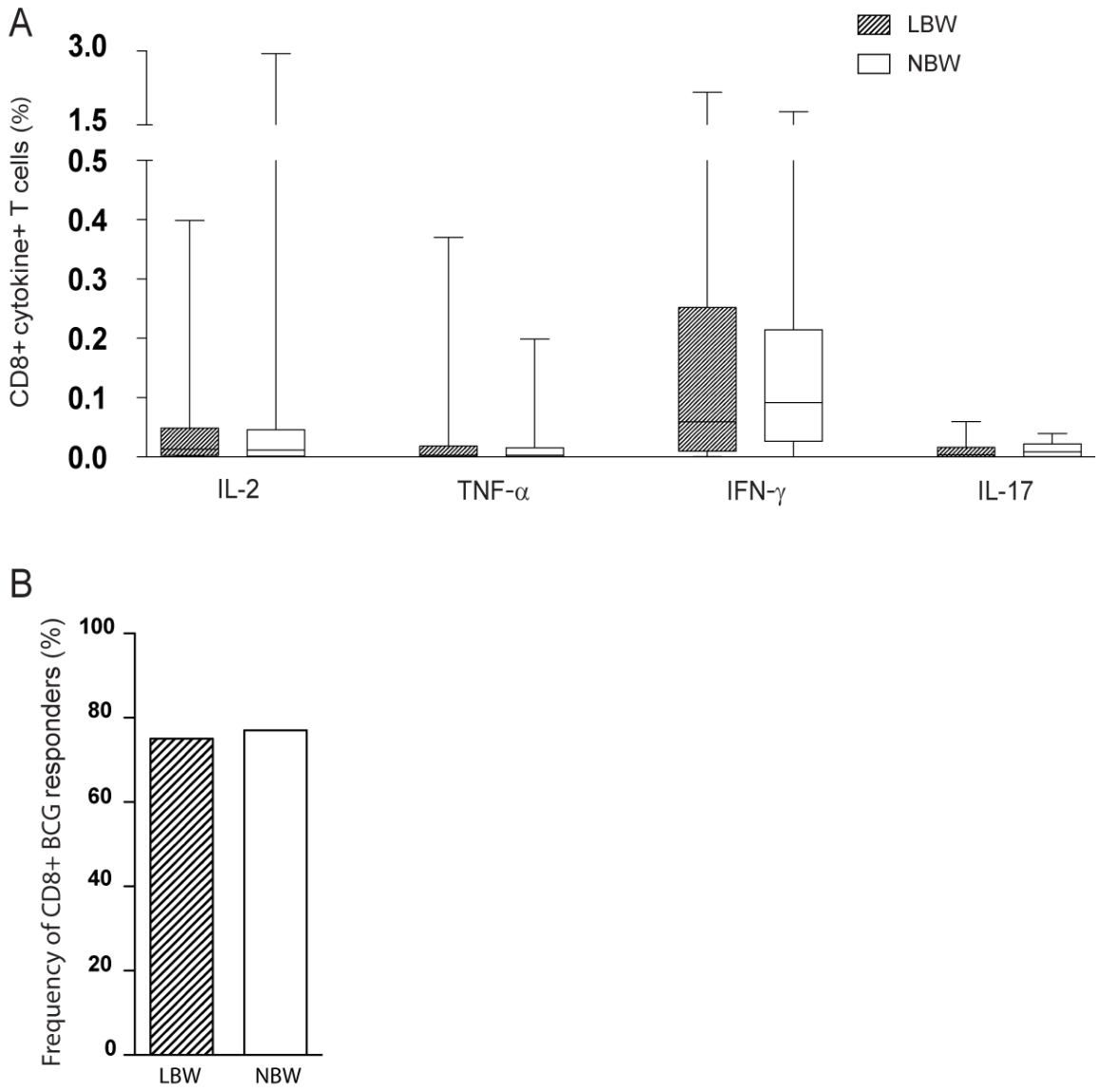

Figure 3.7. (A) Frequency of BCG-specific CD8+ T cells expressing cytokines in LBW and NBW infants. The horizontal line represents the median and the boxes represent the interquartile range. There was no statistical difference as determined by the Mann Whitney test. (B) The percentage of vaccinated infants with a detectable BCG-specific CD8+ T cell response above background levels. Out of a total of 105 infants, 39 LBW (75%) and 41 NBW (77%) infants had a detectable CD8+ T cell response to BCG. There was no statistical difference between the percentages of infants responding to BCG (Fisher's exact test).

3.3.8 There is no association between both birth weight and gestational age and CD8+ T cells expressing IL-2, IFN-γ, TNF-α or IL-17

An association between both birth weight (Fig. 3.8) and gestational age (data not shown) and total frequencies of cytokine-producing, BCG-specific CD8+ T cells in 105 infants was investigated. No correlations were found between birth weight and the total frequency of BCG-specific CD8+ T cells expressing the cytokines IL-2, IFN-γ, TNF-α or IL-17.

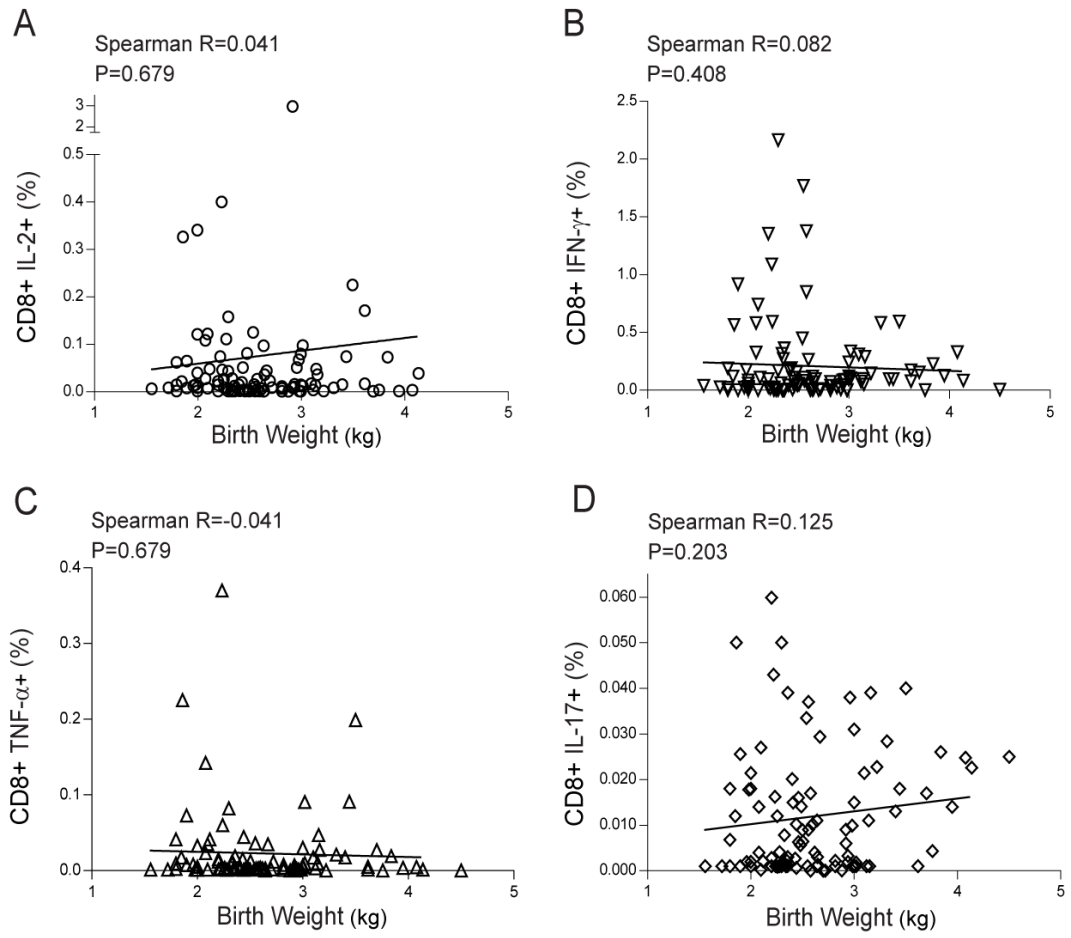

Figure 3.8. Correlation between the birth weight of 105 BCG vaccinated infants and the frequency of BCG-specific CD8+ T cells expressing either IL-2 (A), IFN-γ (B), TNF-α (C), or IL-17 (D). Statistical significance was calculated using the Spearman test.

3.3.9 The quality of BCG-specific CD8+ T cell responses is not affected by birth weight

We compared the frequency of BCG-specific CD8+ T cells expressing all possible combinations of IL-2, IFN-γ, TNF-α or IL-17, and found no difference between NBW and LBW infants (Fig. 3.9A). Within infants with a detectable BCG-specific CD8+ T cell response (see *Materials and Methods,* 2.2.1), we next assessed the contribution of each subset of cells to the total BCG-specific CD8+ T cell response (Fig. 3.9B). There was no difference in the proportion of BCG-specific CD8+ T cells expressing combinations of IL-2, IFN-γ, TNF-α or IL-17. A similar analysis was done for infants stratified according to their size for GA, maturity and gender but no differences in BCG-specific CD8+ T cell responses were found (data not shown). The BCG-specific CD8+ T cell responses were notably smaller in magnitude and the profile less complex compared to BCG-specific CD4+ T cell responses. Consistent with previous reports (Soares, Scriba et al. 2008; Kagina, Abel et al. 2009; Kagina, Abel et al. 2010) the most dominant BCG-specific response was that of single cytokine-producing CD8+ T cells expressing IFN-γ only. These results indicate that the magnitude and quality of the cytokine+ BCG-specific CD8+ T cell response is not affected by birth weight, size for GA, maturity and gender.

