Comparison of Immune Responses Induced by Bacillus Calmette-Guerin When given at Birth or at 6 Weeks of Age in Ugandan Infants

Thesis Presented for the Degree of

Doctor of Philosophy

By

Fredrick Lutwama

In the School of Child and Adolescent Health

Faculty of Health Sciences

University of Cape Town

February 2014

Supervisors: Prof Willem Hanekom

Dr Benjamin Mugo Kagina

Dr Cheryl Day

Prof Harriet Mayanja-Kizza

Abstract

In Uganda, infants delivered at a health care facility receive the tuberculosis vaccine Bacillus Calmette-Guerin (BCG) on the first day of life. Infants delivered at home receive BCG at their first health care facility visit at 6 weeks of age. Our aim was to determine the effect of this delay in BCG administration on the induced immune response. Our hypothesis was that infants who received BCG at 6 weeks of age would show an enhanced BCG-induced T cell immunity compared to infants vaccinated at birth.

We optimised several polychromatic flow cytometry reagent panels to compare BCG-specific immunity in 9 months-old infants who had received the vaccine either at birth or at 6 weeks of age. We used a 12-hour whole blood intracellular cytokine/cytotoxic marker assay to measure T cell-associated cytokine expression and memory phenotypes. We also compared the capacity of BCG-specific T cells to proliferate and produce cytokines upon antigenic stimulation with a 6-day proliferation assay. Finally, we measured plasma soluble cytokines levels in the two groups of infant using multiplex assay.

We enrolled 92 infants: 50 had received BCG at birth and 42 at 6 weeks of age. BCG induced predominantly $CD4^+$ T cell responses, and lesser $CD8^+$ T cell responses, in both groups. Birth vaccination was associated with greater induction of $CD4^+$ and $CD8^+$ T cells expressing either IFN-γ alone, or IFN-γ together with perforin, compared with delayed vaccination. Further, birth vaccination induced proliferating cells that had greater capacity to produce IFN-γ, TNF-α and IL-2 together, compared with delayed vaccination.

In conclusion, distinct patterns of T cell induction occurred when BCG was given at birth and at 6 weeks of age. We propose that this diversity might impact protection against tuberculosis. Our results differ from those of delayed BCG vaccination studies in South Africa and the Gambia, suggesting geographical and population heterogeneity may affect BCG-induced T cell response.

Acknowledgements

This project is a result of hard work from many individuals at the South African Tuberculosis Vaccine Initiative (SATVI), University of Cape Town and Makerere University College of Health Sciences. Without their support, I could not have completed this.

First, I would like to thank my supervisor, Professor Willem Hanekom for giving me the opportunity to pursue my postgraduate studies at SATVI and for his excellent mentorship and support academically, financially and otherwise. I am also grateful to my co-supervisors Dr Benjamin Kagina, Dr Cheryl Day and Professor Harriet Mayanja-Kizza all of whom provided good advice, excellent ideas and stimulating discussions, which helped develop ideas for the work presented here.

I would like to thank the SATVI team in Cape Town for their excellent support throughout my studies. I cannot name all but special thanks go to Dr Thomas Scriba and his academic team for the intellectual discussions, Ms Jane Hughes and her team for all the support in the Laboratory, all the administrative staff: Sarojini Pillay, Keshia Trussels, for facilitating my stay in Cape Town.

I would like to thank the TBVacsin team at the Infectious Diseases Institute, Makerere University College of health Sciences. Special thanks go to Dr Philippa Musoke, Dr Ann Wajja, Zaituni Kanamala, Jackie Kwesiga and Edward Muyombo, for their support in enrolment of study participants. I would also like to acknowledge Prof Moses Joloba, Willium Ssengooba, Sam Kirimunda and Stephen Tukwasibwe for their support at the Makerere University Immunology laboratory.

I would also like to extend my special thanks to all the mothers, infants and healthy donors that participated in this study. Without them this project would not have been possible.

I would like to thank my family and friends for the moral support. Special thanks go to my parents who, despite the limited financial income, still supported me, and my siblings, to get a good education. Even though my father could not live to see this day, he will always remain in my thoughts. I am grateful to my wife Sophie, my son Simon, my daughters Nicole and Denise for the social support during my studies. I would like to thank my siblings and friends both in Cape Town and Uganda for their continuous support and encouragement.

Special thanks to the following funders who provided support to enable successful completion of this work: European and Developing Countries Trial Partnership (EDCTP), SATVI and the University of Cape Town.

Last but not least, I would like to thank the almighty God for his blessings and providing me the strength and knowledge that has enabled me go this far.

Publications

Work directly related to this thesis has been published in the following manuscript and is attached in the appendix.

Lutwama F, Kagina BM, Wajja A, Waiswa F, Mansoor N, Kirimunda S, Hughes EJ, Kiwanuka N, Joloba ML, Musoke P, Scriba TJ, Mayanja-Kizza H, Day CL, Hanekom WA. Distinct T-Cell Responses When BCG Vaccination Is Delayed From Birth to 6 Weeks of Age in Ugandan Infants. J Infect Dis. 2014 Mar;209(6):887-97. doi: 10.1093/infdis/jit570. Epub 2013 Oct 31.
PMID: 24179111.

Table of Contents

List of abbreviations

%	Percent
°C	Degrees Celsius
AIF	Apoptosis-inducing factor
APCs	Antigen presenting cells
APC	Allophycocyanin
BCG	Bacillus Calmette-Guerin
BCL-2	B-cell lymphoma 2
BIS	Brightness index score
BP	Band pass
BSA	Bovine serum albumin
CD	Cluster of differentiation
CCR	C-C chemokine receptor
CFP-10	Culture filtrate protein-10
CFUs	Colony forming units
CO_2	Carbon dioxide
CS&T	Cytometer setup & tracking
CTL	Cytotoxic T lymphocytes
CV	Coefficient of variation
DCs	Dendritic cells
DC-SIGN	Dendritic cells-specific intracellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetra-acetic acid
EPI	Expanded programme on immunisation
ESAT-6	Early secretory antigenic target 6
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence-minus-one
FSC-A	Forward scatter area
FSC-H	Forward scatter height

HICK-2	Human intracellular cytokine 2
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular staining
IFN-γ	Interferon gamma
IL	Interleukin
log	logarithm
LP	Long pass
mABS	Monoclonal antibodies
M. bovis	Mycobacterium bovis
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
ml	Milliliter
mRNA	Messenger ribonucleic acid
MVA85A	Modified vaccinia Ankarra-expressing antigen 85A
M.tb	Mycobacterium tuberculosis
NK	Natural killer
NO	Nitric oxide
NRAMP	Natural resistance-associated macrophage protein
NTM	Non-tuberculosis mycobacteria
OPV	Oral polio vaccine
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy7	Cyanine-7-phycoerythrin
PerCP-Cy5.5	Cyanine-5.5-peridin-chlorophyll
PFC	Polychromatic flow cytometry
PHA	Phytohaemagglutinin
PMT	Photomultiplier tube voltage
PRR	Pattern recognition receptor

Qdots	Quantum dots
RPMI	Rosewell Park Memorial Institute
ROI	Reactive oxygen intermediate
SATVI	South African Tuberculosis Vaccine Initiative
S:N	Signal to noise
SSC	Side scatter
TB	Tuberculosis
TCR	T cell receptor
Th	T helper
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell
TST	Tuberculin skin test
UNS	Unstimulated
WHO	World health organisation
$\gamma\delta$	Gamma delta
μL	microliter

Chapter One: Introduction and literature review

The overall objective of this project was to determine whether delay of Bacillus Calmette-Guerin (BCG) vaccination from birth to 6 weeks of age, due to home birth of Ugandan infants, affects the specific immune response induced. Our hypothesis was that infants who received BCG at 6 weeks of age would show an enhanced BCG-induced T cell immunity compared to infants vaccinated at birth.

1.1. Tuberculosis

Approximately one third of the world's population is infected with Mycobacterium tuberculosis (*M.tb*), the causative agent for tuberculosis (TB). Among persons latently infected with *M.tb*, 10% develop active TB during their lifetime. Therefore, *M.tb* infected persons represent a reservoir that drives the TB epidemic. Annually, an estimated 9 million new cases of active TB occur, whereas 1.3 million people die of the disease. Uganda is among top 22 TB endemic countries listed by the World Health Organisation (WHO) contributing a cumulative 0.52% of the TB cases of the world (WHO, 2013b). Persons within Uganda therefore have a high risk of exposure to *M.tb* infection.

M.tb is an intracellular pathogen transmitted when a person with active pulmonary TB coughs and releases microdroplets containing the *M.tb* bacilli. When persons in the vicinity inhale the microdroplets containing the *M.tb* bacilli, several outcomes may occur depending on bacterial, host or environmental factors: (i) The host immune system may succeed in eliminating the bacilli, (ii) The infection may persist without causing active disease, a situation referred to as latent TB infection (LTBI), or (iii) The infection may progress into active TB. Factors that determine the progression from LTBI to active disease include the age of the individual, host immune status and socio-economic factors.

Regarding age, infants are more susceptible to developing TB disease following infection, compared to adults. This may be explained, in part, by the reduced responsiveness of the infant's immune system to toll-like receptor (TLR) ligand stimulation compared adults (Corbett et al., 2010). As regards immune status,

persons with human immunodeficiency virus (HIV) infection have increased susceptibility to developing TB disease, compared with uninfected individuals (Lawn et al., 2009). HIV causes depletion of $CD4^+$ T cells, which are important in the adaptive immune response to *M.tb* infection. Various socio-economic factors are associated with increased risk of developing TB disease including compromised nutrition and poor quality of housing (Romaszko et al., 2008).

1.2. Approaches to control of TB

In 2006, the Stop TB Partnership set a goal to reduce the incidence of new TB cases globally to less than one case per million population by the year 2050 (Raviglione, 2007). Modeling studies suggest that it will be difficult to achieve this goal using a partially effective vaccine, like BCG, despite wide vaccination coverage (Abu-Raddad et al., 2009). Rather, a multipronged approach may be needed to effectively reduce the TB epidemic. Young et al. used an age structured mathematical model to show that an approach that combines mass vaccination campaigns and effective drug therapy can reduce the incidence of TB to less than 10 cases per million population by the year 2050 (Young and Dye, 2006). The current approaches for control of *M.tb* infection and TB are discussed in greater detail below.

1.2.1. Drugs

Drugs may be used for the treatment of *M.tb* infection or active TB. Persons with LTBI can be treated with isoniazid (INH) for 6 to 9 months (WHO, 1982). The treatment of LTBI is aimed at reducing the reservoir of latent infection, thereby preventing *M.tb* reactivation and development of new cases of active TB. INH prophylaxis can reduce the incidence of TB by up to 90% (Ferebee, 1970). However, the use of single anti-tuberculosis drug may result in drug resistance, especially in persons with undiagnosed active disease (Balcells et al., 2006). Therefore, the diagnosis of active TB should be excluded prior to initiation of chemoprophylaxis.

Successful treatment of active TB disease involves the use of a combination of different anti-TB drugs, plus strategies to secure adherence to the treatment. The Directly Observed Treatment Short course (DOTS) strategy has been used successfully to achieve adherence. First line regimen for treatment of TB consists of isoniazid, pyrazinamide, rifampicin and ethambutol. Persons with resistance to isoniazid and rifampicin, also known as multi drug resistant (MDR) TB patients, are treated with a second line treatment regimen that may include drugs like capreomycin and neomycin (Lalloo, 2010). A third line regimen is used for patients with extensively drug resistant TB (XDR), defined as resistance to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs (amikacin, kanamycin, or capreomycin). Drugs for treatment of patients with XDR TB may include linezolid and rifabutin (Lalloo, 2010).

1.2.2. Diagnostics

Early diagnosis of TB is important for timely initiation of treatment. There are various laboratory methods that can be used for diagnosis of LTBI and active TB. The tuberculin skin test (TST) is used to diagnose persons with LTBI. TST measures immunological response to purified protein derivative (PPD) antigens from *M.tb*. However, the TST is limited in its ability to differentiate between LTBI and previous BCG vaccination (Harada, 2006), given that many proteins in PPD shares homology between *M.tb* and BCG. This challenge can be overcome by use of the interferon gamma release assays (IGRA) that measure the levels of IFN-γ released in response to the proteins early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10)(Ewer et al., 2003). Genes encoding for these two antigens were deleted during the attenuation of BCG (Behr et al., 1999). The sensitivity of IGRAs is however reduced in HIV infection (Raby et al., 2008).

Active pulmonary TB may be diagnosed by use of direct sputum microscopy; however, this method has a low sensitivity in detection of *M.tb* bacilli

(Cattamanchi et al., 2009). Alternatively TB can be detected in a sputum sample by use of Lowenstein Jensen culture (Levidiotou et al., 1999). Also, TB can be diagnosed in a sputum sample, using a mycobacterial growth indicator tube (MGIT). The disadvantage of MGIT cultures is the long time to generate results (Brittle et al., 2009) and the required specialist training. Pulmonary TB may also be diagnosed using chest X-ray radiography. A specialist radiologist is needed for an accurate interpretation of X-rays. The GenXpert MTB/RIF is PCR based technique that uses sputum samples to detect *M.tb* DNA, and the most common genomic determinant of rifampicin resistance. This method produces results within two hours. However, the cost of purchasing the equipment and the skill needed to operate the machine limit the use of this technique in resource limited settings.

1.2.3. Public health measures

Governmental commitment is important for the efficient control of TB epidemic. For example, governments may design policies that ensure efficient delivery of vaccines at a grassroots level. The government may as well play a role in ensuring availability of basic diagnostic tools for TB at the district health centers coupled with additional reference laboratories to support the district facilities in identifying drug resistant cases. There is also need of policies to ensure equitable distribution of anti-tuberculosis drugs, their prescription and adherence monitoring (Abubakar et al., 2013).