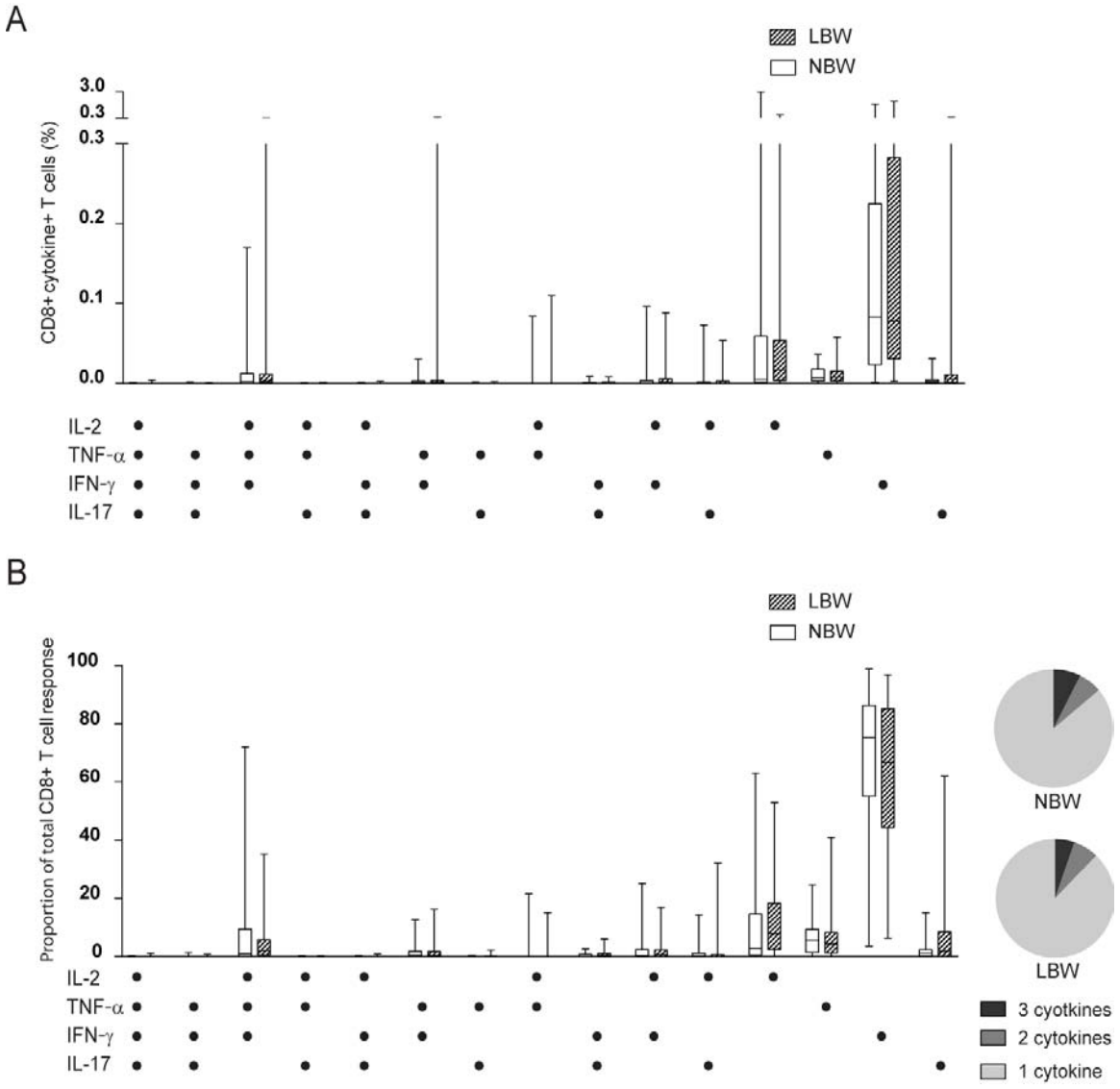

Figure 3.9. The frequency (A) and proportion (B) of BCG-specific CD8+ T cells expressing combinations of IL-2, IFN-γ, TNF-α or IL-17 cytokines in LBW and NBW infants. Only infants with a detectable BCG-specific CD8+ T cell response above background (LBW, n=39 and NBW, n=41) were included in the proportions of the total BCG-specific CD8+ T cell response shown in (B). The horizontal line represents the median and the boxes represent the interquartile range. Each pie is divided into 3 slices that represent the median proportion of the total BCG-specific CD8+ T cell response, a slice each for T cells expressing a combination of 3, 2 or 1 cytokine only (B). Statistical analyses were determined by the Mann Whitney test.

3.4 Discussion

To improve the efficacy of the BCG vaccination program, it is important to evaluate its effects in susceptible populations such as PT and LBW infants. It is not known if maturity or birth weight influences the functional quality of BCG-specific T cell responses. Varying results on the immunogenicity of the vaccine in PT and LBW infants as assessed by BCG scarring and the Mantoux test raises questions about interpretability of the results of these studies. To perform a detailed characterization of BCG-specific T cell responses in PT and LBW infants, we used multiparameter flow cytometry, a more sensitive and specific assay that could measure immune responses on a single cell basis. This is the first study using this assay system to report these responses in PT and LBW infants who were BCG vaccinated at birth. We report that following BCG vaccination, LBW infants have an altered BCG induced immune response, characterised by an increased proportion of monofunctional BCG-specific CD4 T cells producing IFN-γ in LBW infants.

Consistent with previous findings, we were able to measure BCG-specific T cell responses of 10-week-old infants using a WB-ICC assay and multiparameter flow cytometry. Similar to previous findings, BCG-specific CD4+ T cells were predominantly either IFN-γ single positive or polyfunctional cells co-expressing IFN-γ, TNF-α and IL-2, whereas BCG-specific CD8+ T cells were predominately IFN-γ single positive only (Hanekom 2005; Soares, Scriba et al. 2008; Kagina, Abel et al. 2009). It is unlikely that infants enrolled in the study had significant exposure to environmental mycobacteria by the time we collected blood at 10-weeks of age. Kagina *et al's* delayed BCG vaccination study outlined in the introduction section of this chapter supports this view. BCG-specific responses measured in whole blood at 10 weeks of age in the delayed group, before they were BCG vaccinated, were very low or undetectable but were readily detectable in infants BCG vaccinated at birth (Kagina, Abel et al. 2009). We can thus assume that the responses we measured were specific and not affected by environmental mycobacteria. Bacterial superantigens produced by

Staphylococcus aureus such as SEB, our positive control in this study, are potent T cell mitogens activating up to 20% more T cells than conventional Ag (Chaplin 2010). Superantigens including SEB stimulate large numbers of T cells by binding to the TCR and MHC class II molecules outside of their conventional binding sites leading to massive cytokine production, particularly IFN-γ, TNF-α and IL-2. They also have the advantage of activating rare populations of T cells (Fraser and Proft 2008). Conventional Ag on the other hand specifically bind a small subset of TCR and MHC molecules thus activating only small numbers of T cells (Chaplin 2010). All infants in our study responded to SEB stimulation and SEB-specific responses were similar when we compared infants by variables such as birth weight, gestational age, size for GA or gender. These data suggest there was no global functional deficiency in the infant immune systems, at least for the particular Th1 and Th17 cytokines measured here. As SEB responses were similar, then differences in BCG-specific responses could be attributed to either of the aforementioned variables.

We studied CD4+ T cells as they have been shown to be induced upon BCG vaccination and are widely believed to be essential in anti-mycobacterial immunity (Boom, Canaday et al. 2003; Flynn 2004; Cooper 2009). Compared with NBW infants, LBW infants had a lower overall frequency of BCG-specific CD4+ T cells expressing IL-2 and TNF-α. Furthermore, a positive correlation was observed between the frequency of IL-2+ BCG-specific CD4+ T cells and birth weight. Vaccination aims to induce immunological memory and IL-2 expression is important for T cell memory induction and maintenance (Williams, Tyznik et al. 2006). T cells in the elderly express low levels of IL-2 and can be used to illustrate the importance of this cytokine. These cells show a markedly decreased ability to induce effector T cells and functional T cell memory, properties rescued by addition of exogenous IL-2 (Linton, Haynes et al. 1996; Haynes, Linton et al. 1999; Haynes, Eaton et al. 2003; Haynes, Eaton et al. 2004). Presumably the decreased expression of IL-2 in LBW infants is suboptimal, and if it persists this may ultimately result in BCG-induced cells with impaired proliferative capacity.

Our assay system predominantly measures an effector response, thus further studies on the memory T cell phenotype induced by BCG in PT and LBW infants are warranted. Reactivation of latent TB individuals undergoing anti-TNFα treatment for rheumatoid disorders highlights the importance of this cytokine in host resistance against *M.tb* infection (Bruns, Meinken et al. 2009). These data suggests that NBW infants may develop a better qualitative response to BCG vaccination. However, the association of these cytokines with birth weight requires further study. We found no differences in the expression of IFN-γ, a cytokine commonly used as a measure of BCG immunogenicity. Humans with congenital mutations in IFN-γ-associated signalling pathways or receptors are more susceptible to mycobacteria infections, thus providing evidence for the importance of this cytokine (van de Vosse, Hoeve et al. 2004).

Through a more comprehensive analysis of BCG-specific responses on a single cell basis, we showed that LBW infants have a lower proportion of BCG-specific polyfunctional CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 together, and corresponding higher proportions of single cytokine-producing CD4+ T cells expressing IFN-γ only. Our results suggest that birth weight at the time of vaccination may affect the quality of the BCG-induced immune response. Induction of high frequencies of polyfunctional T cells co-expressing IFN-γ, TNF-α and IL-2 together have been associated with improved outcome in murine models following experimental TB infection (Forbes, Sander et al. 2008). Interestingly, there was no direct correlation between the proportion of polyfunctional cells and birth weight, although there was a trend towards higher proportions of polyfunctional CD4+ T cell responses by birth weight. Thus the association of birth weight and the proportion of BCG-specific polyfunctional CD4+ T cells require further study. As discussed in chapter 1, neonatal APCs have been shown to express low levels of costimulatory molecules. Although we were not able to measure expression of costimulatory molecules in this study, we hypothesise that expression of these molecules may have been reduced in our cohort of LBW infants. Priming of BCG specific responses in LBW infants may

have led to sub-optimal differentiation of BCG-specific T cell responses that were more monofunctional than polyfunctional in nature. This may explain our observation that LBW infants expressed higher monofunctional responses, characterised by a higher proportion of BCG-specific cells producing IFN-γ only.

We also measured BCG-specific CD4+ T cells expressing IL-17. To our knowledge this cytokine has never been studied in BCG vaccinated PT and LBW infants. This cytokine triggers chemokine expression in the lung, mediating the recruitment of protective Th1 cells and inducing inflammation in a murine vaccination model against TB (Khader, Bell et al. 2007). We did not observe a difference in the frequency of BCG-specific CD4+ IL-17+ T cells, however we did find an association between the frequencies of CD4+ T cells expressing IL-17 and gestational age. We do not know whether the presence of IL-17 in older infants reflects a more optimal immune response as IL-17 induced inflammation may be detrimental. The WHO policy is that PT infants should be vaccinated when they reach the gestational age of 40 weeks, presumably when their immune systems have matured more. Interestingly, there was no association with gestational age and the Th1 cytokines IL-2, IFN-γ or TNF-α that are routinely used as measures of BCG vaccine immunogenicity.