1.3. The BCG vaccine

BCG is currently the only vaccine licensed for the prevention of TB (Fine PEM, 1999). The vaccine was developed from a virulent strain of *Mycobacterium bovis* (*M.bovis*), isolated from the milk of a heifer with TB mastitis. Calmette and Guérin subjected this pathogenic strain to 230 passages over glycerinated bile potato medium at the Institut Pasteur in Lille, France, to generate BCG. In 1921, the first reports of the successful use of BCG for prevention of TB emerged when oral

administration of the vaccine to a baby born to a mother with TB resulted in protection of this infant from development of active disease (Bonah, 2005).

In the United Kingdom, BCG vaccination was included into the routine vaccination programme in 1953 after studies demonstrated efficacy of BCG in prevention of TB disease (WHO, 1972). In 1974, this was followed by a World Health Organisation (WHO) recommendation for universal BCG vaccination resulting in incorporation into the expanded program for infant immunization (EPI). Currently, the WHO recommends administration of BCG soon after birth in TB endemic settings, where the risk of exposure to *M.tb* infection is high (WHO, 2004). To date, over 80 percent coverage of BCG vaccination has been achieved in countries where the vaccine is used (WHO, 2013a). Approximately 100 million doses of BCG vaccine are administered worldwide each year.

1.3.1. BCG use in Uganda

The Uganda national expanded program on immunization (UNEPI) guidelines recommend that BCG be routinely administered soon after birth (UNEPI, 2003). It is practical to administer BCG to newborn babies while still in the health care center where birth takes place. However, about 40% of Ugandan infants are born at home (Fadnes et al., 2011, Nankabirwa et al., 2011), resulting in a missed opportunity to administer BCG. Infants who miss BCG vaccination at birth commonly receive the vaccine at their first visit to a health center, usually at 6 weeks of age, for further expanded program on immunization (EPI) vaccination (WHO, 2004). Therefore, a large number of Ugandan infants born at home receive BCG at 6 weeks of age. It remains unclear how administration of BCG at 6 weeks of age may affect vaccine-induced immunity, when compared with infants who receive BCG shortly after birth. This is the focus of this project.

1.3.2. Efficacy of BCG in prevention of TB disease

Multiple clinical trials have assessed efficacy of BCG in prevention of TB disease. In 1930, a Norwegian trial involving student nurses showed 80% efficacy of BCG

in the prevention of TB disease (Aronson, 1948). Subsequently, in the United Kingdom, a clinical trial involving 14-year-old tuberculin skin test (TST)-negative individuals showed a 78% efficacy of the Danish strain of BCG in prevention of TB disease (Hart and Sutherland, 1977). No evidence of protection was observed when the Tice strain of BCG was administered to TST-negative individuals in Georgia and Albama, U.S.A. (Comstock and Palmer, 1966). Similarly, in the Chingleput trial conducted in South India, no protection was observed in individuals who received either the Paris (Pasteur) or the Danish strains of BCG (Baily, 1980). Meta-analyses have shown the BCG vaccine is between 73 to 77% effective in protecting infants against severe forms of TB, such as meningitis and military disease (Bonifachich et al., 2006, Trunz et al., 2006, Walker et al., 2006). However, the vaccine's efficacy in preventing childhood pulmonary tuberculosis varies in different settings, ranging from 0 to 80% (Fine, 1995). A meta-analyses of studies indicated that BCG vaccination on average is 50% effective in prevention of TB in adults previously vaccinated at birth (Brewer, 2000). Also, Mangtani et al., in a recent systematic review of randomized controlled trials showed that absence of prior infection with *M.tb* or environmental mycobacteria was associated with higher BCG efficacy against pulmonary TB in all ages (Mangtani et al., 2014). There are various other factors that have been put forward to explain this variability, which are discussed in greater detail below.

1.3.2.1. Vaccine strain

The original BCG vaccine strain developed by Calmette and Guerin was distributed to laboratories all over the world. Since then, several genetically different BCG strains have evolved through culturing of the original BCG strain under different conditions (Behr et al., 1999). Compared to *M. bovis* and *M. tuberculosis*, all BCG strains lack the region of deletion one (RD1), which appears to be important in mycobacterial pathogenesis. The vaccine strains obtained from the Pasteur Institute before 1926 (BCG-Japan and BCG-Moreau)

have two copies of the IS6110 insertion, compared with one copy of this sequence found in strains obtained from the same institute after 1931 (BCG-Denmark, BCG-Tice and BCG-Glaxo). In addition, the latter strains lack the region of difference two (RD2). Currently, there are more than 20 different BCG vaccine strains in use worldwide (Ritz and Curtis, 2009). The most widely used BCG strains are BCG-Denmark (Statens Serum Institute, Denmark), BCG-Russia (Bulbio, Bulgaria) and BCG-Japan (Japan BCG Laboratory) (Stefanova et al., 2003). Although not clear, it is possible that efficacy of the BCG vaccine in protection against TB may be partly attributed to these strain differences (Milstien and Gibson, 1990). Currently, there are insufficient data to suggest that different BCG strains could result in differential efficacy in prevention of TB disease (Brewer and Colditz, 1995). Aronson et al. demonstrated a lower protective efficacy of BCG-Phipps (44%), compared with BCG-Pasteur (59%) in a randomized controlled trial involving 3025 children and adults followed up for 50 years (Aronson et al., 2004). The vaccine strains that were used by Aronson et al., study are not currently in use.

1.3.2.2. Environmental mycobacteria

In animal studies, exposure to environmental mycobacteria was shown to confer a degree of protection against subsequent challenge with *M.tb* (Young et al., 2007). Furthermore, in clinical studies, individuals with evidence of exposure to environmental mycobacteria show some level of protection against TB (Edwards et al., 1973). Moreover, several species of environmental mycobacteria share antigens with the BCG vaccine (Tortoli, 2003). Therefore, prior exposure to environmental mycobacteria may lead to diminished protection conferred by subsequent BCG vaccination (Brandt et al., 2002). Furthermore, Flaherty et al. demonstrated that, in mice, exposure to environmental mycobacteria post BCG vaccination resulted in diminished vaccine-induced immunity to *M.tb* (Flaherty et al., 2006). In areas closer to the equator, prevalence of environmental mycobacterial infection is high (Comstock et al., 1974): in these areas BCG

appears to have a lower efficacy, compared with temperate regions (Fine, 1995). For example, Palmer, et al. demonstrated that guinea pigs previously exposed to *M. fortuitum*, *M. avium* or *M. kansasii* had reduced protection imparted by BCG (Palmer and Long, 1966). Also, in the Chingleput trial conducted in South India, an area with a high prevalence of environmental mycobacteria exposure, no protection was observed in individuals who received either the Paris (Pasteur) or the Danish strains of BCG (Baily, 1980).

1.3.2.3. Genetic variability of different populations

Multiple clinical studies have assessed the association of several genes controlling cellular immune mechanisms and susceptibility to development of TB (Bellamy et al., 1998, Brahmajothi et al., 1991, Goldfeld et al., 1998). A clinical study by Stead, et al. conducted in the USA showed infection occurred twice as often in black as in white individuals who had equal exposure to active TB in nursing homes (Stead 1990). Also, it is postulated that population genetic differences might explain the performance of BCG. In the United States Public Health Service (USPHS) trials, slightly higher BCG-induced protection against TB was observed among black, compared with white participants (Comstock and Palmer, 1966). Genetic differences in immune genes may underlie population-wide differences, as shown by Randhawa et al., who demonstrated an association between polymorphisms in TLR6 and altered innate immune responses to *M.tb* and adaptive immune responses to BCG (Randhawa et al., 2011).

1.3.3.4. Operational factors

BCG requires storage at 4^0C prior to administration (Lawn et al., 2009). A break in the cold chain may compromise the viability of the vaccine. In resource poor countries, it is often challenging to maintain the cold chain during vaccine transport (Samant et al., 2007). Although clever innovations such as heat

stabilization of vaccines have been introduced, it is likely that this remains a significant factor in delivery of a quality vaccine product.

1.3.4. Efficacy of BCG in prevention of *M.tb* infection

Mathematical models have demonstrated that prevention of transmission would have the greatest impact on the TB epidemic (Dye et al., 2013); therefore, vaccination aimed at reducing *M.tb* infection may have the same effect as prevention of disease. Results from various uncontrolled studies suggest that BCG may protect against *M.tb* infection (Eriksen et al., 2010, Young and O'Connor, 2005). In study conducted in Turkey among 979 children who were household contacts of adults with active TB disease, BCG vaccination was associated with a 24% protection against infection with *M.tb* (Soysal et al., 2005). Eisenhunt, et al. demonstrated a 38% effectiveness of prior BCG vaccination in prevention of *M.tb* infection, when 199 pupils exposed to a 9-year-old pupil with active pulmonary tuberculosis were evaluated (Eisenhut et al., 2009). However, in a study conducted in the Gambia among household contacts of adults with active tuberculosis, geographical proximity and the degree of shared activities predicted the risk of a positive TST rather than presence of BCG vaccination (Lienhardt et al., 2003). This indicates that multiple factors may confound the ability of BCG to protect against *M.tb* infection.

1.4. New vaccination approaches for prevention of TB

The main focus of new vaccines for prevention of TB is to induce a stronger cellular immune response compared with BCG. There are two main strategies that are being used to achieve this goal. The first is to replace BCG with improved recombinant live BCG constructs or an attenuated strain of *M.tb* as a prime vaccine. The second strategy aims at enhancing the protective efficacy of BCG using a prime-boost subunit vaccine administered after BCG vaccination.

1.4.1. Vaccines aimed at replacing BCG

Among the approaches aimed at substituting the BCG vaccine is the

development of an attenuated strain of *M.tb* having deletions in two or more independent virulence genes (Larsen et al., 2009, Martin et al., 2006). An example of such a vaccine is the *M. tuberculosis* SO2 vaccine. In animal models, a high dose aerosol challenge of *M. tuberculosis* SO2 vaccinated guinea pigs resulted in superior levels of protection when compared with BCG vaccination, as measured by guinea pig survival and reduction in disease severity in the lung (Martin et al., 2006).

A second approach aimed at replacing the BCG vaccine involves use of non-pathogenic mycobacteria. An example is *Mycobacteria w*, which has been used for immunotherapeutic intervention against TB. The vaccine is designed to enhance *M.tb* specific T cell responses, thus potentiating the efficacy of standard chemotherapy. In patients with active TB, improved rates of cure was reported when standard chemotherapy was combined with the vaccine (Patel and Trapathi, 2003). Another whole cell preparation based vaccine candidate is *Mycobacterium vaccae* (*M. vaccae*). In a randomised controlled efficacy trial conducted in Tanzania, five doses of *M. vaccae* showed an overall efficacy of 39% in protection against TB disease (von Reyn et al., 2010).

The third rational approach aimed at replacing BCG is the generation of a recombinant BCG vaccine that overexpresses key genes. The rBCG**D**ureC:Hly vaccine (VPM1002) expresses the listeriolysin enzyme of *Listeria monocytogenes*. This enzyme enables BCG to escape from the phagosome into the cytoplasm. This could result in cross-priming of $CD8^+$ T cells via the MHC class I pathway (Winau et al., 2006). In addition, this vaccine is urease C-deficient, allowing for optimal acidification of the phagosome containing the mycobacterium, and therefore presumed enhanced antigen processing. The VPM1002 vaccine is currently being evaluated in a Phase IIa clinical trial in South African infants. Another recombinant BCG vaccine, rBCG30, is engineered to overexpress the immunodominant Ag85B antigen from *M.tb* (Horwitz et al., 2000). A phase I clinical trial in adults randomized to receive either rBCG30 or

parenteral Tice BCG showed that rBCG30 was safe and significantly increased the number of Ag85b-specific T cells capable of inhibiting intracellular mycobacteria (Hoft et al., 2008). The third BCG recombinant vaccine, Aeras 422 was engineered to expresses a pore-forming bacterial molecule perfringolysin. The phase I trial of Aeras 422 was discontinued following adverse effects in phase I trials (Kaufmann, 2012).

1.4.2. Vaccines aimed at boosting BCG immune responses

An alternative strategy to replacing the BCG vaccine is to administer a booster vaccine aimed at enhancing or modulating BCG-induced immunity. This is achieved through use of a protein vaccine co-administered with an adjuvant to induce high levels of cellular immunity. An example of such a vaccine is M72, which consists of the fused *M.tb* proteins Mtb32 and Mtb39 formulated in the AS01 adjuvant (Brandt et al., 2004, Leroux-Roels et al., 2013). A second subunit vaccine, Hybrid I, is a fusion protein of two secreted antigens: the early secreted antigenic target 6 (ESAT-6) and antigen 85B (Ag85B). However, the inclusion of ESAT-6 in Hybrid I subunit vaccine confounds the use of interferon gamma release assays (IGRAs) for the diagnosis of latent *M.tb* infection (Pai et al., 2004). The HyVac4 subunit vaccine exchange ESAT-6 for TB10.4. Therefore unlike Hybrid 1, the HyVac4 vaccine maintains the advantage of allowing for the use of ESAT-6-specific diagnostic tests in participants vaccinated with the vaccine. The fourth subunit vaccine candidate currently in clinical trial is H56. Similar to Hybrid 1, this vaccine comprises Ag85B and ESAT-6 antigens in addition to a latency-associated protein Rv2660c. The H56 vaccine has a unique advantage in its potential ability to target dormant *M.tb* bacilli.