After observing differences in CD4+ T cell responses by birth weight we also analyzed BCG-specific CD8+ T cell responses. CD8+ T cells have been detected previously in whole blood of BCG vaccinated infants (Davids, Hanekom et al. 2006; Murray, Mansoor et al. 2006). Experimental models of TB infection support the role of CD8+ T cells in protection against TB disease. For example, in BCG vaccinated rhesus macaques, depletion of CD8+ T cells results in increased bacterial counts and in a loss of BCG-induced protective immunity upon challenge with Mtb (Chen, Huang et al. 2009). The frequencies of CD8+ T cell responses, though lower than CD4+ T cell responses, were still readily detectable, consistent with previous reports (Soares, Scriba et al. 2008; Kagina, Abel et al. 2010). Mycobacterial Ags are primarily presented in the context of class II MHC molecules thus stimulating mainly CD4+ T cells. The contribution of

CD8+ T cells is thus likely to be under represented. No differences by birth weight were observed, suggesting that birth weight may have less of an effect on priming CD8+ T cell responses to BCG.

Caution should be exercised when interpreting the biological and clinical significance of our results, which require further study. In a study by Kagina *et al*, blood was collected from 10-week old term, NBW infants who were BCG vaccinated at birth and where then followed up for at least 2 years for development of TB disease. They identified a group of infants that developed culture positive TB, despite having received BCG at birth. The BCG-specific T cell responses at 10 weeks of these unprotected infants were compared to that of control groups of infants that did not develop TB (Kagina, Abel et al. 2010). Kagina *et al*, using a whole blood assay and multiparameter flow cytometry, showed that the presence of polyfunctional BCG-specific CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 did not correlate with protection against development of pulmonary TB in this cohort. In addition, the overall frequency of BCG-specific CD4+ T cells expressing IFN-γ, TNF-α, IL-2 and IL-17 did not correlate with protection. In this study we did not measure $\gamma\delta$ T cells, which constitute a large proportion of neonatal T cells, and may play an important role in BCG-induced immunity. Kagina et al however found that the frequency of BCG-specific $\gamma\delta$ T cells did not correlate with protection (Kagina, Abel et al. 2010). These studies suggest that the quantitatively greater BCG-specific T cell responses we measured in NBW infants may not necessarily afford a greater degree of protection against development of TB. Kagina *et al* enrolled infants from the same parent cohort that we selected our study participants from. This parent cohort consisted of 11,670 infants enrolled as part of a phase IV randomized control trial (RCT) testing the effect of vaccination route on BCG vaccine efficacy (Hawkridge, Hatherill et al. 2008). Infants chosen for our study were in the group of infants that did not develop TB when followed up for at least 2 years. We are therefore unable to address the question of whether BCG protects PT and LBW infants against TB disease. Our study can only address

BCG-induced immunogenicity, and large-scale efficacy trials would be required to address the effects of maturity and birth weight on BCG-mediated protection against TB. However, the responses we measured, if based on the common opinion that qualitatively greater responses are protective, suggest that NBW infants may have a more optimal response to BCG than LBW infants.

Overall, most cytokine expression patterns of BCG-specific cells were not affected by birth weight, gestational age, size for GA or gender. Our results reveal the complexity of BCG-induced immunity and of the relationship between birth weight, maturity and BCG-specific responses. These findings, in part, support our hypothesis that PT and LBW infants have a lower frequency of BCG-specific T cells, compared to term and NBW infants. As discussed previously, immaturities in the neonatal immune system may be even more pronounced in PT and LBW infants and may potentially reduce vaccine specific responses. This may be the main reason explaining the altered BCG-specific immune response in PT and LBW infants observed in this study.

Our findings may be unique to the selected cohort and thus we propose that these results should be confirmed in larger cohorts before future studies are performed to determine the effects of maturity and birth weight on BCG-mediated protection against TB. Another limitation of our study is that we only assessed BCG-specific responses at 10 weeks post vaccination. There is a possibility that when we collected blood at 10-weeks of age the immune systems of PT and LBW infants had matured sufficiently enough to result in the masking of differences in BCG-specific responses compared to term and NBW infants. It would be interesting in future studies to perform a longitudinal analysis of the cytokine profiles of BCG-induced T cell responses in LBW and NBW infants. This will address the question of whether the altered BCG-specific immune response we observed in PT and LBW infants is short-lived or if this alteration persists. Furthermore, analysis of BCG-specific T cell responses at time points earlier than 10 weeks post-vaccination may shed light on important events early in priming of BCG-specific immune responses that may be influenced by either birth weight or

maturity. Despite these limitations, the findings of this preliminary study represent an important first step in helping policy makers optimize BCG vaccination policies. Optimal vaccination of PT and LBW infants will ultimately lead to better control of the TB epidemic.

CHAPTER FOUR

The effect of birth weight and gestational age on the proliferative potential and cytokine producing capacity of BCG-specific T cells

4.1 Summary

Proliferation and clonal expansion upon secondary antigen exposure is an important feature of protective immunological memory. Measurement of antigen-specific proliferative potential following vaccination is therefore important in assessing vaccine immunogenicity. We showed differences in BCG immunogenicity by birth weight on a single cell basis using a WB-ICC assay and multiparameter flow cytometry. This short-term assay measured cytokine production directly ex vivo, which is only one aspect of cellular responses. A different aspect is the proliferative potential and cytokine producing capacity of T cell, critical functions that may be affected by birth weight. We hypothesized that BCG vaccination at birth induces antigen-specific T cells with a greater proliferative and cytokine producing capacity in NBW infants. In assessing this attribute we compared 2 flow cytometry based methods of measuring proliferation, a Ki67 proliferation assay and dye dilution of Oregon green. Ki67 expression allowed greater detection of antigen-specific proliferating and cytokine producing cells. Cryopreserved PBMC from 10-week old infants were thawed and cultured in a 6-day KI67 proliferation assay, followed by multi-parameter flow cytometry analysis. No differences were found in the proliferative response and the capacity of proliferating BCG-specific T cells to express IFN-γ, TNFα and IL-2 in LBW and NBW infants. This preliminary data suggests that birth weight may not directly influence the proliferative response and cytokine production capacity of BCG-specific T cells as measured in a 6-day proliferation assay.

4.2 Introduction

Proliferation and clonal expansion upon secondary antigen exposure is an important feature of protective immunological memory and is the basis of vaccination (Sprent and Surh 2002; Janeway 2005). Measurement of antigen-specific proliferative potential following vaccination is therefore important, and has been widely used in assessing vaccine immunogenicity. The methods summarized in Chapter 2 have been commonly used to measure vaccine immunogenicity *in vitro*. These techniques, coupled with multiparameter flow cytometric analysis of antigen-specific proliferating cells, allow delineation of different cell types, cell subsets and the detection of cytokine expression. These include techniques based on uptake of radioactive nucleotides such as tritiated thymidine (^{3}H), DNA incorporation of nucleotide analogues such as bromodeoxyuridine (BrdU), dye dilution assays and intracellular detection of the nuclear protein Ki67 (see *Materials and Methods 2.1.2*).

The neonatal immune system differs from the adult immune system in certain regards and is widely considered to be immature. For example, a large proportion of neonatal T cells are in cycle and are susceptible to apoptosis, indicating a high rate of cell turn over (proliferation) (Hassan and Reen 2001; Schonland, Zimmer et al. 2003; Szabolcs, Park et al. 2003). Telomere length shortens with successive replication cycles (Harley, Futcher et al. 1990), but despite the high rate of proliferation, neonatal T cells maintain long telomere length. In addition, neonatal T cells display characteristics of recent migration from the thymus. Compared to adults, neonatal T cells have high concentrations of T-cell-receptor excision circles (Trecs), a marker of newly synthesized T cells (Hassan and Reen 2001; Schonland, Zimmer et al. 2003).

The underlying differences in the proliferative potential of neonatal T cells may be even more pronounced in pre-term (PT) and low birth weight (LBW) infants, and may affect vaccine immunogenicity. Raqib *et al* found that compared to normal birth weight (NBW) infants, LBW infants showed a trend towards lower percentage of CD3 T cells, shorter telomere length and higher concentrations of

Trecs. PBMCs stimulated with phytohemagglutinin (PHA) and assessed for ^{3}H incorporation showed a trend towards higher proliferative capacity in children who were born with LBW (Raqib, Alam et al. 2007). Most studies in PT and LBW infants have largely focused on Ab responses, but a recent study on post-vaccination responses to inactivated polio vaccination at 2 months of age measured T cell responses (Klein, Gans et al. 2010). Even though levels of poliovirus type 3-specific memory T cells were comparable at 2 months of age, poliovirus type 3-specific proliferation was lower in PT infants compared to term infants. At 7 months of age, levels of circulating poliovirus-specific memory T cells were lower in PT infants implying a waning immune response in PT infants. In addition, at 2 and 7 months of age, levels of CD4+CD69+IFN-γ+ T cells induced by staphylococcus enterotoxin B (SEB) stimulation were significantly reduced in PT infants compared to term infants (Klein, Gans et al. 2010). This study suggests that PT infants may have both vaccine-specific and nonspecific T cell deficiencies.

BCG-induced immunity is 80% efficacious in protecting against severe forms of childhood TB, however efficacy against development of adult pulmonary TB varies from 0 to 80% (Trunz, Fine et al. 2006). The effect of birth weight and maturity on the proliferative capacity of T cells following BCG vaccination has not been completely defined, and may be a contributing factor to the variable efficacy of BCG. A few studies have looked at purified protein derivative (PPD)-specific proliferative responses in these infants. One study measured PBMC lymphoproliferation post-BCG vaccination and found no difference in PPD-specific responses when 30 LBW infants were compared to 56 NBW infants (Ferreira, Bunn-Moreno et al. 1996). Mussi-Pinhata *et al* also measured PBMC lymphoproliferation to PPD in a study assessing the effects of intra uterine growth restriction (IUGR) on BCG immunogenicity. No differences were found between 57 SGA and 52 AGA term infants vaccinated at birth, 3 and 6 months of age. Furthermore, no difference was observed in proliferative responses between SGA infants vaccinated when they reached a birth weight of 2,500g, compared to AGA infants vaccinated at birth (Mussi-Pinhata, Goncalves et al. 1993).

Neonatal immunity studies suggest that proliferative responses in LBW infants may be functionally immature. However, there is a paucity of data in the current literature characterizing vaccine-induced proliferative responses in PT and LBW infants. Previous studies have been limited in that a detailed analysis of the specific T cell subsets and cytokine-producing capacity of BCG-specific proliferating cells in PT and LBW infants has not been performed. In addition, we observed in a WB-ICC assay that LBW infants had a lower overall frequency of BCG-specific CD4+ T cells expressing IL-2, which may translate into reduced proliferative capacity. This prompted us to comprehensively characterize these responses in PT and LBW infants.