The second approach in the boost strategy involves the use of a recombinant viral vector expressing key *M.tb* antigens. One such vaccine is the adenovirus 35 based Aeras 402, which expresses a fusion protein of the mycobacterial antigens Ag85A, Ag85B, and TB10.4. The Aeras 402 vaccine is immunogenic and protective in mice and non-human primates (Magalhaes et al., 2008, Radosevic

et al., 2007). In a phase I clinical trial conducted in South Africa among BCG-vaccinated adults, this vaccine was shown to be safe and to induce polyfunctional $CD4^+$ and $CD8^+$ T cells (Abel et al., 2010). The modified vaccinia virus Ankara (MVA) expressing antigen 85A from *M.tb* is another viral-vectored vaccine candidate. This vaccine is composed of a safe replication-deficient strain of vaccinia virus, as a delivery system for the mycobacterial antigen Ag85A. In phase I and phase IIb clinical trials, this vaccine candidate was shown to be safe and highly immunogenic (McShane et al., 2004, Scriba et al., 2012). MVA85A induced a sustained antigen-specific multifunctional cytokine producing $CD4^+$ and $CD8^+$ T cells. However in a recently completed phase IIb clinical trial of healthy South African infants, no efficacy against TB disease or *M.tb* infection was observed with this vaccine (Tameris et al., 2013).

1.5. Immune control of *M.tb*

The great majority (90%) of persons infected with *M.tb* do not develop active TB during their lifetime. This indicates that most individuals' host response is able to control the replication of the bacilli. Our understanding of this immunological control of *M.tb* infection remains incomplete. A more comprehensive understanding of the role played by different components of the immune system is needed for rational design of vaccines and therapeutic interventions. This section focuses on the different aspects of the immune system involved in the control of *M.tb*.

1.5.1. Innate immune response to *M.tb*

Innate immunity refers to the host responses that occur upon first encounter with a pathogen. The cell types that play a role in this frontline of defense against *M.tb* infection include macrophages and dendritic cells. The innate immune arm is non-specific for antigens and lacks immunological memory (Kurtz, 2005). However in a recent study, Kleinnijenhuis et al., showed that BCG vaccination of in healthy volunteers resulted in a twofold-enhanced release of monocyte-derived cytokines, such as TNF and IL-1β (Kleinnijenhuis et al., 2012). This suggests

that mycobacterial components including BCG can induce trained immunity through epigenetic reprogramming of innate cells. In this section, various components and processes that make up the innate host response to *M.tb* are discussed in greater detail.

1.5.1.1. Phagocytosis and recognition of M.tb

In the lungs, *M.tb* is primarily taken up by alveolar macrophages. Subsequently, monocyte derived macrophages and dendritic cells take part in phagocytosis of the inhaled *M.tb* (Henderson et al., 1997). Scavenger receptors mediate uptake of opsonised *M.tb*, while mannose and complement receptors take up non-opsonised *M.tb* (van Crevel et al., 2002). Also, various C-type lectin receptors including DC-SIGN can recognise *M.tb* and initiate phagocytosis of the pathogen (Berrington and Hawn, 2007, Kleinnijenhuis et al., 2011).

Different classes of pattern recognition receptors (PRRs) that play a role in the detection of *M.tb* by phagocytes have been identified (**Figure 1**). These include the Toll-like receptors (TLRs) TLR2, TLR6, TLR9 (Bafica et al., 2005, Berrington and Hawn, 2007), and TLR4 (Means et al., 1999). TLRs are composed of a cytoplasmic domain that is homologous to the signaling domain of IL-1 receptor that links to IL-1R-associated kinase (IRAK) through the adapter molecule MyD88 (Oddo et al., 1998). This serine kinase activates the transcription factors like NF-kb to signal the production of pro-inflammatory cytokine, IL-12 (Brightbill et al., 1999, Oddo et al., 1998). The importance of TLRs in the initial host response to *M.tb* is evidenced by an increased susceptibility to *M.tb* in myeloid differentiation protein 88 (MyD88)-deficient mice (Fremond et al., 2004).

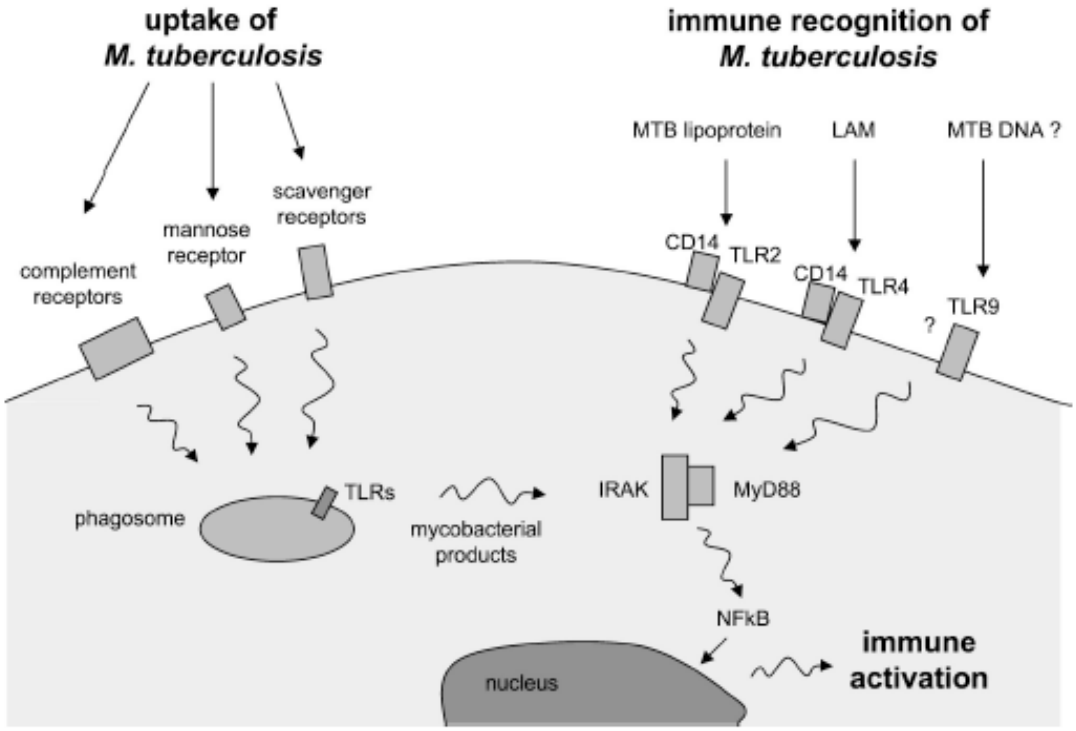

Figure 1: Phagocytosis and immune recognition of *M.tb*. Various receptors for phagocytosis of *M.tb* by macrophages and dendritic cells include the complement receptors Mannose receptors and scavenger receptors. TLRs are pattern recognition receptors that play a central role in immune recognition of M. tuberculosis. Binding of mycobacterial antigens to TLRs results in intracellular signaling pathways lead to cell activation and cytokine production (**Adapted from Van Cravel, *et al* 2002**).

1.5.1.2. Intracellular control of M.tb

Macrophages are the major cells of the immune system involved in killing of *M.tb*. The mechanisms by which the macrophages mediate the growth inhibition or killing of intracellular *M.tb* are discussed in greater detail below.

Phagolysosome fusion: Upon phagocytosis of *M.tb*, the bacilli reside within a phagosome. Subsequently, the phagosome fuses with the lysosome, a complex vacuolar organelle (Kornfeld, 1987). The lysosome contains hydrolytic enzymes that are capable of degrading the phagocytosed microbe (Kornfeld, 1987). Fusion of the phagosome and the lysosome is followed by maturational changes characterised by acidification of the phagolysosome for optimal function of the

hydrolytic enzymes (Desjardins et al., 1994). Upon acidification of the phagolysosome, the lysosomal hydrolytic enzymes degrade the microbe.

Reactive oxygen intermediates: The second mechanism by which macrophages control intracellular *M.tb* is by generation of free radicals. For example, hydrogen peroxide is a reactive oxygen intermediate (ROI) generated via an oxidative burst. ROI are produced by activated macrophages and are mycobactericidal (Walker and Lowrie, 1981). Also, macrophages generate nitric oxide and reactive nitrogen intermediates (RNI) in a nitric oxide synthesase-2 (NOS2) dependent mechanism. In mice, RNI are protective against infection with *M.tb* as demonstrated by increased disease burden in lungs of NOS2 double knockout mice, compared with wild type controls (MacMicking et al., 1997). In humans, patients with active pulmonary TB have increased levels of exhaled NO compared to healthy controls (Wang et al., 1998).

Autophagy: Autophagy is a process in which long-lived cytosolic macromolecules and whole organelles are delivered to lysosomes for degradation. Autophagy also plays a key role in immune responses to *M.tb* (Harris et al., 2009). In an experimental study *in vitro* killing of intracellular bacilli was observed when autophagy was induced in *M.tb*-infected macrophages (Gutierrez et al., 2004). Autophagy is stimulated by the Th1 cytokines TNF-α and IFN-γ and is inhibited by the Th2 cytokines interleukin IL-4, IL-13 (Harris et al., 2007) and the anti-inflammatory cytokine IL-10 (Van Grol et al., 2010). Also, vitamin D induces autophagy via cathelicidin (Roth et al., 2004). Autophagy plays a key role in antigen processing and presentation (Jagannath et al., 2009). Therefore, autophagy enhances the presentation of antigens to T cells and the control of *M.tb* infection. Our understanding of the cellular mechanisms involved in the autophagic processes would be potentially useful in designing vaccines that can induce autophagy to improve control of infection.

1.5.1.3. Cytokines produced by innate cells

IL-12**:** Dendritic cells and macrophages produce IL-12 in response to phagocytosis of *M.tb* (Oppmann et al., 2000). IL-12 drives type 1 T cell (Th1) differentiation important in control of *M.tb*. The importance of IL-12 in immunity towards *M.tb* was demonstrated in animal studies that showed increased bacterial burden and reduced survival time in IL-12p40 gene deficient mice, compared with controls (Cooper et al., 1997). In humans, functional genetic mutations have been identified in the genes encoding IL-12p40 (Remus et al., 2004), IL-12Rβ1 (de Jong et al., 1998) in patients with recurrent or fatal non-tuberculous mycobacterial infections. This indicates that this cytokine is likely important in control of *M.tb*.

IL-6: IL-6 is produced at the site of infection in response to infection with *M.tb* (Law et al., 1996). IL-6 has both anti- and pro-inflammatory properties. For example, this cytokine may inhibit the production of TNF-α (Schindler et al., 1990). In mice, the role of IL-6 in immunity towards *M.tb* is evidenced by an early increase of bacterial burden coupled by a delay in the production of IFN-γ following aerosol challenge with *M.tb*, in IL-6 double knockout mice compared to controls, indicating the importance of this cytokine in early infection with *M.tb* (Saunders et al., 2000).

IL-1: IL-1 is another pro-inflammatory cytokine produced by macrophages, monocytes and dendritic cells during infection with *M.tb* (Dahl et al., 1996) at the site of disease (Law et al., 1996). In mice, IL-1α and IL-1β double knock out mice have increased *M.tb* growth and impaired granuloma formation compared to the wild type (Yamada et al., 2000). Further more, mice lacking IL1-receptor type I have increased disease burden compared to the wild type indicating the importance of this cytokine in protection against TB (Juffermans et al., 2000).

IFN-γ: Natural killer (NK) cells, alveolar macrophages (Wang et al., 1999) and $CD4^+$ and $CD8^+$ T cells produce IFN-γ. The detailed role of this cytokine in control of *M.tb* is discussed in section 1.5.2.3.

TNF-α: TNF-α is another cytokine that plays a key role in immune response to *M.tb*. The Macrophages, dendritic cells, and T cells cytokine produce TNF-α (Henderson et al., 1997, Ladel et al., 1997). The detailed role of this cytokine in control of *M.tb* is discussed in section 1.5.2.3.

IL-10: IL-10 is an anti-inflammatory cytokine important in control of immunopathology resulting from excessive inflammatory response to infection (O'Garra and Vieira, 2007). Macrophages produce IL-10 in response to infection with *M.tb*. This cytokine inhibits production of IL-12 resulting in downregulation of T cell expression of IFN-γ (D'Andrea et al., 1993). Also, IL-10 downregulates surface expression of molecules involved in antigen presentation (Moore et al., 2001). Furthermore, IL-10 produced by macrophages inhibits T cell proliferation (Gong et al., 1996). Although, IL-10 is important for prevention of immunopathology due to excessive inflammatory response, excessive production of IL-10 may result in the inability of the host to control infection. However, in animal studies, mice deficient in IL-10 showed no difference in control of *M.tb* infection compared to the wild type (Jung et al., 2003). In this project, we assessed IL-10 production in 9-month old infants vaccinated with BCG at birth or at 6 weeks of age.

Transforming growth factor-β: TGF-β is another anti-inflammatory cytokine produced by monocytes in response to *M.tb* infection (Toossi et al., 1995). This cytokine inhibits T cell response to *M.tb* (Rojas et al., 1999). In macrophages, TGF-β inhibits IFN-γ induced NOS2 production (Ding et al., 1990).