In this preliminary study, we aimed, for the first time, to use multiparameter flow cytometry and a 6-day PBMC Ki67 proliferation assay with intracellular cytokine staining to measure BCG-specific proliferative responses in infants stratified by birth weight and maturity. A Ki67 proliferation assay has advantages over standard methods of being non-toxic, non-radioactive and simple to use (Shedlock, Talbott et al. 2010; Soares, Govender et al. 2010). The use of multiparameter flow cytometry in this context would allow the detailed study on a single cell basis of Ki67+ proliferating cells expressing cytokines in response to BCG stimulation. We hypothesized that BCG vaccination at birth induces antigen-specific T cells with a greater proliferative and cytokine producing capacity in NBW infants, compared with LBW infants. Measurement of BCG-specific proliferative responses in the context of birth weight and maturity will advance our understanding of important host factors affecting BCG immunogenicity.

4.3 Results

4.3.1 Flow cytometric detection of proliferating T cells in the Ki67 and Oregon Green proliferation assays

Previously, we showed an alteration in the cytokine production profile of BCG-specific responses in LBW infants. Proliferation is an important assessment of vaccine immunogenicity and T cell function, thus we next assessed the effect of birth weight on BCG-specific proliferative responses. In our aim to study the proliferative and cytokine producing capacity of BCG-specific T cells in LBW and NBW infants in more detail, we required a PBMC proliferation assay to optimally measure these responses in cryopreserved cells. We compared a Ki67 proliferation assay with an Oregon Green (OG)-based dye dilution proliferation assay. Ki67 is expressed in the nuclei of actively dividing cells and can thus be used as a marker of proliferation, whilst the fluorescence intensity of OG is halved with each cell division, thereby allowing measurement of populations of proliferating cells (see *Materials and Methods*, 2.1.2).

Based on reports of potential OG toxicity to PBMC cultures (Wallace, Tario et al. 2008), we first compared the frequency of viable CD3+ T cells from a Ki67 proliferation assay with a dye dilution OG proliferation assay. Recovery of sufficient numbers of viable cells following long-term storage of cryopreserved PBMC would ultimately determine if we could reliably compare different outcomes in our study.

An aliquot of cryopreserved cells from a 10-week old infant were thawed and cultured for 6 days with or without antigen in either a Ki67 proliferation assay or OG proliferation assay (see *Materials and Methods*, 2.2.4). On day 6, cells were labeled with the viability dye ViViD to enable identification of viable cells. We then analysed cells by multiparameter flow cytometry and a sequential gating strategy (Fig. 4.1A) was used to delineate total live CD3+ T cells into different subsets (CD4+ and CD8+). Live CD3+ T cells were selected by gating on $ViViD^{low}$ stained cells (Fig. 4.1A). Representative data for proliferating live CD4+

T cells from an OG proliferation assay (Fig. 4.1B) and from a Ki67 proliferation assay (Fig. 4.1C) are shown. Gates were based on the unstimulated sample and were kept constant for each infant's corresponding SEB and BCG stimulated condition. The percentage of viable cells after 6-day culture for all conditions was significantly higher when PBMC were stained on day 6 with Ki67 only, compared to cells labeled on day 0 with OG (Fig. 4.1D). These results indicate that labeling cryopreserved PBMC with OG may contribute to increased toxicity in the 6-day culture, as evidenced by decreased frequencies of live CD3+ T cells on day 6, compared to cells incubated in the Ki67 proliferation assay. The Ki67 assay was favored as it enabled recovery of a greater percentage of viable cells that could be reliably delineated into proliferating and cytokine producing cell subsets.

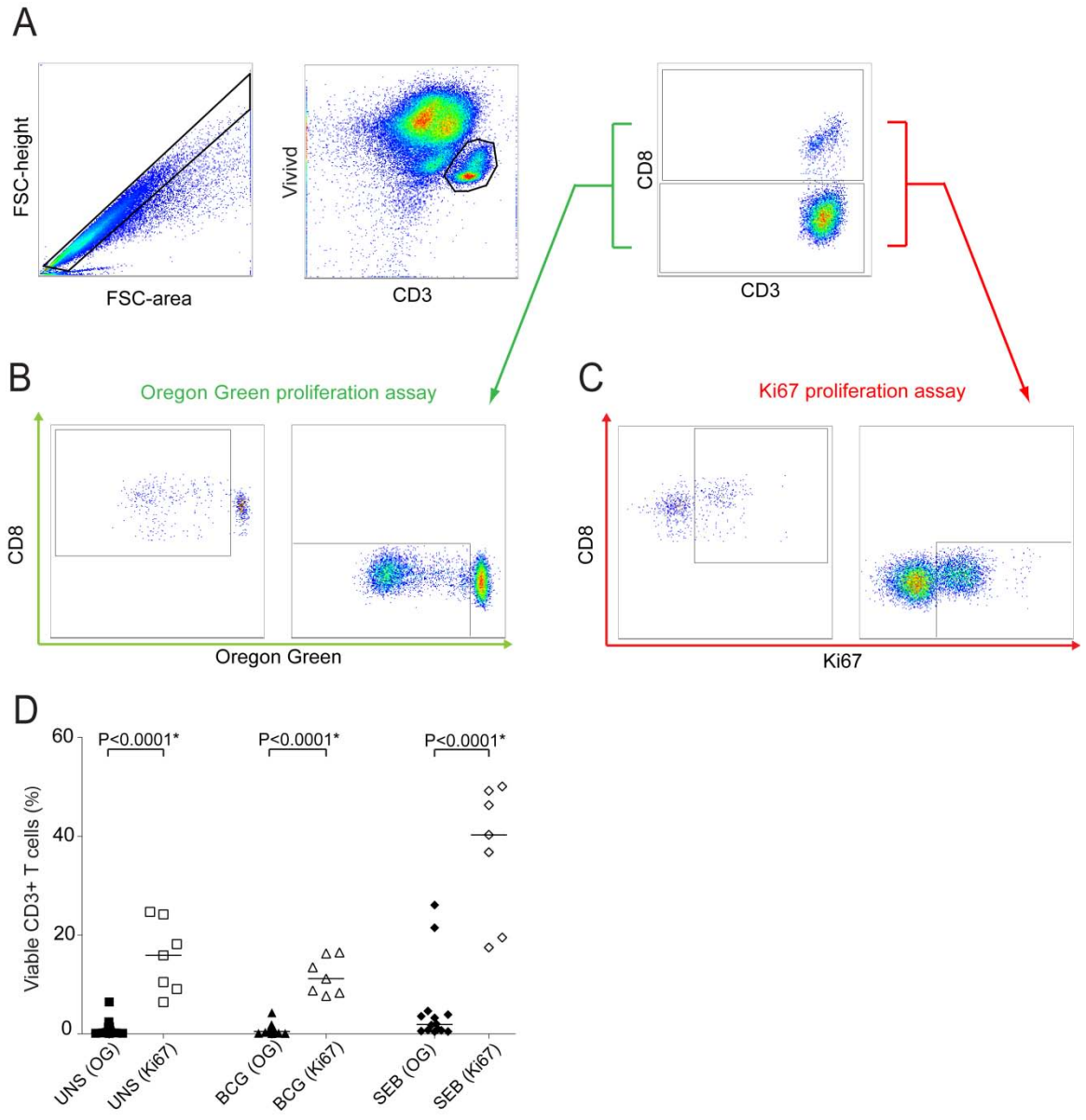

Figure 4.1. Flow cytometry gating strategy and analysis of lymphocyte viability in the Ki67 and Oregon Green (OG) proliferation assays. Cryopreserved PBMC were thawed and cultured with BCG, SEB or medium (UNS; unstimulated) in either a 6-day Ki67 proliferation assay (without OG), or a 6-day OG-based proliferation assay (see *Materials and Methods*, 2.2.5). (A) Gating is first performed on singlets then on live lymphocytes by gating on ViViD^{low} CD3+ cells, from which total CD3+ T cells are differentiated into CD3+CD8+ and CD3+CD8- T cell subsets. This is followed by gating on CD8+ and CD8- OG^{low} (B) or CD8+ and CD8- Ki67+ (C) proliferating cells. CD4+ T cells were defined as CD3+CD8- lymphocytes. Representative data are shown from a BCG stimulated sample. (D) Frequency of viable CD3+ T cells gated on ViViD^{low} CD3+ live cells from Ki67 (n=7) and OG (n=14) proliferation assays. The horizontal line represents the median; the Mann Whitney test was used for comparisons between two groups.

4.3.2 Kinetics of Ki67 expression in proliferating T cells over 6 days

Next we investigated the kinetics of proliferating cells detected by Ki67 expression over 6 days in the presence or absence of antigen. Aliquots of cryopreserved PBMC from 5 infants were thawed, and cultured for 6 days. Using multiparameter flow cytometry we investigated proliferating cells by quantifying Ki67 expression in cells from 5 donors harvested on days 2, 4 and 6. The dot plots in Fig. 4.2A are representative of proliferating cells detected by intracellular Ki67 expression.

In unstimulated cells, the frequency of proliferating cells detected was low on day 2, with a slight increase on day 4 and a peak in proliferation at day 6 (Fig. 4.2B). Background proliferation on day 6 was an unexpected result, as most lymphocytes isolated from peripheral blood are in the resting phase until stimulated. Repeated experiments (data not shown) ruled out contamination of unstimulated cells. We concluded that long-term cryopreserved infant PBMC samples, when thawed and cultured for 6 days in the absence of antigen, may spontaneously proliferate and express Ki67 at low levels. We then quantified antigen-specific proliferation upon BCG stimulation; specific proliferating cells were detected at very low levels on day 2. On day 4 we saw an appreciable increase in BCG-specific proliferating cells with peak expression detected on day 6 (Fig. 4.2C). Although background proliferation was present on day 6 in unstimulated cells, BCG-specific cell proliferation was above levels detected in unstimulated cells. We observed the same pattern in unstimulated and BCG stimulated PBMC cultured over 6 days in an OG assay (data not shown). These results together with increased frequencies of live CD3+ T cells we observed on day 6 in the Ki67 assay prompted us to continue with this assay to determine the effects of birth weight on different outcomes selected in our study.