1.5.1.4. Antigen processing and presentation

Dendritic cells are the most efficient antigen presenting cells (APC), and the bridge between innate and the adaptive immune response (Steinman and Hemmi, 2006). Dendritic cells directly take up *M.tb* or phagocytose dead innate immune cells containing tuberculosis bacilli. Thereafter, the *M.tb* antigens are recognised by PRRs resulting in intracellular signalling with concomitant maturation: upregulation of co-stimulatory molecules (CD80 and CD86), major histocompatibility complex (MHC) class I or class II molecules, and the chemokine receptor CCR7 (Trombetta and Mellman, 2005). Mature DCs are also able to process antigen for ultimate presentation via MHC class I and/or class II molecules (Inaba et al., 2000, Turley et al., 2000). DCs migrate to draining lymph nodes. (Humphreys et al., 2006). In the lymph node, the following events occur during the priming of the naïve T cells: (i) presentation of the cognate peptide on the MHC class I and MHC class II molecules of the dendritic cells to $CD8^+$ and $CD4^+$ T cells, respectively; (ii) provision of co-stimulatory signals through interaction of CD80 (B7-1) and CD86 (B7-2) on the dendritic cell with CD28 on the T cell; (iii) signaling from pro-inflammatory cytokines produced by the dendritic cells such as IL-12 (Curtsinger et al., 1999).

1.5.1.5. Immune evasion by M.tb

Despite the various effector mechanisms aimed at growth inhibition or killing of the intracellular *M.tb*, the pathogen may survive and continue replicating within the macrophage. *M.tb* has developed several strategies to circumvent the host immune responses thereby enabling the pathogen to survive within the macrophage. For example, *M.tb* can prevent fusion of the phagolysosome (Hart et al., 1972) and the maturation of the phagolysosome (Sturgill-Koszycki et al., 1994). In addition, *M.tb* produces two enzymes, urease (Clemens and Horwitz, 1995) and glutamine synthases (Harth and Horwitz, 1999), which prevent acidification of the phagolysosome therefore preventing the optimal function of the hydrolytic enzymes. The *M.tb* component lipoarabinomannan has neutralising

properties for oxygen radicles (Chan et al., 1991). Finally, *M.tb* preferentially induces production of IL-10 which inhibits TNF-α mediated apoptosis of the macrophage. Apoptosis of the macrophage results in the death of the intracellular *M.tb* thus limiting the spread of the pathogen (Molloy et al., 1994). Therefore, by inhibiting apoptosis, *M.tb* is able to survive and infect other APCs.

1.5.2. Adaptive immune response to *M.tb*

The presentation of the mycobacterial antigens to the T cell results in priming of $CD4^+$ and $CD8^+$ T cells. Upon successful activation, T cells differentiate, undergo clonal expansion and develop effector function (Macatonia et al., 1995). Subsequently, the differentiated T cells migrate from the lymph nodes towards the site of infection to execute their effector functions (Mempel et al., 2004). The adaptive immune response is specific for *M.tb* and is characterized by development of immunological memory. In this section, cells and effector molecules involved in specific immune responses to *M.tb* are discussed in greater detail.

1.5.2.1. Classically restricted T cells

$CD4^+$ T cells: In mice, the importance of $CD4^+$ T cells in a protective immune response against *M.tb* is supported by increased mortality rates observed in *M.tb* infected $CD4^+$ T cell deficient mice, compared with wild type mice (Caruso et al., 1999). *M.tb* infected cynomolgus monkeys treated with anti-$CD4^+$ T cell antibodies showed increased pathology and bacterial burden in comparison to the controls (Chen 2012). Furthermore, an association between reactivation of TB in cynomologus monkeys with LTBI and co-infected with SIV correlated with depletion of peripheral $CD4^+$ T cell counts (Diedrich et al., 2010). In HIV-infected humans, progression to TB disease correlates with $CD4^+$ T cell decline (Aaron et al., 2004, Lawn et al., 2009).

Specific $CD4^+$ T cells recognise *M.tb* peptides on the MHC class II molecule of APCs, which may result in multiple effector functions, including production of Th1 cytokines such as IFN-γ, TNF-α and IL-2. $CD4^+$ T cells may also produce cytotoxic molecules in response to *M.tb* antigens (Bastian et al., 2008). Canaday et al., reported upregulation of mRNA expression for granzyme A and B, granulysin and perforin in $CD4^+$ T cells from healthy tuberculin skin test (TST) individuals following incubation of PBMC with *M.tb*. Moreover, $CD4^+$ T cells from these individuals lysed *M.tb* infected monocytes *in vitro* (Canaday et al., 2001).
As $CD4^+$ T cells are important in immune response to *M.tb*, we planned to investigate this subset in assessment of BCG induced immunity in infants vaccinated at birth or at 6 weeks of age.

$CD8^+$ T cells: In animal models, the importance of $CD8^+$ T cell in immunity against *M.tb* is demonstrated by the lesser control of *M.tb* infection in mice lacking MHC I molecules, compared with wild type mice (van Pinxteren et al., 2000). In BCG-vaccinated rhesus macaques, $CD8^+$ T cells depletion results in compromised BCG-induced immune control of *M.tb* replication. Further, in the rhesus macaques previously infected with *M.tb* and cured by chemotherapy, depletion of $CD8^+$ T cells resulted in increased risk of development of active TB upon re-infection with *M.tb* (Chen 2009). In humans, Bruns et al., reported decreased antimicrobial activity mediated by effector memory $CD8^+$ T cells in patients receiving anti-TNF-α treatment for rheumatoid arthritis, suggesting that $CD8^+$ T cells contribute to host defense against tuberculosis. (Bruns et al., 2009). The $CD8^+$ T cells recognise cytosolic antigens loaded as peptides onto MHC class I molecules of APCs (Bachmann et al., 1996).

The $CD8^+$ T cell effector function is mediated through production of cytolytic molecules such as granzymes, granulysin and perforin (Woodworth et al., 2008, Canaday et al., 2001). In addition, $CD8^+$ T cells produce the Th1 cytokine IFN-γ (Vesosky et al., 2009), among others. Given the putative role of $CD8^+$ T cells in

control of *M.tb*, we therefore planned to investigate this subset in assessment of BCG induced immunity in infants vaccinated at birth or at 6 weeks of age.

1.5.2.2. Non-classically restricted T cells

Gamma delta (γδ) T cells: γδ T-cells are a subset of lymphocyte that express γ and δ chains as the T cell receptor on their surface, compared with the conventional $CD4^+$ and $CD8^+$ T cells which express a T-cell receptor composed of a dimer of α and β protein chains. γδ T-cells are activated by non-peptide phosphoantigens, are not restricted by classical MHC molecules and may respond rapidly upon activation (Hayday, 2000, Holtmeier and Kabelitz, 2005). The Vγ9Vδ2 subset of Adult γδ T-cells are known for their capacity to express cytotoxic molecules such as perforin and granzymes, and their high production of the Th1 cytokine, IFN-γ upon in vitro stimulation. Spencer, et al. in a clinical study demonstrated that granzyme A produced by γ9δ2 T cells was essential for TNF-α production by monocytes resulting in control of intracellular mycobacterial growth (Spencer et al., 2013). In viral models, functional fetal γδ T cell responses have been shown to be present as early as 21 weeks *in utero*, suggesting an important role of these cells against infection in the prenatal period (Vermijlen et al., 2010). In a clinical study, specific functional γδ T cell responses have been demonstrated in adults following BCG vaccination (Hoft et al., 1998, Spencer et al., 2008). Furthermore, Vermijlen et al. showed that γδ T cells (especially the $Vγ9^+$ subset) from PBMC derived from South African 10 week-old infants vaccinated at birth with BCG, showed greater capacity to proliferate compared with cord blood PBMC from infants. In addition, the γδ T cells from these 10-week old infants showed high expression of cytotoxic markers (perforin, granzyme A, granzyme B, granulysin), were more highly differentiated, and expressed more IFN-γ and TNF-α upon stimulation with BCG (David Vermijlen, unpublished data). Although γδ T cells contribute to immunity towards *M.tb*, our focus in this project was on BCG specific αβ T cell subsets, for practical reasons.

NKT cells (CD1-restricted ab T cells): The natural killer T (NKT) cell subset express the α and β receptor dimer, are stimulated by mycobacterial lipids and are restricted by the CD1 molecule. CD1 is an MHC-like molecule composed of the a chain and non-covalently bound to β_2-microglobulin (Zeng et al., 1997). Mycobacterial lipids stimulate CD1-restricted cells to produce cytokine and cytolytic molecules (Stenger et al., 1997). In experimental studies using guinea pigs, an enhanced CD1-restricted ab T cell response was observed in BCG vaccinated animals, compared with the placebo group (Hiromatsu et al., 2002). Also, a clinical study, PPD-positive individuals had higher mycobacterial lipid specific CD-1 restricted proliferative response, compared with PPD-negative controls. This indicates the presence of memory *M.tb*-specific CD1-resticted ab T cells (Ulrichs et al., 2003). In this project our focus was on BCG-specific conventional ab T cell subsets. Therefore we did not assess for BCG induced CD1-resticted ab T cells, again, for practical reasons.

Mucosal associated invariant T cells (MAIT): Mucosal associated invariant T cells (MAIT) constitute up to 4% of peripheral blood T cells. These cells are characterized by the expression of Vα7.2/Jα33 TCR (Martin 2009). MAIT cells are frequent in the lungs and may be important as first line defense during primary infection with *M.tb*. Lewinsohn, et al. in a clinical study showed that a proportion of MAIT $CD8^+$ T cells are restricted by MHC-related molecule 1 (MR1); *M.tb* induced increased expression of MR1 on the surface of lung epithelial cell. Finally, MAIT cells recognize lung epithelial cells infected with *M.tb* (Lewinsohn 2000). However, the *M.tb*-reactive MAIT cells are non-specific for *M.tb* and can detect cells infected with other pathogenic organisms. In this project we did not assess MAIT cells, for practical reasons.

1.5.2.3. Th1 cytokines

IFN-γ: IFN-γ is a key effector cytokine in immune response to *M.tb*. In an experimental study where mice were vaccinated with BCG and boosted with

modified vaccinia Ankara expressing-antigen 85A (MVA85A), followed by an *M.tb* challenge, frequencies of Ag85A-specific IFN-γ-expressing T cells were positively correlated with reduced bacterial load in the lungs, supporting the role of IFN-γ in immunity towards *M.tb* (Goonetilleke et al., 2003). In addition, humans with mutations in genes associated with IFN-γ production or signaling show an increased risk of disease due to mycobacterial disease (Bogunovic et al., 2012, Newport et al., 1996, Ottenhoff et al., 2000).

During induction of *M.tb* immunity, IL-12 is produced by antigen bearing APCs to drive Th1 differentiation and IFN-γ production by T cells (Manetti et al., 1994). IFN-γ promotes Th1 differentiation, production of IL-12, and activation of APCs through a positive feedback mechanism. In addition, IFN-γ inhibits the differentiation of naïve $CD4^+$ T cells to Th2 subsets. Furthermore, IFN-γ enhances the initiation and amplification of T cell-dependent immune response by stimulating expression of MHC molecules and B7 co-stimulatory molecules on APCs (Zhou, 2009). In the event of *M.tb* exposure and infection, it is proposed that BCG-specific IFN-γ produced by T cells activates infected macrophages (**Figure 2**), to control intracellular *M.tb* directly. (Herbst et al., 2011).

TNF-α: The role of TNF-α in control of *M.tb* has been shown by increased bacterial burden and delayed granuloma formation in mice deficient for the 55 kDa TNF-α receptor or following anti-TNF-α monoclonal antibody (mAb) administration (Bean et al., 1999). In humans, the role of mycobacteria-specific TNF-α in *M.tb* immunity has been shown by an increased risk of TB reactivation in rheumatoid arthritis patients receiving anti TNF-α treatment (Nacci and Matucci-Cerinic, 2011, Wallis, 2007, Keane et al., 2001). In TB immunity, TNF-α enhances chemotaxis of APCs such as, neutrophils and DCs to the site of infection. Furthermore, TNF-α supports formation and maintenance of granulomas (Algood et al., 2005).

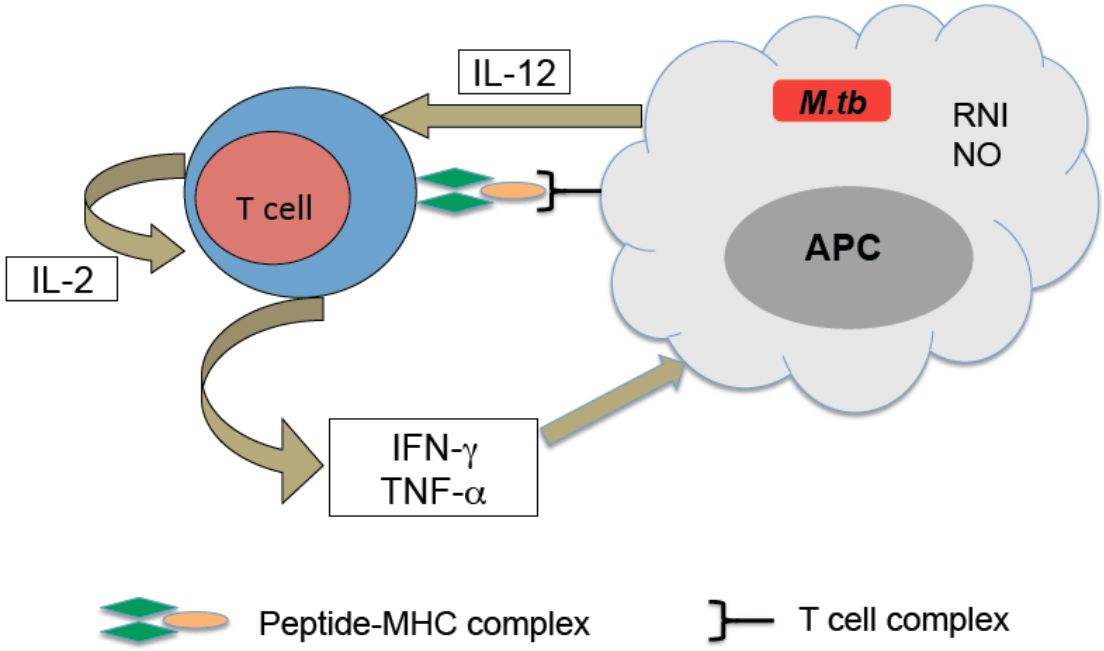

Figure 2. A simplified model of the *M.tb* induced immune response: (A) Mycobacterial peptides are presented on MHC molecules of a BCG infected APC to the T cell receptor leading to activation of the T cell. In addition, IL-12 produced by the infected macrophage provides signals to further activate the T cell, which then produces IFN-γ. This cytokine in turn activates the infected macrophage. The activated macrophage produces reactive oxygen intermediates (ROI) and nitric oxide (NO), which can directly kill the intracellular bacillus.