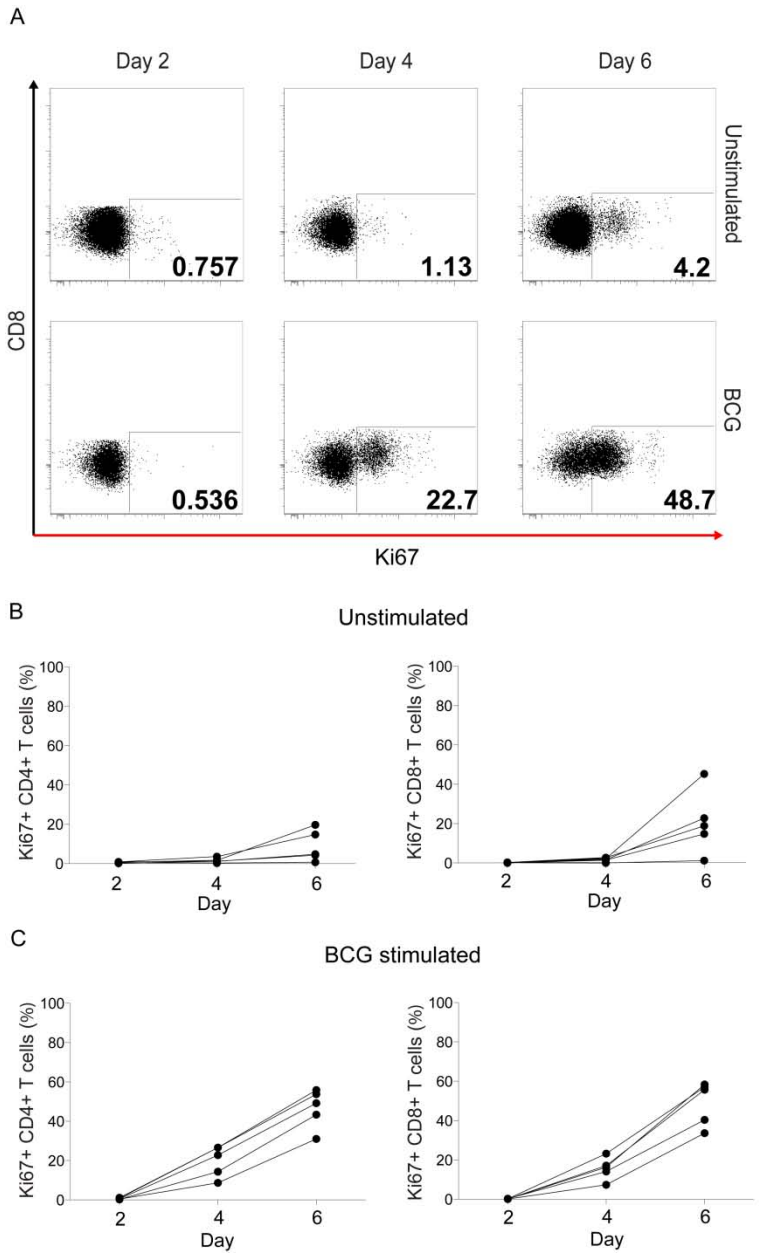

Figure 4.2. Kinetics of Ki67 expression in proliferating T cells over 6 days. Cryopreserved PBMC were thawed and cultured with BCG or medium only (unstimulated) in a 6-day Ki67 proliferation assay (see *Materials and Methods*, 2.2.5). Representative dot plots of proliferating CD8-Ki67+ live cells from one infant incubated with medium only or BCG and quantified on days 2, 4 and 6 are shown (A). The frequency of proliferating CD8- Ki67+ live cells incubated with medium only (B) or BCG (C) were quantified on days 2, 4 and 6 by multiparameter flow cytometry. CD4+ T cells were defined as CD3+CD8- lymphocytes. Data shown are from 5 infants. Dot plots shown are from one infant and data are shown as a percentage of CD4+ T cells.

4.3.3 Frequency of BCG-specific proliferating T cells in LBW and NBW infants

To investigate whether the BCG-specific proliferative response was affected by birth weight, cryopreserved PBMC from a 6-day Ki67 proliferation assay (see *Materials and Methods*, 2.2.5) were thawed and anlaysed by multiparameter flow cytometry. Proliferating cells were detected by quantifying intracellular expression of Ki67 in viable CD3+CD8+ and CD3+CD8- (CD4+) lymphocytes. We compared BCG-specific CD4+ and CD8+ T cell responses in 40 infants grouped categorically as either LBW (n=20) or NBW (n=20) (Fig. 4.3). No difference was demonstrated in the frequency of proliferating BCG-specific (Fig. 4.3) and SEB-specific (data not shown) T cell responses between LBW and NBW infants.

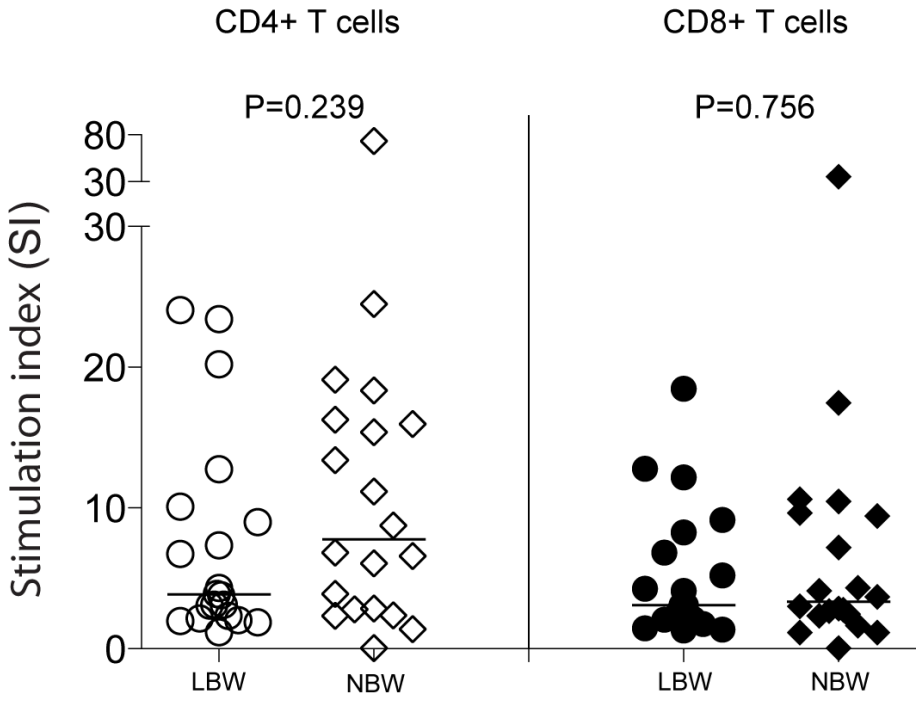

Figure 4.3. Proliferative response of BCG-specific Ki67+CD4+ and CD8+ T cells in LBW (n=20) and NBW (n=20) infants. Proliferative response is expressed as a stimulation index (SI). The horizontal line represents the median. Statistical differences between the proliferative BCG-specific T cell responses were determined by the Mann Whitney test.

After assessing the effect of birth weight categorically, we next investigated the effect of birth weight as a continuous variable on the frequency of proliferating cells. We found no correlation between the frequency of either BCG-specific CD4+ or CD8+ T cells expressing Ki67 and birth weight in 40 infants (Fig. 4.4). Our results indicate that BCG and SEB induce substantial proliferation of T cells, but that birth weight does not affect the ability of these cells to proliferate and express Ki67.

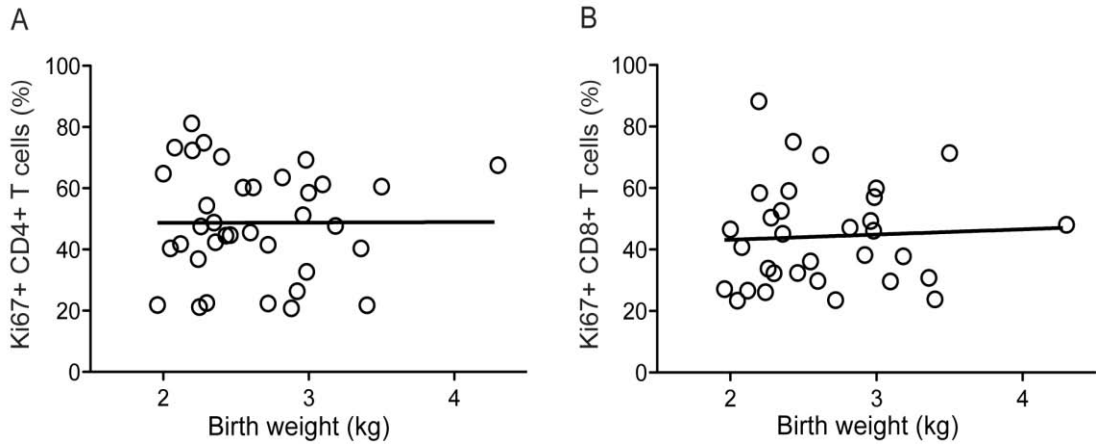

Figure 4.4. Correlation between BCG-specific T cell proliferation and birth weight. (A) Correlation between BCG-specific Ki67+CD4+ T cell expression and birth weight (n=40). (B) Correlation between BCG-specific Ki67+CD8+ T cell expression and birth weight (n=40). Statistical analyses were determined by the Spearman test.

4.3.4 Effect of birth weight on the total cytokine producing capacity of Ki67+ BCG-specific T cells

The cytokine producing capacity of BCG-specific Ki67+ proliferating T cells from a 6-day Ki67 proliferation assay was assessed by adding PMA/I for the last 5 hours of culture, followed by analysis by flow cytometry (see *Materials and Methods*, 2.2.5). Short-term restimulation with PMA/I stimulates cytokine production, providing further information on the functional capacity within the population of antigen-specific, proliferating T cells. Although PMA/I restimulation on day 6 was able to induce some cytokine production by cells incubated without antigen from day 0 (Unstimulated), this was low compared to the appreciable amounts IL-2, IFN-γ or TNF-α produced by proliferating BCG-specific Ki67+ CD4+ T cells in LBW and NBW infants (Fig. 4.5A). All infants had a proliferative response to the positive control, SEB. We excluded infants who failed to meet criteria for a CD4+ BCG-specific proliferative response (n=2; see *Materials and Methods*, 2.2.8). Within the total CD4 T cell population, we compared the total cytokine expression of proliferating BCG-specific CD4 T cells expressing either IL-2, IFN-γ or TNF-α cytokines between LBW and NBW infants. There were no differences in the total CD4+ T cell expression of any of the cytokines by birth weight (NBW, n=18 and LBW, n=18) (Fig. 4.5B). These results indicate that total cytokine expression of BCG-specific Ki67+ proliferating cells is not affected by birth weight.