1.5.2.4. Th17 cytokine

IL-17 is a pro-inflammatory cytokine (Harrington et al., 2006). The role of IL-17 in TB immunity is supported by an experimental *M.tb* challenge mouse model that demonstrated IL-17 associated enhanced protection against TB development via recruitment of neutrophils and monocytes to the lungs, with concomitant granuloma formation in wild type mice compared to IL-17 KO mice (Umemura et al., 2007). Additionally, IL-17 has been shown to enhance recruitment of the Th1 cells to the lungs in mice (Khader et al., 2007, Wozniak et al., 2006).

1.5.2.5. Th2 cytokines

High levels of Th2 cytokines may attenuate Th1 immune responses and impede induction of anti-mycobacterial immunity (Rook, 2007). The Th2 cytokines, IL-4 and IL-13, cause alternate macrophage activation, which may negatively impact the generation of protective immunity against *M.tb* (Martinez et al., 2009). Indeed, Elias et al., reported a decrease in Th2 cytokine levels, as well as enhanced PPD-specific IFN-γ expression, in individuals who received anti-helminth medication compared to the untreated controls (Elias et al., 2001).

1.5.2.6. T regulatory cytokines

Regulatory T cells (Tregs) are important in regulation of immune responses to prevent immunopathology (Sakaguchi, 2005). Tregs produce IL-10 and transforming growth factor (TGF)-β, both of which inhibit Th1 and cytotoxic responses (Fiorentino et al., 1991, O'Garra and Vieira, 2004). In addition, increased levels of IL-10 and TGF-β have been reported in the lungs of TB patients, compared with controls (Bonecini-Almeida et al., 2004).

1.5.2.7. Cytotoxic T cell function

Cytotoxic T lymphocytes (CTLs) mediate their effector functions through the death receptor pathways (Kagi et al., 1994). Furthermore, CTL and NK cells secrete cytolytic molecules such as perforin, granulysin and granzymes that are important in the control of intracellular infections. *In vitro* studies have demonstrated that cytotoxic $CD8^+$ T cells (Turner and Dockrell, 1996) as well as $CD4^+$ T cells (Canaday et al., 2001) play a cytolytic role in immunity against *M.tb*.

Perforin: The proposed role for perforin in mediating immunity against *M.tb* involves perforation of cell membrane of an infected cell to permit entry of the cytolytic granzymes that directly kill *M.tb* or the infected cell (Thiery et al., 2011). Supporting the role of perforin in TB immunity is a study by Rahman *et al.* that

showed an association between frequencies of perforin-expressing cells and protection when non-human primates vaccinated with a novel TB vaccine combination (rBCG/rAD35) and subsequently challenged with *M.tb* (Rahman et al., 2012). Furthermore, in a clinical study, Andersson *et al*, demonstrated decreased mRNA expression levels of perforin in lung lesions from patients with pulmonary TB, compared with those from uninfected controls (Andersson et al., 2007), suggesting that perforin-mediated cytotoxicity is an important aspect of T cell immunity (Stenger and Modlin, 1998).

Granulysin: Granulysin is a cytotoxic and proinflammatory molecule belonging to the saposin-like family of lipid binding proteins. Granulysin is found in association with perforin in cytolytic granules of CTLs and NK cells (Pena and Krensky, 1997, Clayberger and Krensky, 2003). This molecule has a broad cytolytic activity against tumors cells and microbes including *M.tb* (Stenger and Modlin, 1998). The cytotoxic effect of granulysin is dependent on perforin (Stenger and Modlin, 1998). The mechanism of granulysin cytotoxicity involves disruption of the target cell membrane leading to influx of extracellular calcium (Kaspar et al., 2001), the release of cytochrome c and the apoptosis-inducing factor (AIF), leading to the death of the target cell (Okada et al., 2003). In addition, granulysin has chemoattractant properties for T cells and monocytes (Deng et al., 2005). In a clinical study, patients with active TB showed decreased serum levels of granulysin, compared with healthy controls, suggesting the importance of granulysin in protective immunity against *M.tb* (Di Liberto et al., 2007). Furthermore, Klucar *et al*, demonstrated perforin and granulysin dependent lysis and growth inhibition of *M.tb* by cytolytic $CD4^+$ T cells (Klucar et al., 2008).

Granzymes: Granzymes are serine proteases that are contained in cytoplasmic granules of CTLs. In humans, 5 granzymes encoded by 5 genes have been described; grzA, grzB, grzK, grzH, and grzM. Among these, grzA and grzB are well characterised and associated with induction of apoptosis in the target cell (Zhang 2001). Perforin is essential for translocation of granzymes A and B to the

nucleus to induce apoptosis of the target cell. Granzyme A induces apoptosis through stimulating the generation of ROS resulting in DNA damage and cell death (Chowdhury and Lieberman, 2008). Granzyme B directly activates executioner caspases 3 and 7, which results in apoptosis of the target cell (Thomas et al., 2000). Healthy individuals from households of patients with TB disease demonstrate greater levels of granzymes A and B in blood stimulated with CFP-10 peptides, compared with the patients themselves (Madhan Kumar and Raja, 2010).

1.6. Granuloma formation

Formation of the granuloma begins with the migration of the *M.tb*-specific T cells from the regional lymph nodes to the infection site, in the lungs. At the infection site, *M.tb* specific T cells interact with infected macrophages. This results in the production of proinflammatory cytokines resulting in recruitment of additional mononuclear cells and T cells leading to formation of a granuloma (Davis and Ramakrishnan, 2009, Kaufmann, 2001). The granuloma, the hallmark of *M.tb* infection, is a well organised structure consisting of a central area with infected macrophages, giant foamy cells and Langhans cells, surrounded by T cells and B cells (Russell et al., 2009). The granuloma enables containment of the pathogen (Walzl et al., 2011). In the granuloma, the bacilli may be killed or prevented from replicating thereby persisting in a latent state. In 5% of cases, the intracellular *M.tb* bacilli continue to replicate within the granuloma causing death of the infected macrophages. This results in formation of caseous necrotic area at the center of the granuloma. The destruction due to necrosis may spread to the rest of the granuloma resulting in lung tissue damage and cavitation. Dissemination of the *M.tb* bacilli may occur when the cavity erodes into a blood vessel.

1.7. Immune correlates of protection

Correlates of protection could be defined as markers that predict protection against TB disease when vaccinated persons are compared with unvaccinated

control persons. In the presence of a known partially efficacious vaccine like BCG, it is not possible to conduct placebo-controlled trials to identify correlates of protection for ethical reasons. We therefore use the term correlates of risk of developing TB disease. (Qin et al., 2007). Our knowledge of the immune correlates for protection against development of TB remains limited. Therefore, in TB vaccine trials, we measure markers of immune responses that are critical for protection against *M.tb*, e.g., Th1 cytokines production. In an experimental study, boosting BCG-primed mice with a recombinant adenovirus expressing *M.tb* Ag85A resulted in greater median fluorescence intensities of polyfunctional $CD4^+$ T cells, expressing multiple Th1 cytokines together, that correlated with protection against development of TB (Forbes et al., 2008). However, in a clinical study conducted in South Africa, the frequency of polyfunctional $CD4^+$ T cells expressing multiple Th1 cytokines together, did not correlate with the risk of development of TB (Kagina et al., 2010). This may be explained by the presence of other unknown function of $CD4^+$ T cells that may be important in protection against TB other that production of IFN-γ and TNF-α (Gallegos et al., 2011).

1.8. BCG-induced immunity

1.8.1. BCG induced cytokine response

Although the correlates of protection against TB are not known, we proposed to investigate $CD4^+$ and $CD8^+$ T cell immunity thought to be critical for control of *M.tb*. In infants and adults, previous studies have reported that vaccination with BCG at birth mainly induces specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ (Hoft et al., 2012, Murray et al., 2006, Smith et al., 1999, Soares et al., 2008). Also, neonatal BCG vaccination induces specific TNF-α production by both $CD4^+$ and $CD8^+$ T cells (Lalor et al., 2011, Soares et al., 2008). We therefore evaluated the frequencies of BCG-specific IFN-γ and TNF-α expressing $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth and 6 weeks of age.

Moreover, neonatal BCG vaccination also induces IL-2, another Th1 associated cytokine (Smith et al., 1999, Soares et al., 2008). Antigen experienced T cell

memory pools require IL-2 for maintenance and expansion. The mechanism involves IL-2 induced production of the anti-apoptotic marker, Bcl-2 (Miyazaki et al., 1995). In addition, IL-2 enhances production of the Th1 cytokine, IFN-γ. A study done in our laboratory showed measurable frequencies of BCG-specific IL-2, produced by conventional T cells in 10 week-old infants, vaccinated with BCG at birth (Kagina et al., 2009). We therefore investigated whether frequencies of BCG-specific IL-2 expressing $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth would be greater than those of infants vaccinated at 6 weeks of age.

BCG vaccination also induces IL-17 producing $CD4^+$ T cells. In a clinical study conducted by our laboratory, BCG-specific IL-17 producing $CD4^+$ T cells were detected in healthy adults, previously vaccinated with BCG at birth (Scriba et al., 2008). Kagina et al. also showed that BCG induces IL-17-producing T cells (Th17 cells) (Kagina et al., 2010). In this project, we were therefore interested in establishing whether frequencies of BCG-specific IL-17 $CD4^+$ and $CD8^+$ T cells would be greater when the vaccine is administered at birth than at 6 weeks of age.

BCG is an intracellular organism that induces primarily Th1-mediated immune responses. However, studies conducted among infants in Malawi reported PPD-induced IL-4 production in BCG vaccinated infants. (Lalor et al., 2010). Furthermore, PPD-induced IL-13 production among BCG vaccinated infants was reported in this study, as well as in BCG vaccinated infants from the Gambia (Lalor et al., 2010, Burl et al., 2010).

Also, BCG vaccination induces perforin producing $CD4^+$ T cells. Semple *et al*, demonstrated BCG-induced cytotoxic T cells (Tc) producing perforin in 10-weeks infants (Semple et al., 2011). We therefore compared BCG-specific perforin-expressing $CD4^+$ and $CD8^+$ T cells when the vaccine is administered at birth and 6 weeks of age based on evidence showing that this cytotoxic molecule is induced in infants following the administration of the vaccine (Semple et al.,

2011). We used CD69 antibody staining to identify BCG-activated, perforin expressing $CD4^+$ and $CD8^+$ T cells.

In animal studies, BCG vaccination has been shown to induce granulysin-producing $CD4^+$ T cells (Endsley et al., 2004). Semple et al., showed that BCG vaccination of newborns does induce increased production of granulysin-producing $CD4^+$ and $CD8^+$ T cells (Semple et al., 2011). However, in this project, our focus was not on assessing BCG-specific granulysin expressing $CD4^+$ and $CD8^+$ T cells in our study participants.

1.8.2. BCG induced antibody responses

Evidence from animal studies indicates that BCG vaccination results in detectable levels of lipoarabinomannan (LAM)-specific immunoglobulin (Ig) G (Watanabe et al., 2006, Maglione et al., 2008). Furthermore, Brown *et al* in a clinical study demonstrated elevated LAM-specific antibodies in BCG vaccinated individuals (Brown et al., 2003, de Valliere et al., 2005), indicating that BCG induces specific antibody (Ab) responses. In addition, BCG vaccination has been shown to induce long-lived memory B cells in individuals lacking ESAT-6 and CFP-10 responses (Sebina et al., 2012). A clinical study by de Valliere, et al. investigating the role of BCG-induced Ab responses in serum samples from BCG vaccinated volunteers showed enhanced phagocytosis of BCG in post vaccination samples, enhanced growth inhibitory effects of neutrophils and monocytes by BCG-induced antibodies, as well as an enhanced proliferative and IFN-γ production by specific $CD4^+$ and $CD8^+$ T cells compared to the unvaccinated controls (de Valliere et al., 2005). In this project, we did not focus on assessing BCG-specific Ab production.

1.8.3. Memory phenotypes of BCG-specific T cells

Effective vaccines induce long-lived immunity by generating immunological memory (Cellerai et al., 2007, Seder et al., 2008, Miller et al., 2008). Therefore, quantification of BCG-induced memory T cells is important. Completed studies

from our laboratory and others have shown that BCG vaccination induces immunological memory (Soares et al., 2013, Weir et al., 2008).

Different subsets of memory T cells can be defined by evaluating expression of different surface markers. For example, CD45RA expression may be used to differentiate naïve from memory T cells (Michie et al., 1992). In addition, subsets of memory T cells can be classified based on expression of chemokine receptors, for example CCR7 (Sallusto et al., 1999). $CCR7^-$ T cells have immediate effector function and home to sites of inflammation. On the contrary, $CCR7^+$ T cells home to secondary lymphoid organs (Campbell and Butcher, 2000). On the basis of CD45RA and CCR7 co-expression, T cells may be classified as naïve-like ($CD45RA^+CCR7^+$, T_{Naive}) which are non-antigen experienced; central memory ($CD45RA^-CCR7^+$, T_{CM}) which are long lived and have the potential to expand rapidly upon subsequent encounter with specific antigen; effector memory ($CD45RA^-CCR7^-$, T_{EM}) are associated with preferential production of immediate effector molecules, such as IFN-g and terminally differentiated effector memory ($CD4^+5RA^+CCR7^-$, T_{EMRA}) are the most differentiated subset based on telomere length (Harari et al., 2005).