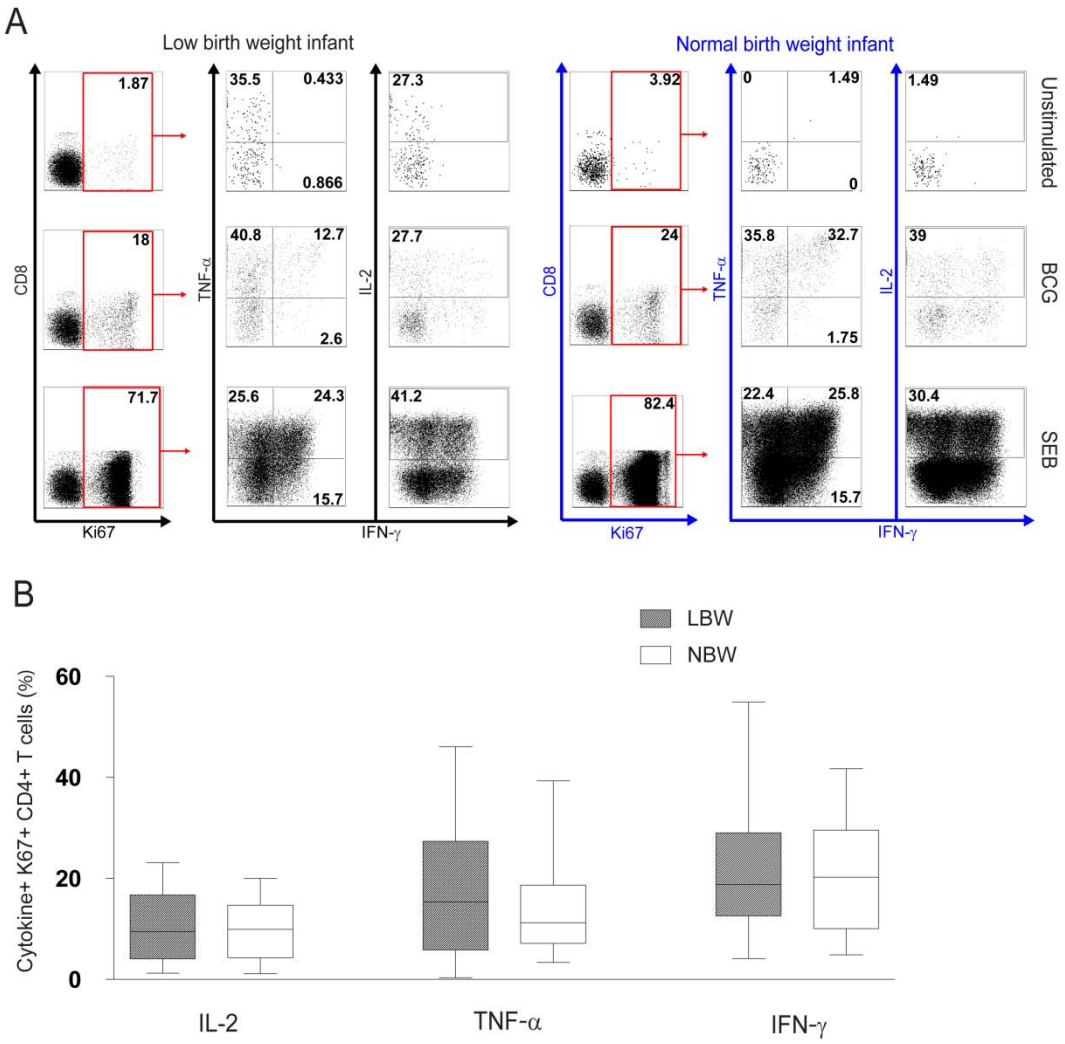

Figure 4.5. Cytokine production capacity of BCG-specific proliferating CD4+ T cells. Cryopreserved PBMC were cultured for 6 days with BCG, SEB or medium (UNS; unstimulated) (see *Materials and Methods*, 2.2.5). To detect cytokine expression, cells were restimulated for 5 hrs on day 6 with PMA and ionomycin (PMA/I) and analyzed by flow cytometry. Cells gated on proliferating CD3+CD8- Ki67+ live cells were differentiated into T cells expressing combinations of IL-2, IFN-γ or TNF-α. CD4+ T cells were defined as CD3+CD8- lymphocytes. (A) Representative dot plots of cytokine expressing cells gated on live CD4+ proliferating cells in one LBW and NBW infant are shown. Percentages shown on the plots are prior to subtraction of cytokine production in the PMA/I negative control sample. The percentage of BCG-specific CD4+ T cells expressing cytokines in LBW (n=18) and NBW (n=18) infants is shown in (B). The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). The horizontal line represents the median and the boxes represent the interquartile range. Data shown in (B) are after background subtraction of the PMA/I negative control sample (see *Materials and Methods*, 2.2.8). Statistical analyses were determined by the Mann Whitney test.

4.3.5 Effect of birth weight on the cytokine expression profiles of proliferating BCG-specific T cells

After assessing total cytokine expression in the analysis above, we next investigated the cytokine expression profile of individual proliferating T cells restimulated with PMA/I. Using multiparameter flow cytometry we CD4+ T cells and their capacity to express combinations of IL-2, IFN-γ or TNF-α. After excluding infants who failed to meet criteria for a CD4+ BCG-specific proliferative response (n=2; see *Materials and Methods*, 2.2.8), we then compared cytokine expression between LBW and NBW infants.

Within the total T cell population, we compared the frequencies (Fig. 4.6A) and the proportions (Fig. 4.6B) of proliferating, cytokine producing BCG-specific CD4+ T cells. The most dominant response was that of BCG-specific CD4+ T cells co-expressing IFN-γ and TNF-α. Short-term PMA/I restimulation did not induce any cytokine expression in a large proportion of BCG-specific T cells, and no difference was observed between LBW and NBW infants (right panels of Fig. 4.6). Similar frequencies and proportions (left panels of Fig. 4.6A and B, respectively) of T cell subsets expressing 7 different combinations of IL-2, IFN-γ or TNF-α were observed in LBW and NBW infants. We concluded that the magnitude and qualitative response as measured by a Ki67 proliferation assay is not affected by birth weight.

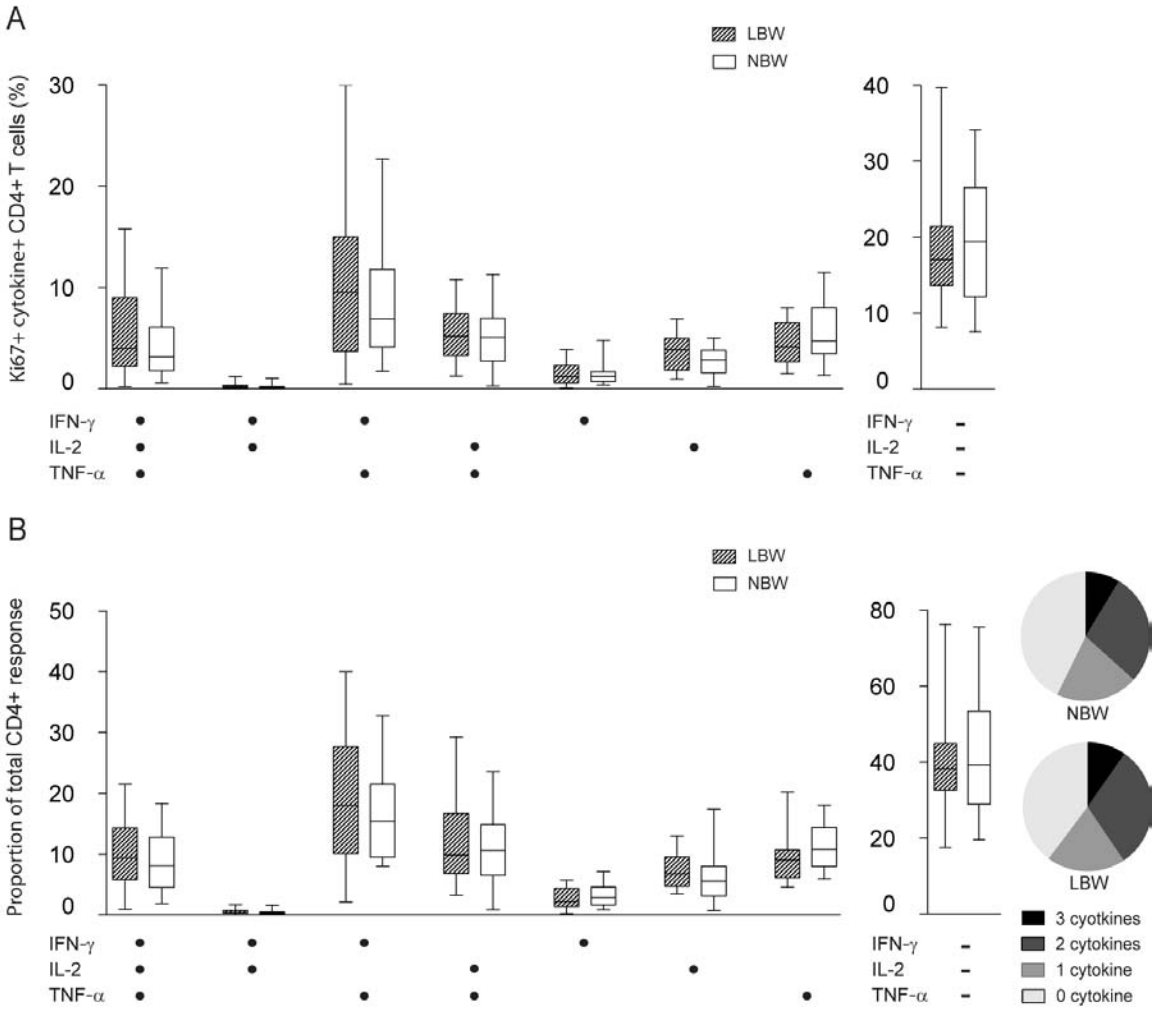

Figure 4.6. The frequency (A) and proportion (B) of proliferating BCG-specific CD4+ T cells expressing combinations of IL-2, IFN-γ or TNF-α cytokines in LBW and NBW infants. Data shown are after background subtraction of the PMA/I negative control sample, and only infants who met criteria for a positive response and minimum numbers of viable CD3+ cells are shown (LBW, n=18 and NBW, n=18) (see *Materials and Methods*, 2.2.8). The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). CD4+ T cells were defined as CD3+CD8- lymphocytes. The horizontal line represents the median and the boxes represent the interquartile range. Each pie is divided into 4 slices that represent the median proportion of the total BCG-specific CD4+ T cell response, a slice each for proliferating T cells that are not expressing any cytokines, or a combination of 3, 2 or 1 cytokine only (B). Statistical analyses were determined by the Mann Whitney test.