In addition to CD45RA and CCR7 markers, memory T cell subsets can also be classified based on expression of CD27 (Fritsch et al., 2005). CD27 functions as a co-stimulatory molecule (Croft, 2009, Nolte et al., 2009). CD27 is expressed on naive and less differentiated memory T cells (Hintzen et al., 1994). Repeated antigenic stimulation of T cells leads to irreversible loss of CD27 expression (De Jong et al., 1992). BCG-specific memory T cells previously described include naive, effector, central and terminally differentiated T cells (Kagina et al., 2009, Soares et al., 2013, Tena-Coki et al., 2010). In this project, we assessed the memory and maturational phenotypes of BCG-specific cells in infants vaccinated with BCG at birth and 6 weeks of age.

1.8.4. Kinetics of BCG induced immune responses

Current novel vaccination strategies against TB under development involve use of BCG as the prime vaccine, followed by boosting with a second vaccine. Therefore, knowledge of the kinetics of BCG induced immunity is of critical importance in determining the optimal time to administer boost vaccines. In a study conducted in the UK among adolescents, Weir, et al. showed that BCG-induced IFN-γ responses waned after 3 months post vaccination among adolescents. In addition, in the same study, adolescents vaccinated at birth showed detectable BCG induced immune responses up to 14 years of age (Weir et al., 2008). In another study conducted in our laboratory among infants, Soares et al., reported BCG induced immunity peaked between 6-10 weeks of age; the $CD4^+$ T cell responses at the peak were characterised by predominant production of IFN-γ; after the contraction phase, BCG specific T cells appeared to be a combination of central and effector memory phenotypes, and produced combinations of the Th1 cytokines IFN-γ, IL-2 and TNF-α (Soares et al., 2013). Therefore it may be ideal to boost BCG immune responses at 14 weeks post BCG vaccination, after the peak effector immune response phase. This would reduce the risk of activation induced cell death that may occur when boosting is done at the peak (6-10 weeks) of the immune response, when cells are prone to exhaustion (McKinstry et al., 2007). Furthermore, boosting at 6 weeks or at 10 weeks would mean co-administering the vaccine with other EPI vaccines, which may result in reduced Th1 responses to the novel TB vaccine (Ota et al., 2011).

1.8.5. Co-variates in BCG-induced immunity

Studies evaluating immune response to BCG indicate that various factors may impact the vaccine-induced immunity. These covariates are discussed in greater detail below.

1.8.5.1. Route of vaccination

Upon administration of BCG intradermally or percutaneously, BCG is taken up by resident APCs, processed and presented to T cells present in regional lymph nodes. Different populations of APCs predominate in different tissues (Knight and Stagg, 1993). Also, different APCs have distinct antigen presenting properties (Vidard et al., 1992). Therefore, administration of a vaccine via different routes may result in differential vaccine-induced immunity. For example, studies of acute and chronic viral infection show differential immune responses when vaccines are administered using different routes (Holland et al., 2008). In a clinical study by Davids et al*.,* greater frequencies of BCG-specific IFN-γ expressing $CD4^+$ and $CD8^+$ T cells were found in whole blood from infants who received percutaneous Japanese BCG, compared to those who received intradermal Danish BCG at birth (Davids et al., 2006). This indicates that the route of vaccination may have an effect on vaccine-induced immunity.

1.8.5.2. Vaccine strain

Various clinical studies have investigated the differences in immunogenicity in individuals vaccinated with different BCG strains (Hussey et al., 2002, Davids et al., 2006, Gorak-Stolinska et al., 2006) (Aguirre-Blanco 2007). For example, Gorak-Stolinska et al., reported similar immunogenicity as measured by the PPD-specific IFN-γ responses, among school children one year post-vaccination with either the Danish-SSI 1331 or the Glaxo-Evans 1077 vaccine (Gorak-Stolinska et al., 2006). In another clinical study from South Africa, 10 week-old infants vaccinated with percutaneous Japanese BCG showed greater frequencies of IFN-γ expressing $CD4^+$ and $CD8^+$ T cells, and greater $CD4^+$ and $CD8^+$ T cell proliferation compared with infants who had received intradermal Danish BCG (Davids et al., 2006). However when followed up for two years post BCG vaccination, there was no difference in efficacy against TB between infants randomized to receive Tokyo 172 BCG through the intradermal or percutaneous route (Hawkridge et al., 2008). In a recent randomized clinical trial, Ritz et al*.,*

compared the immune response to BCG-Denmark, BCG-Japan and BCG-Russia. Infants in the BCG-Denmark and BCG-Japan groups had a higher proportion of polyfunctional $CD4^+$ T cells compared to infants in the BCG-Russia vaccination group (Ritz et al., 2012). Similarly, greater IFN-γ responses to mycobacterial antigens were observed in infants previously vaccinated with BCG Danish strain compared to those who received either BCG-Russia or BCG-Bulgaria strains (Anderson et al., 2012).

1.8.5.3. Co-infection with helminths

Infection with helminths mainly induces a Th2 immune response characterised by production of IL-4 and IL-5 (Bundy et al., 2000). In addition, helminths induce production of IL-10, an anti-inflammatory cytokine. Therefore, immune responses induced by helminth infection may result in attenuation of BCG-induced Th1 responses. In support of this hypothesis is a study by Elias et al., showing that BCG-specific Th1 responses were greater in adults who received anti-helminth medication prior to BCG vaccination, compared with those in the placebo group (Elias et al., 2001). The reduced Th1 responses were associated with greater frequencies of TGF-β producing $CD4^+$ T cells in the placebo group (Elias et al., 2008). In another clinical study, the BCG-specific immune responses of children born to mothers with helminth infection were reduced compared to those born to mothers without the infection (Malhotra et al., 1999). Moreover, Webb et al., in a randomised clinical trial, showed that infants born to mothers randomised to receive anti-helminth medication showed decreased IL-5 and IL-13 levels compared to those in the placebo group (Webb et al., 2011). On the contrary, no effect was observed on mycobacterial specific Th1 responses among Ugandan infants born to helminth-infected mothers (Elliott et al., 2010). It is important to note that in young infants, the likelihood of helminth infection is lower compared to that in observed in adults (Belyhun et al., 2010).

1.8.5.4. Host immune deficiency

The immune response to live BCG is mediated through the adaptive arm of the immune system. Therefore, in an immune deficiency state, such as during HIV infection, characterized by depletion of $CD4^+$ T cells, the immune response to BCG is compromised. In a clinical study conducted in our laboratory, Mansoor et al*.,* showed that HIV infection in infants severely impairs BCG-induced immune responses (Mansoor et al., 2009). Furthermore, there have been case reports of BCG disease (BCGosis) following administration of BCG to immune compromised babies (Hesseling et al., 2009). Therefore, WHO current recommends that BCG not be given to HIV-exposed babies, if public health structures allow this (Hesseling et al., 2008). Data from our study on effect of delaying BCG vaccination will inform if there may be an immunological beneficial effect of administering BCG to HIV-exposed uninfected infants later in life.

1.8.5.5. Age at which BCG is administered

Neonatal cell-mediated immune responses are quantitatively and qualitatively different from those of adults. This is evidenced by the increased susceptibility to intracellular infections such cytomegalovirus and TB in infants, compared with adults (Siegrist, 2001). Compared with adults, neonates' innate cells produce less of the Th1-promoting cytokine IL-12 (Goriely et al., 2004, Corbett et al., 2010), and display diminished TLR4 expression (Sadeghi et al., 2007) and signaling (Yan et al., 2004). In addition, the adaptation of the neonatal immune system to the *in utero* environment is associated with a bias towards type 2 immune responses in newborns (Warner, 2004). This may potentially result in a suboptimal response to BCG. During the first 6 weeks of life, the infant immune system would have started adapting to the new an ex-utero environment (Yazdanbakhsh et al., 2002) and might therefore be better primed to respond to BCG if given at 6 weeks of age.

Data from our laboratory has previously shown that delaying BCG vaccination to 10 weeks of age resulted in higher frequency of BCG-induced polyfunctional T cells at one year of age (Kagina et al., 2009).

1.9. Rationale of the study

Currently, our knowledge of the factors constituting optimal protection against *M.tb* infection and active disease remain limited (Hanekom, 2005). Therefore, BCG vaccination remains critical in understanding the nature of vaccine-induced immunity. What we learn from the current BCG is likely to apply also to modified BCG vaccines of the future. Also, BCG is likely to form the cornerstone of future TB vaccination strategies as the prime vaccine in the prime-boost paradigm of new vaccines (Parida and Kaufmann, 2010). Therefore, studies designed to enhance the understanding of the optimal schedule to vaccinate infants with BCG for optimal priming are research priorities.

Multiple studies have investigated the effect of delaying BCG vaccination (**Table 1**). Comparable host responses were shown between birth and delayed vaccination when PPD-induced IFN-γ production was used as the outcome (Burl et al., 2010, Hussey et al., 2002, Marchant et al., 1999). In contrast, a more detailed study from our group in South Africa showed that delaying BCG from birth to 10 weeks of age resulted in induction of a greater frequency of BCG-specific polyfunctional $CD4^+$ T cells, i.e., cells that express IFN-γ, TNF-α and IL-2 together (Kagina et al., 2009). Importantly, in all previous studies, infants were randomized to receive BCG either at birth or later age after birth. In contrast, we wished to address effect on BCG-induced immune response, in the setting where delayed vaccination occurred due to home delivery of infants. We hypothesized that BCG vaccination at 6 weeks of age would result in an enhanced specific T cell response, compared with administration at birth.

Previous studies investigating the effect of delaying BCG vaccination on BCG-induced immunity have either used the tuberculin skin test (Ildirim et al., 1992),

soluble cytokine levels (Burl et al., 2010, Hussey et al., 2002, Ildirim et al., 1992) or T cell cytokine responses (Kagina et al., 2009) as the immunological readouts. In our study, we simultaneously assessed different immunological readouts as detailed in the aims section below. This multipronged approach allowed for a comprehensive comparative picture of BCG-induced immunity in infants vaccinated with BCG at birth and at 6 weeks of age. Our report is of the most comprehensive immunological assessment of delayed BCG vaccination reported to date.

In this study, we looked at 6 weeks of age, because we propose that earlier BCG vaccination in high endemic areas may be advantageous. This may as well be the optimal time to vaccinate infants that are exposed to HIV infection.

We hypothesized that infants who received BCG at 6 weeks of age will show an enhanced BCG-induced T cell immunity compared to infants vaccinated at birth.

Study	**Duration of BCG delay**	**Method to measure outcome**	**Key findings**
Ildrim et al. 1992	12 weeks	TST	Greater PPD specific induration at one year in delayed BCG group
Marchant et al. 1999	8 and 16 weeks	Elisa	No difference in PPD specific Th1 responses at one year in both groups
Hussey et al. 2002	10 weeks	Elisa	No difference PPD specific Th1 cytokine response at one year in both groups
Kagina et al. 2009	10 weeks	WB-ICS	Greater Th1 cytokine responses at one year in delayed group
Burl et al. 2010	18 weeks	Elisa	No difference Th1 and Th17 PPD specific responses at 9 months

Abbreviations used: Purified protein derivative, PPD; Tuberculin skin test, TST; Enzyme linked immunosorbet assay, Elisa.

Table 1: Delayed BCG vaccination studies. The duration of delay in BCG vaccination from birth, assay used as well as the outcomes of the studies are show.

1.10. Objectives of this project

1. To optimise a 9-colour flow cytometry panel to measure BCG-specific $CD4^+$ and $CD8^+$ T cell responses in infants vaccinated with BCG at birth or at 6 weeks of age (Chapter 3).

2. To compare the frequency of BCG-specific IFN-γ, TNF-α, IL-2, IL-17 and/or perforin expressing $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth or at 6 weeks of age (Chapter 4).

3. To evaluate memory phenotypes of BCG-specific $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth or at 6 weeks of age (Chapter 5).

4. To compare levels of soluble cytokines in plasma from whole blood stimulated with BCG in infants vaccinated with BCG at birth or at 6 weeks of age (Chapter 6).

5. To compare the ability of $CD4^+$ and $CD8^+$ T cells to proliferate, and the capacity of the proliferating cells to produce cytokines, in infants vaccinated with BCG at birth or at 6 weeks of age (Chapter 7).

Chapter two: Materials and methods

2.1. Study setting and design

This was an observational study. Healthy 9-month old infants were enrolled at the Child Health and Development Centre (CHDC) in Mulago National Referral Hospital, Kampala, Uganda. This hospital is located in Kampala, the country's capital city. The CHDC provides free vaccination as part of the national immunization program. Over 95% of the mothers coming in with their babies to the CHDC reported having had voluntary counseling and testing during pregnancy or had taken part in prevention of mother to child transmission of HIV program.

2.2. Participant recruitment

At the CHDC, a trained health worker conducted the recruitment of healthy 9-months old infants into this study. Mothers who brought their infants for measles vaccination at 9 months of age were approached and informed verbally about the study. Informed consent was obtained from infant's mothers interested in participating in the study.

2.3. Enrollment

Infants who satisfied the inclusion criteria were enrolled in the study.

2.3.1. Inclusion criteria

1. Infants with evidence of having received BCG vaccination, either through documentation in the immunization card, or by presence of a BCG scar.
2. Infants whose parent or guardian gave informed consent.

2.3.2. Exclusion criteria

We used the following criteria to exclude infants from enrollment into this study.

1. Infants whose mothers did not take part in a maternal to infant transmission of HIV prevention program during pregnancy.
2. Infants or infants with mothers with documented evidence of HIV infection.

HIV exposure or infection of infants may affect the BCG-induced immune response.

3. Infants who had received any vaccine within a month prior to enrolment.
4. Infants who had a low birth weight as defined by weight less than 2.5 kilograms as recorded on growth card or on delivery discharge note.
5. Infants born before term (less than 38 weeks of gestation).
6. Infants who had a history of significant perinatal complications, such as an emergency cesarean section or severe neonatal jaundice.
7. Infants exposed to TB disease in the household in the first 9 months of life.
8. Infants who had any acute condition such as febrile illness, diarrhea or a cold, at the time of enrollment.
9. Infants who had a history of any chronic disease including TB over first 9 months of life.
10. Infants taking any immunosuppressive therapy like steroids within four weeks of enrollment.
11. Infants who had clinically apparent anemia.
12. Infants who had low weight for age or other signs of malnutrition.

2.4. Clinical and demographic assessment

A child health growth card was used to identify infants who received BCG at birth or at 6 weeks of age as well as other EPI vaccines. A questionnaire was administered to prospective volunteers to capture basic demographic data. Infants' weight was measured followed by a clinical examination to assess for any clinical signs and symptoms.

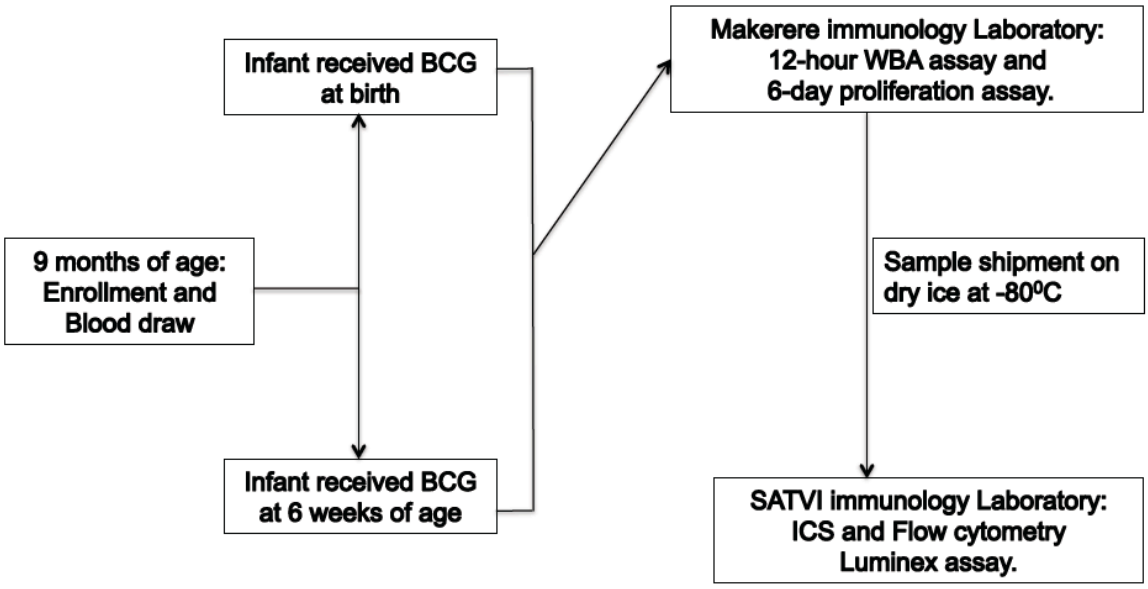

Figure 3. Overview of the study procedures. We enrolled 9-months old infants previously vaccinated with BCG at birth or at 6 weeks of age. 5 ml whole blood was drawn from each participant and transported to the Makerere university immunology laboratory for the 12-hour WBA and 6-day proliferation assays. White cells and plasma were harvested, frozen at -80^{0}C then later shipped to SATVI laboratory for ICS and multiplex assays.

2.5. Blood collection and initial processing

5ml of peripheral whole blood was drawn from the infant into a sodium heparin tube by a trained phlebotomist. To ensure the safety of study participants, we followed the national guidelines for maximum volumes of blood that may safely be collected. This is less than 3 ml per kg body weight, the limit that can be drawn from a healthy infant (Howie, 2011). A maximum of 3 attempts were allowed for blood collection. The collected blood was transported to the immunology laboratory, Makerere University College of Health Sciences, within an hour of being drawn for processing. The collected blood was allocated into five tubes with or without antigen as indicated in **Table 2**.

Total volume of blood taken	6-Day whole blood assay	12-hour whole blood assay				Total volume used
		WBA-BCG	WBA-UNS	WBA-PHA	WBA-TB10.4	
6	1	1	1	1.0	1	5.0
5	1	1	1	0.5	1	4.5
4	1	1	1	0.5	0.5	4
3	1	1	0.5	0.5	0	3
2.5	1	0.5	0.5	0.5	0	2.5

Table 2. Prioritization of blood collected for assays used. A maximum of 5 ml whole blood collected from 9-month old infants and stimulated in the 12-hour WBA and 6-day proliferation assays. Blood was allocated to the different assay conditions depending on the blood volumes collected from the infants. The assay conditions included unstimulated, BCG, PHA, and TB10.4. The quantities of the antigen (BCG, PHA, and TB10.4) added were adjusted according to blood volume incubated.

2.6. Whole blood incubation and cryopreservation

2.6.1. Twelve-hour (short-term) whole blood assay

One ml heparinised whole blood was incubated with either BCG (Danish strain 1331, Statens Serum Institut, 1.2X10^{6}cfu/ml), phytohaemagglutinin (PHA, Sigma-Aldrich, 5μg/ml, positive control) or left unstimulated, as previously described (Hanekom et al., 2004). The co-stimulatory antibodies anti-CD28 and anti-CD49d (at 1μg/ml each, BD Biosciences, San Jose, CA) were added to all assay condition to enhance the specific response (Waldrop et al., 1997). Blood was incubated at 37^{o}C for 7 hours, after which plasma was removed and stored at -80^{o}C for later measurement of soluble cytokine levels as described in in chapter 6. Thereafter, Brefeldin-A (Sigma-Aldrich, 10μg/ml) was added and the blood was incubated for a further 5 hours. Cells were harvested, fixed in BD FACS

Lysing Solution (BD Biosciences) and frozen at -80^{0}C. Later, the samples were shipped on dry ice to the South African Tuberculosis Vaccine Initiative (SATVI) laboratory for further processing to measure T cell-associated cytokine expression and memory phenotypes as described in subsequent chapter 4 and 5 respectively.

2.6.1.1. Immune outcomes evaluated in the short-term assay to assess for BCG-Specific T cell responses.

I. The total frequency of $CD4^+$ and $CD8^+$ T-cells expressing Th1 (IFN-γ, IL-2 and TNF-α), Th17 (IL-17) cytokines and perforin.
II. The frequency of $CD4^+$ and $CD8^+$ T-cell subsets expressing either one or a combination of IFN-γ, IL-2, TNF-α and IL-17, singly of in combination.
III. The soluble levels of Th1 (IFN-γ and IL-2) cytokines
IV. The soluble levels of Th2 (IL-4, IL-5 and IL-13) cytokines
V. The soluble levels of the immunoreguratory cytokine, IL-10.

2.6.1.2. Immune outcomes evaluated in the twelve-hour whole blood assay to characterise BCG-specific T cell phenotypes.

I. The frequency of BCG specific CD4 T-cell expressing either one or a combination of CD45RA, CCR7 and CD27.
II. The frequency of BCG specific CD8 T-cell expressing either one or a combination of CD45RA, CCR7 and CD27.

2.6.2. Six-day whole blood proliferation assay

A further 1ml whole blood was diluted in 9 ml of RPMI media and mixed in a sterile polypropylene tube. One ml of diluted blood was incubated with either BCG (Danish strain 1331, Statens Serum Institut, 1X10^5 cfu/ml) at day zero or PHA (Sigma-Aldrich, 1μg/ml, positive control) at day three, or left unstimulated. Incubation continued for 6 days at 37°C in 5% CO_2(Soares et al., 2010). Four hours prior to the end of the cell culture phorbol 12-myristate 13-acetate (PMA,

Sigma-Aldrich, 20ng/mL), ionomycin (Sigma-Aldrich, 10μg/mL) and Brefeldin-A (Sigma-Aldrich, 2μg/mL) were added to induce cytokine expression. At the end of culture, red cells were lysed with BD FACS Lysing Solution (BD Biosciences) white blood cells were fixed and frozen at -80^{0}C. Later, the samples were shipped on dry ice to the SATVI laboratory for further processing to measure the ability of T cell to proliferate and the capacity of proliferating cells to produce cytokines.

2.6.2.1. Immune outcomes evaluated in the six-day assay

I. Frequency of BCG- specific Ki67 expressing $CD4^+$ and $CD8^+$ T cells.
II. Proportion of BCG-specific (i.e., Ki-67+) $CD4^+$ and $CD8^+$ T cells producing Th1 cytokines (IFN-γ, IL-2, TNF-α) and IL-17, singly or in combination.

2.7. Sample size calculation

Sample size calculation was based on a study done in Cape Town that investigated the effect of delaying BCG vaccination to 10 weeks on the induced $CD4^+$ and $CD8^+$ T cell responses (Kagina et al., 2009). In this pilot study, the mean $\log_{10}$ difference of the frequency of $CD4^+$ T cells co-expressing IFN-γ, TNF-α, and IL-2 between children vaccinated at birth and 10 weeks of age was 0.55. Assuming a linear change in the frequency of $CD4^+$ T cells co-expressing IFN-γ, TNF-α, and IL-2 when the vaccine is delayed from to 10 weeks of age, at 6 weeks we would expect a lower difference of 0.4. We therefore needed to study 36 subjects in both arms of our study to be able to reject the null hypothesis that the population means of the two study groups are equal with probability (power) 0.8. The type I error probability associated with this test of this null hypothesis is 0.05 and a Z of 1.96.

2.8. Ethical considerations

2.8.1. Informed consent

A trained member of the clinical study team conducted the written informed consent process. Informed consent was administered in English or in the home language (Luganda) using appropriately translated consent forms. The person taking the consent explained the protocol to the infants' parents or guardian and ensured that they fully understood the research protocol before obtaining their signature. One copy of the consent forms was kept under appropriate lock with the questionnaire. A copy of the consent form was given to the parent or guardian.

2.8.2. Ethical approvals

The study was approved by the institutional review board of the School of Public Health, Makerere University College of Health Sciences (IRB00005876FWA-HDREC066), the Uganda National Council for Science and Technology, as well as the University of Cape Town Research Ethics Committee (HREC Ref Number : 176/2012).

2.9. Contributions

Dr. F. Lutwama wrote the study proposal, designed and conducted the experiments and wrote this chapter under supervision of Dr. B.M.N. Kagina, Prof. H. Mayanja-Kizza and Prof. W.A. Hanekom.

Chapter 3: Optimisation of a 9-colour flow cytometric assay to measure BCG-induced cytokine and cytotoxic marker expression in T cells

3.1. Introduction

Our aim was to optimise a 9-colour flow cytometry assay to measure BCG-specific $CD4^+$ and $CD8^+$ T cell responses in thawed, fixed white blood cells, generated by incubation of whole blood with BCG, as described in Chapter 2.

We chose polychromatic flow cytometry (PFC) to assess BCG-specific T cell responses for our studies for the following reasons. First, PFC allows for assessment of multiple immune characteristics using small blood volumes. This was of particular advantage in our study where it was only possible to safely collect small blood volumes. Second, PFC allows for simultaneous evaluation of multiple parameters on a single cell basis (Mahnke and Roederer, 2007, Roederer et al., 2004). Third, our laboratory has vast experience with the use of PFC in assessment of BCG-specific T cell responses (Kagina et al., 2010, Mansoor et al., 2009, Soares et al., 2008). Finally, similar assays, although more limited, had been used in a previous project of our laboratory, when the specific T cell outcomes of delaying BCG from birth to 10 weeks of age in South African infants were compared (Kagina et al., 2009).

Several challenges arise in designing large PFC panels, where more than 4 variables are detected per cell. These include limited availability of antibody conjugates, limitations in specific flow cytometer configurations, and spectral overlap of emission from sub-optimal antibody-fluorochrome combinations that may lead to measurement errors (Perfetto et al., 2004, Roederer, 2001). It is therefore paramount to perform careful optimisation of PFC panels prior to their application in clinical studies. This includes choice of the most optimal antibody-fluorochrome combinations for the particular application, and introduction of excellent quality control procedures for both staining and analysis (Baumgarth and Roederer, 2000).

Here, we describe optimization of a PFC panel to measure BCG-specific immunity in $CD4^+$ and $CD8^+$ T cells.

3.2. Rationale for selection of phenotypic and functional T cell markers

We considered several T cell markers that are induced by BCG vaccination of infants, and which may play a role in protection against TB. These included CD3, CD4, CD8, CD69, IFN-γ, TNF-α, IL-2, IL-4, IL-13, IL-17 and perforin (**Table 3**).

We included CD3 to allow us identify T lymphocytes. From T lymphocytes, we were interested in selecting $CD4^+$ and $CD8^+$ T cells. Both $CD4^+$ and $CD8^+$ T cells are induced by BCG vaccination, and both subsets may play a role in protection against TB (see Chapter one, section 1.7.1).