4.3.6 Effect of birth weight on cytokine expression of proliferating BCG-specific CD8 T cells

After assessing the effect of birth weight on cytokine expression of BCG-specific proliferating CD4+ T cells, we next performed a similar analysis of CD8+ T cell responses. We used multiparameter flow cytometry to measure the cytokine producing capacity of BCG-specific Ki67+ proliferating T cells restimulated with PMA/I from a 6-day Ki67 proliferation assay. Proliferating BCG-specific Ki67+ CD8+ T cells expressing appreciable amounts IL-2, IFN-γ or TNF-α above background were detected in LBW and NBW infants (Fig. 4.7A). All infants had a proliferative response to the positive control, SEB. We excluded infants who failed to meet criteria for a CD8+ BCG-specific proliferative response (n=4; see *Materials and Methods*, 2.2.8). There was no difference in the total cytokine expression of proliferating BCG-specific CD8 T cells between LBW and NBW infants (Fig. 4.7B).

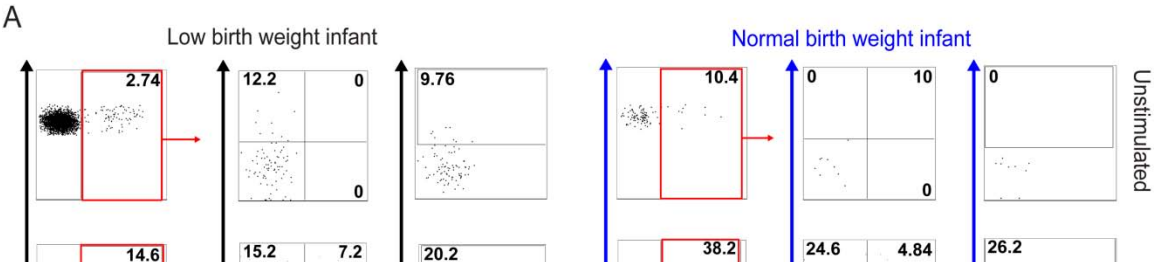

Figure 4.7. Cytokine production capacity of BCG-specific proliferating CD8+ T cells. Cryopreserved PBMC were cultured for 6 days with BCG, SEB or medium (UNS; unstimulated), restimulated for 5 hrs on day 6 PMA/I and analyzed by flow cytometry (see *Materials and Methods*, 2.2.5). Cells gated on proliferating CD3+CD8+ Ki67+ live cells were differentiated into T cells expressing combinations of IL-2, IFN-γ or TNF-α. (A) Representative dot plots of cytokine expressing cells gated on live CD3+CD8+ proliferating cells in one LBW and NBW infant are shown. Percentages shown on the plots are prior to subtraction of cytokine production in the PMA/I negative control sample. (B) Frequency of BCG-specific CD8+ T cells that are proliferating and expressing cytokines in LBW and NBW infants. The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). The horizontal line represents the median and the boxes represent the interquartile range. There was no statistical difference as determined by the Mann Whitney test.

We next performed a detailed analysis of the cytokine expression profile of individual proliferating CD8+ T cells found in 7 distinct subsets expressing combinations of IL-2, IFN-γ or TNF-α (left panels, Fig. 4.8). We compared the frequency (left panels, Fig. 4.8A) and proportion (left panels, Fig. 4.8B) of proliferating BCG-specific CD8+ T cells expressing all combinations of IL-2, IFN-γ, TNF-α, and found no difference between NBW and LBW infants. The main populations of cells expressed either IL-2 or IFN-γ only. However the majority of CD8 T cells did not express any cytokine at all, and no difference in this subset was found between LBW and NBW infants (right panels of Fig. 4.8A and Fig. 4.8B). These results indicate that the magnitude and quality of the proliferating BCG-specific CD8+ T cell response is not affected by birth weight as measured by a Ki67 proliferation assay.

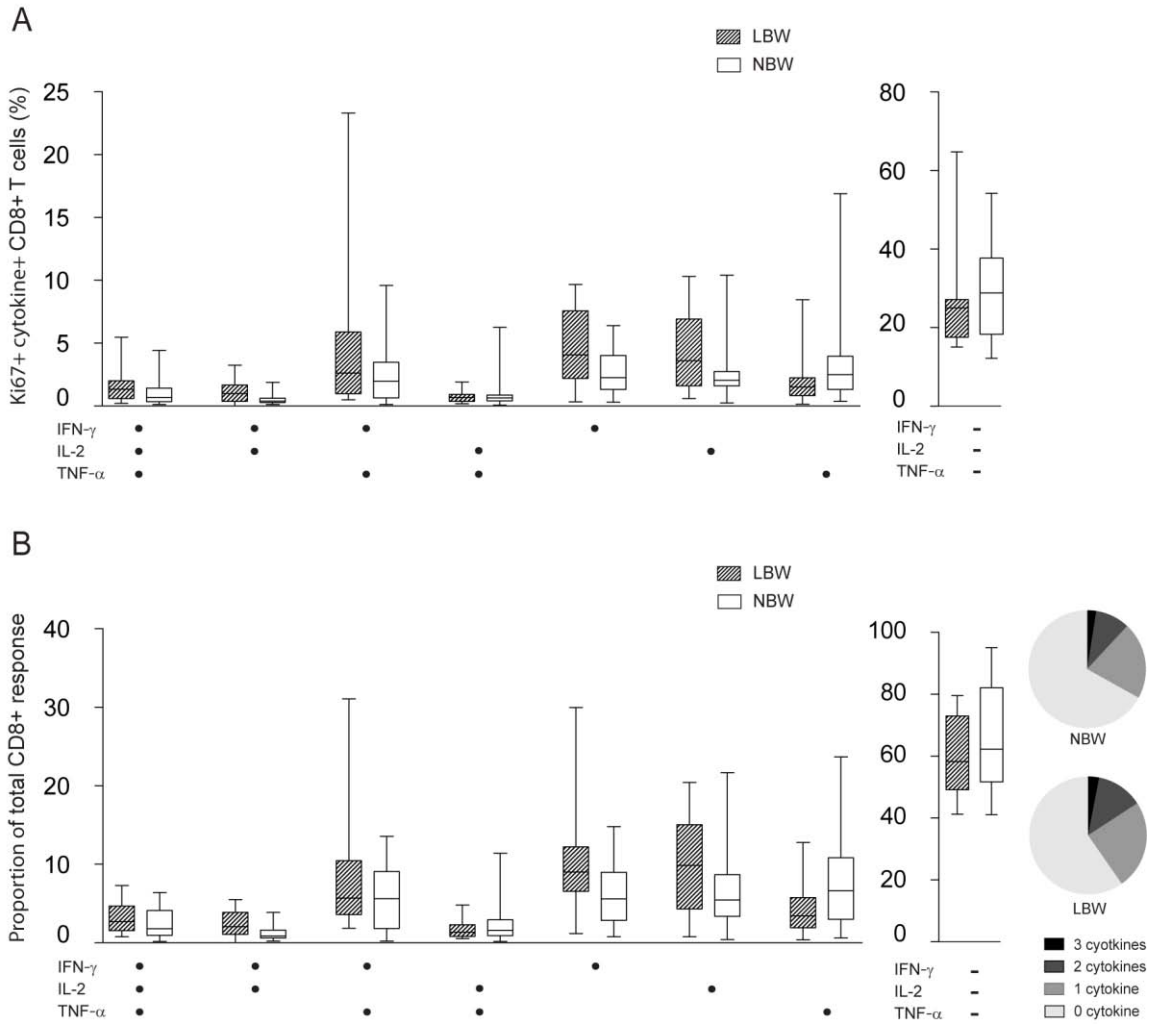

Figure 4.8. The frequency (A) and proportion (B) of BCG-specific CD8+ T cells that are proliferating and expressing combinations of IL-2, IFN-γ or TNF-α cytokines in LBW and NBW infants. Data shown are after background subtraction of the PMA/I negative control sample, and only infants who met criteria for a positive response and minimum numbers of viable CD3+ cells are shown (LBW, n=16 and NBW, n=16) (see *Materials and Methods*, 2.2.8). The data is shown as the frequency of grandparent (see *Materials and Methods*, 2.2.7). The horizontal line represents the median and the boxes represent the interquartile range. Each pie is divided into 4 slices that represent the median proportion of the total BCG-specific CD8+ T cell response, a slice each for T cells that are not expressing any cytokines, or a combination of 3, 2 or 1 cytokine only (B). Statistical analyses were determined by the Mann Whitney test.

4.4 Discussion

In the previous chapter, we showed differences in BCG immunogenicity by birth weight on a single cell basis using a WB-ICC assay and multiparameter flow cytometry. This short-term assay measured cytokine production *ex vivo*, which is only one aspect of cellular immune responses. To gain a greater comprehension of BCG-induced immunity, it is important to further characterize immune responses in susceptible populations such as PT and LBW infants. We thus measured the proliferative potential and cytokine producing capacity of BCG-specific T cells, critical functions that may be affected by birth weight. Evidence of neonatal functional immaturities and the lack of previous detailed studies on BCG-specific proliferative responses prompted us to perform this study. We used a 6-day Ki67 lymphoproliferation assay and multiparameter flow cytometry, a more sensitive and specific assay that could measure BCG-specific responses on a single cell basis. To the best of our knowledge this is the first time such assay systems have been used to characterise BCG specific proliferative responses in LBW infants. We report that following BCG vaccination, BCG-specific proliferative responses were not affected by birth weight or maturity.

We complemented our initial WB-ICC results by measuring the ability of lymphocytes to proliferate and express cytokines in response to BCG stimulation *in vitro*. We used a 6-day Ki67 proliferation assay that has been shown to be antigen specific, reproducible and comparable to other conventional methods such as dye dilution of Oregon Green (OG) (Shedlock, Talbott et al. 2010; Soares, Govender et al. 2010). Ki67 is a nuclear protein that can be used as a marker of proliferation *in vitro* as it is upregulated and expressed in actively dividing cells but not in resting cells (Jeurink, Vissers et al. 2008; Soares, Govender et al. 2010). During long-term culture, cell death occurs thus we incorporated a viability dye in the assay enabling the differentiation of live cells from dead cells by flow cytometry. The Ki67 proliferation assay has been studied in detail before (Shedlock, Talbott et al. 2010; Soares, Govender et al. 2010) and we chose this assay over an OG proliferation assay as we found it to be less

toxic. Thus we were able to exclude dead cell artifacts, and to measure sufficient numbers of proliferating cells isolated and expanded from the small volume of blood that we could collect from 10-week-old infants.

Similar to a study by Soares et al, we used multiparameter flow cytometry to quantify the kinetics of Ki67 expression in live proliferating T cells over 6 days (Soares, Govender et al. 2010). However, in contrast to their findings, we found high background frequencies of Ki67+ proliferating T cells in our unstimulated samples on day 6 of the assay. This may be due to difference in methodology, as we used cryopreserved infant PBMCs isolated from peripheral blood, whilst Soares et al used fresh whole blood from either adults or toddlers. Jeurink et al has reported a difference in cytokine production of Ki67+ proliferating cells in cryopreserved PBMC compared to freshly isolated PBMC. PMA and calcium ionophore (Ca-I) stimulated cells expressing Ki67 had reduced levels of IL-4, IL-13 and IL-1-β after cryopreservation compared to freshly isolated cells (Jeurink, Vissers et al. 2008). In addition, the high frequencies we observed could be due to the high rate of cell turn over in neonates (Hassan and Reen 2001; Schonland, Zimmer et al. 2003; Szabolcs, Park et al. 2003). However, the background Ki67 expression did not impact on our results, as after BCG stimulation and 6-day culture, BCG-specific Ki67+ proliferative responses were higher than background.

In comparison, NBW and LBW infants had similar frequencies of BCG-specific proliferating T cells and we found no association with birth weight. These results are consistent with a previous study by Ferreira et al, who reported that birth weight did not affect the ability of PPD-specific cells to proliferate (Ferreira, Bunn-Moreno et al. 1996). All infants responded equally to SEB stimulation showing that global T cell proliferation was functional in this cohort. This is in contrast with a study by Raqib *et al* that found a trend towards greater proliferative responses to PHA in LBW infants compared to NBW infants, whilst a study by Klein *et al* suggested reduced proliferative responses to poliovirus type 3 in PT infants compared to term infants (Raqib, Alam et al. 2007; Klein, Gans et

al. 2010). Use of cryopreserved PBMCs in our study compared to freshly isolated PBMC in Klein *et al's* study may explain the contrasting results. On the other hand, Raqib *et al's* cohort consisted of children who were formerly LBW and proliferation was assessed by ^{3}H uptake in 3-day PHA-stimulated cells. These differences in methodology compared to our BCG-stimulated 6-day Ki67 proliferation assay may be the underlying reasons for our contrasting results.

The functional capacity of BCG-specific proliferating cells from a 6-day Ki67 proliferation assay were determined by polyclonal re-stimulation with PMA/I during the last 5 hours of culture. PMA/I induces T cell activation and cytokine production by directly activating protein kinase C-θ in a TCR independent process. This reflects the intrinsic ability of a T cell to proliferate and produce cytokines regardless of TCR expression (Kim, Lancki et al. 1989; Kim, St John et al. 2009). We measured the expression of IFN-γ, IL-2 and TNF-α, cytokines that we found to differ by birth weight as measured by a WB-ICC assay and multiparameter flow cytometry (see *Chapter 2*).

We measured the total expression and the capacity to express combinations of the cytokines IFN-γ, IL-2 or TNF-α in BCG-specific proliferating cells. Birth weight did not affect the ability of BCG-specific cells to proliferate and express cytokines, in particular IL-2 which is important for immunological memory (Chaplin 2010). These cytokines, as discussed in previous chapters, are induced upon BCG vaccination and play important roles in host defense against TB disease.

These preliminary results do not support our hypothesis that BCG vaccination at birth induces antigen-specific T cells with a greater proliferative and cytokine producing capacity in NBW infants compared with LBW infants. BCG strongly activates DCs, and unlike other childhood vaccines, seems to be able to overcome immaturities in the neonatal immune system that are mainly of a Th2 phenotype by skewing them towards a Th1 phenotype (Marchant and Goldman 2005). This may be a reason why differences were not seen between

LBW and NBW infants. This potential masking of responses as a result of BCG induced innate cell activation may not be seen if individual mycobacterial antigens (Ags) such as *M.tb* peptides or recombinant proteins had been used to stimulate cells.

Our preliminary results suggest that birth weight at the time of vaccination does not affect the magnitude and quality of the BCG-induced proliferative responses in a Ki67 assay. Due to the small sample size, we were unable to determine the effects of gestational age, size for GA or gender on BCG-specific proliferative responses. We thus propose that further studies should be performed in larger cohorts to determine the effects of these host factors on BCG-specific proliferative responses. In addition, the persistence of the proliferative responses we measured should be further investigated in longitudinal studies. The importance of this is highlighted by Kagina *et al's* study on delaying BCG vaccination from birth to 10 weeks. Differences between birth vaccinated infants and those vaccinated at 10 weeks were most significant at 1 year of age (Kagina, Abel et al. 2009). In particular, the frequency of polyfunctional BCG-specific T cells was greater in the delayed group (Kagina, Abel et al. 2009) and it may be possible that in our study, the proliferative capacity of BCG-specific T cells in LBW infants is impaired at later time points. Future studies will ultimately lead to optimal vaccination strategies in susceptible populations such as PT and LBW infants. This is important, as the basis of immunisation is to induce memory cells, which upon exposure to the actual pathogen will proliferate more rapidly and effectively respond to provide protection.

CHAPTER FIVE

Overall discussion and conclusion

Overall, we have shown that LBW infants may have an altered immune response to BCG. In particular, using a WB-ICC assay, we found that LBW infants had a lower proportion of polyfunctional BCG-specific CD4+ T cells and corresponding higher proportions of single cytokine-producing CD4+ T cells expressing IFN-γ only, compared to NBW infants. No differences were found in the proliferative potential of BCG-specific T cells from a 6-day Ki67 proliferation assay. In addition, most cytokine expression patterns of specific cells were not affected by BW, GA, size for GA or gender. The clinical and biological significance of our results is unknown. In the absence of large-scale efficacy trials, our results are too preliminary to suggest that optimal protection afforded by BCG vaccination can be obtained by vaccinating all infants at birth regardless of birth weight or gestational age. Our results are also presented in light of several limitations that we acknowledge.

The risk factors that may contribute to IUGR and LBW are numerous (Valero De Bernabe, Soriano et al. 2004) and were not controlled for in our study. Our study participants come from the rural Worcester area of the Western Cape and data is available for this region, which should provide a basic socio economic history. Infants enrolled in our study were born predominantly to mixed race ("colored") mothers who were low-income workers. The Western Cape is a wine-producing region and due to maternal alcohol consumption, certain rural areas have reported the highest incidence of fetal alcohol syndrome (FAS) ever worldwide (May, Gossage et al. 2005; May, Gossage et al. 2007). In rural areas up to 51% of mothers abuse alcohol during pregnancy and in addition, an estimated 48% of mixed race adults (>18 years) in the Western Cape region of South Africa smoke tobacco (Reddy, Meyer-Weitz et al. 1996; May, Gossage et al. 2005). Ethnicity, low socioeconomic level, maternal smoking and FAS are possible risk factors for LBW (Valero De Bernabe, Soriano et al. 2004), and any of these may be confounders that could have potentially affected BCG-induced T cell responses in our study participants.

Our study participants were formerly enrolled as part of a larger randomised control trial (RCT; n=11,670). As part of the RCT, unhealthy infants were excluded. It is possible that exclusion of ill or symptomatic infants resulted in potential selection for a healthier population of LBW infants, as most LBW infants are generally more susceptible to infection and therefore more likely to have been excluded at enrolment. More differences may have been observed if these infants were included in this study. The blood products we used in this study had been collected in the RCT investigating the incidence of TB over 2 years in infants vaccinated at birth intradermally or percutaneously with Japanese BCG. Our study participants did not develop TB over a 2-year follow up and were thus excluded from the RCT. However as part of the RCT, whole blood collected was immediately stimulated and processed in a WB-ICC and cryopreserved. Therefore, we had no control in this study over the antigen stimulus used, and upon thawing the cryopreserved whole blood samples we could not explore any other Ags and mitogens. On the other hand, multiple aliquots of PBMC were collected and cryopreserved from each infant enrolled. Production of cytokines following short-term stimulation of these PBMC with mycobacterial antigens other than BCG, such as Mtb protein or peptides, could have been performed. These assays could have been complemented by assessment of proliferation. Soluble cytokines levels from the supernatant of these cultured PBMC could have been measured as well. These different assays would be of interest in future studies on BCG immunogenicity in PT and LBW infants.

Other aspects of BCG-induced immunity may be affected by birth weight and maturity. In this study, we focused only on the cytokines IFN-γ, TNF-α, IL-2, and IL-17. Various other cytokines, Tregs, $\gamma\delta$ T cells and memory T cell phenotype induced by BCG vaccination may be differentially affected by birth weight and maturity, and would be interesting to investigate in future studies. In addition, host innate immunity also plays an important role in BCG-induced immunity, although the analysis of multiple immune mechanisms was beyond the scope of this study. Thus our aim was focused on certain aspects of T cell

immunity and on measuring BCG-induced T cell responses. In this study it would have been interesting to correlate proliferation and whole blood assay cytokine responses. Due to the limited availability of stored PBMC and whole blood of each enrolled infant, both assays were performed only a small number of infants, and we were thus unable to directly compare these two assays in the same infants.

Our study highlights the complexity of birth weight and maturity on the immune response to BCG vaccination. This represents the first detailed analysis of the functional capacity of BCG-induced T cell responses in healthy PT and LBW infants. These data are currently lacking in the literature, especially in highly endemic TB areas such as South Africa. Statistical correction for multiple comparisons was not performed and it is possible that significance will not be maintained after such correction. Although we cannot draw firm conclusions, our preliminary findings raise the possibility that healthy PT and LBW infants may not develop optimal BCG-induced immunity. However, as immune correlates of protection against TB have not been defined to date, we suggest caution in interpreting our preliminary results, which have unknown implications in terms of protection against TB disease. As shown in a recent human study, the quality of the polyfunctional BCG-specific CD4+ T cell response that we found to be elevated in NBW infants may not be an immune correlate of protection (Kagina, Abel et al. 2010). Future studies confirming our results, as well as optimization of vaccine strategies and efficacy trials in PT and LBW infants are required before current BCG vaccination policies are altered. Future studies may potentially improve the efficacy of BCG. Our study has potentially important implications for infant vaccination practices worldwide.

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