We also considered inclusion of CD69, an early activation marker expressed on the surface of antigen stimulated cells (Testi et al., 1994), and may play a co-stimulatory role in T cell activation and proliferation (Ziegler et al., 1994). Previous studies have used CD69 to successfully identify *M.tb*-specific T cells (Avgustin et al., 2005, Hughes et al., 2005). In our experiments, we used CD69 to identify BCG-activated, perforin expressing $CD4^+$ and $CD8^+$ T cells. Perforin is contained in cytolytic granules, which degranulate upon T cell stimulation releasing this cytolytic molecule (Makedonas et al., 2009). Therefore, inclusion of CD69 in our ICS panel enabled us to measure newly formed perforin upon T cell stimulation. We therefore incorporated CD69 in our ICS antibody panel to identify BCG-activated and perforin expressing T cells in infants vaccinated with BCG at birth of at six weeks of age.

Previous studies conducted in our laboratory show that BCG vaccination induces specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α and IL-2. We therefore considered inclusion of these cytokines (IFN-γ, TNF-α and IL-2) in our PFC panel. A detailed discussion of the role of these cytokines in immunity against

M.tb and BCG vaccination is discussed in detail in chapter one (section 1.5.2.3 and 1.8).

BCG vaccination also induces IL-17 producing $CD4^+$ T cells. (Scriba et al., 2008). (Kagina et al., 2010). A detailed role of IL-17 in immunity against *M.tb* is discussed in chapter 1 (section 1.8). Investigators in our laboratory have reported detectable IL-17 expression in specific CD4+ T cells. We therefore included this marker in our PFC panel.

A previous study from our laboratory showed that, BCG-specific expression of IL-4 and IL-13 by $CD4^+$ and $CD8^+$ T cells was very low, in 10-weeks old infants (Soares et al., 2008). However, there is no reported data on the expression levels of these cytokines in 9-months old infants. We therefore chose to assess the expression levels of IL-4 and IL-13 cytokines in this age group.

Moreover, BCG vaccination also induces IL-10 (Marchant et al., 1999, Ota et al., 2002), an immunoregulatory cytokine that may inhibit *M.tb* induced immune responses (O'Garra and Vieira, 2004). However, a previous study in our laboratory showed that this cytokine is expressed at very low levels in our preferred assay system. We therefore did not consider IL-10 for inclusion in our PFC panel.

Finally, we considered inclusion of perforin, a cytotoxic molecule induced by BCG vaccination (Semple et al., 2011). We used the newly introduced anti perforin-PE (B-D48 antibody clone) capable of detecting both the preformed perforin in the cytotoxic granules as well as newly formed perforin residing in the endoplasmic reticulum (Hersperger et al., 2008). **Table 3** below shows a summary of the 11 T cell markers considered for evaluation in our PFC panel for the study.

Surface marker	Description
CD3	T cell marker
CD4	T cell marker
CD8	T cell marker
CD69	Early activation T cell marker
IFN-γ	Th1 cytokine
IL-2	Th1 cytokine
TNF-α	Th1 cytokine
IL-17	Th17 cytokine
IL-4	Th2 cytokine
IL-13	Th2 cytokine
Perforin	Cytolytic molecule

Table 3: Candidate T cell markers for PFC panel. The table shows the phenotypic and functional T cell markers considered for inclusion in the PFC panel, and the rationale for consideration of each marker.

3.3. Capacity and configuration of our flow cytometer

Our laboratory is equipped with a LSRII flow cytometer (BD Biosciences), a multi colour instrument configured with red, blue and violet lasers. The red laser has three detectors, the blue laser has 8 detectors while the violet laser has 3 detectors. Based on this configuration, the cytometer would allow a 12 colour PFC panel, at least, excluding side scatter (SSC) and forward scatter (FCS) detection. **Table 4** shows a summary of this configuration and capacity. We therefore proposed that one panel would suffice for all anticipated measurements.

3.4. Rationale for selection of optimal antibody-fluorochrome combinations

Next, we proceeded to select antibody-fluorochrome combinations. We considered the flow cytometer configuration, anticipated expression levels of the proposed T cell, cytokine and cytotoxic markers, as well as the brightness of the

fluorochromes (BIS). This strategy is critical to minimize spectral overlap inherent in large PFC panels (Baumgarth and Roederer, 2000).

First, we considered antibody-fluorochrome constructs available in our laboratory. Next, we consulted existing data of the expression levels of BCG-induced T cell markers (Kagina et al., 2010, Mansoor et al., 2009, Soares et al., 2013). We then selected the antibody-fluorochrome combination proposed to be most optimal for each marker (Maecker et al., 2004). The ideal match would be a highly expressed marker with a fluorochrome with low BIS, and vise versa. Using this criterion, the 11 T cell markers considered could be classified in to three different categories, (a) antigens that are well characterized and are either expressed or not a cell surface (CD3, CD4 and, CD8). (b) Antigens that are expressed in a relatively high molecular density per cell but exhibit a continuous expression (CD69, IFN- γ, TNF$-\alpha$ and perforin). (c) Antigens that are expressed at a low density or by a low frequency of cells (IL-17, IL-2, IL-4 and IL-13). We considered matching bright flourochomes to dim T cell markers and vice versa (**Table 4**).

Laser	Detector	LP (nm)	BP (nm)	Fluorochrome	BIS
Blue	FSC		488/10		
(488nm)	SSC				
	A	735	780/60	PE-CY7	4
	B	685	695/40	PerCP-CY5.5	3
	C	635	670/14	PE-Cy5, PerCP	3, 2
	D	600	610/20	PE-Texas Red	NR
	E	550	576/26	PE	5
	F	505	530/30	FITC	3
Violet	A	580	605/10	QDot 605	NR
(405nm)	B	505	560/40	QDot 565	NR
	C		440/40	PacBlue	1
Red	A	735	780/60	APC-Cy7	2
(633nm)	B	690	720/40	Alexa Fluor 700	2
	C		660/20	APC, Alexa Fluor 647	5, 5

Table 4: Flow cytometer configuration and fluorochrome brightness index score. The SATVI LSR II has 3 lasers and 14 detectors. Each detector has long pass (LP) and band pass (BP) filters. The possible fluorochromes that can be used for the different detectors are shown. The fluorochromes brightness index score (BIS) are indicated from lowest (1=dimmest) to highest (5=brightest). The BIS for PE-Texas Red, Qdot-565 and -605 are not reported (NR).

3.5. Materials and methods

3.5.1. Study participants and blood collection

Whole blood was collected from four healthy adult participants. We recruited and consented these adults at the University of Cape Town, South Africa. All participants provided written informed consent. The protocol was approved by the Research Ethics Committee of the University. Participants were excluded from the study for the following reasons: presence of any acute or chronic disease, use of any immunosuppresive treatment, pregnancy and no history of BCG vaccination. From each healthy participant, 3 ml whole blood was collected into heparinized tubes and transferred to the laboratory for stimulation with viable BCG in a short-term whole blood assay.

3.5.2. Antigens

Bacillus Calmette-Guérin (BCG, Danish 1331) was obtained from Statens Serum Institut, Copenhagen. Each lyophilised vial contained 2-8x10^6 colony forming units (CFUs), and was reconstituted with RPMI and used at a final concentration of 1.2x10^6 CFU/ml. Phytohemagglutinin (PHA, Sigma-Aldrich), a positive control, was used at a final concentration of 5 μg/ml.

3.5.3. Short-term whole blood stimulation and cryopreservation

From each study participant, 1 mL whole blood was left unstimulated or stimulated with either BCG (Danish strain 1331, Statens Serum Institut, 1.2 x 10^6cfu/ml) or PHA and incubated at 37^{o}C for 7 hours. Brefeldin-A (Sigma-Aldrich, 10μg/ml) was then added and the blood was incubated for a further 5 hours. Red cells were lysed and white cells fixed in FACS lysing solution (BD Biosciences). Multiple vials of the stimulated and fixed white cells were cryopreserved for later intracellular staining and flow cytometry analysis. **Figure 4** shows a summary of the whole blood assay procedures that we used.

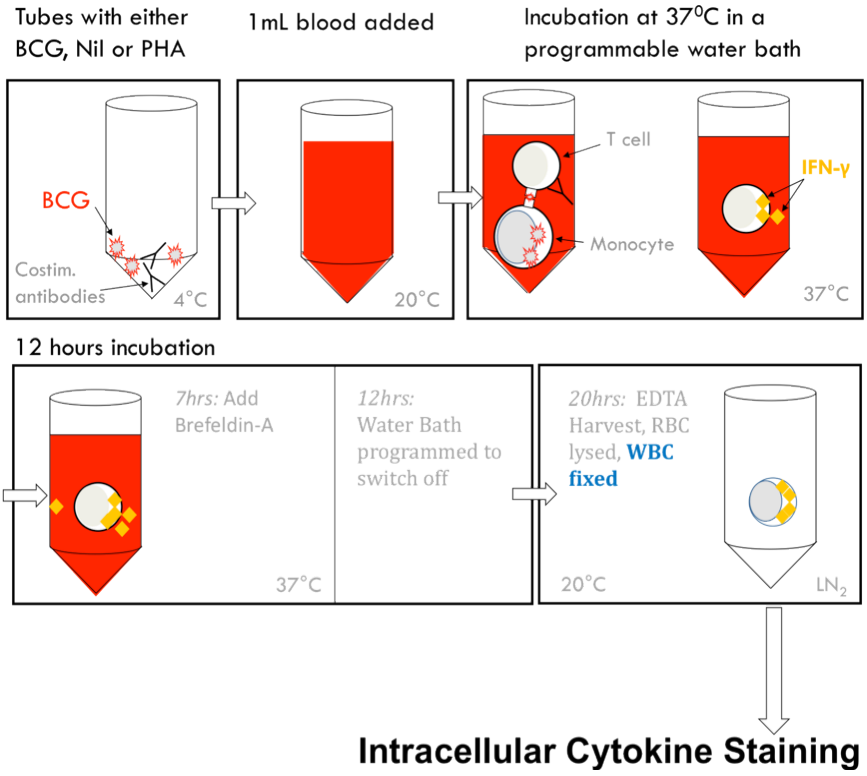

Figure 4. Procedures for the 12-hour whole blood assay. BCG and co-stimulatory antibodies (anti-CD28 and anti-CD49d) were prepared and placed in tubes and kept at 2° to 8°C in the laboratory. Blood, collected in a sodium-heparinized syringe, was added drop wise to an opened tube, up to a level marked on the side of the tube. The tube was closed, vortexed then incubated at 37°C in a water bath. Brefeldin-A was added at 7 h. After 12 h, water bath was preprogrammed to switches off. The temperature gradually dropped to room temperature. After 20 h, cells were harvested following incubation with EDTA. Red cells were lysed and white cells fixed with FACS lysing solution. The cells were cryopreserved in FBS/DMSO solution. (**modified from Hanekom *et al*, 2005**)

3.5.4. Intracellular cytokine staining assay and flow analysis

We used a “one step” staining method, previously optmised in our laboratory, to assess the functional profiles of T cells. In this assay, fixed, cryopreserved white cells from the stimulated whole blood were thawed, washed in phosphate buffered saline (PBS, BioWhittaker), permeabilised in Perm/Wash Buffer (BD Biosciences) and stained with combinations of monoclonal antibodies (mAbs) for 1 hour at 4°C, as previously described (Hanekom et al., 2004). Cells were then washed and acquired on the flow cytometer (BD Biosciences). For quality assurance, compensation was done with positive and negative anti-mouse Ig

kappa-beads (BD Biosciences) labeled with the respective fluorochrome-conjugated antibodies. Cytometer Setting and Tracking (CST) beads (BD Biosciences) were used for daily settings. After acquisition, data were compensated and analysed using FlowJo software (v9.4.11; Treestar). Flow data were exported to Pestle v1.7 (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Spice (v5.1) for further analysis (Roederer et al., 2011).

3.5.5. Antibody titration

We titrated each of the commercially obtained antibody-fluorochrome conjugates by serial dilution to determine the optimal antibody titer. We used between 6 and 8 two-fold serial dilutions for each antibody-fluorochrome combination. Our starting titer volume was twice the volume recommended by the manufacturer. Outcomes included visual separation between the negative and positive populations using dotplots, frequency of positive events and signal-to-noise ratio using MFIs. We used either or both the following parameters to determine the optimal antibody titer for each antibody: (i) a titer at which optimal resolution between unstained (noise) and stained (signal) cells was observed, (ii) the antibody volume at the plateau or peak area of plots.

3.6. Results

3.6.1. IL-4 and IL-13 expression

Our laboratory has successfully used different 7 and 8 PFC panels to characterize the BCG-specific T cell responses (Kagina et al., 2010, Mansoor et al., 2009, Soares et al., 2008). In these studies, the following antibody-fluorochrome conjugates were used: CD3-Pac Blue, CD4-Qdot 605, IFN-γ-Alexa Fluor 700, IL-2-FITC, TNF-α-PECy7, IL-17-Alexa Fluor 647 and CD8-PerCP Cy5.5 (Kagina et al., 2010, Soares et al., 2008, Scriba et al., 2008). We therefore considered the use of these optimized antibody-fluorochrome combinations before proceeding to explore additional antibody-fluorochrome conjugates for inclusion in our study PFC panel.

We aimed to additionally evaluate the expression levels of IL-4, IL-13, Perforin and CD69 in our assay system. This was critical in choosing the optimal antibody-fluorochrome combinations for our 9-colour PFC panel because markers expressed at low levels should be assigned to fluorochromes with the greatest BIS.

To address this aim, we used the fixed white cells from BCG-stimulated whole blood, as described in the methods section. We used human intracellular cytokine-2 (HICK-2) cells (BD Biosciences), which are fixed lymphoid cells that express detectable levels of IL-4 and IL-13 as positive control because PHA-induced IL-4 and IL-13 producing T cells were barely detectable (data not shown). We thawed, washed and stained the cells using a basic 4-colour PFC panel: CD3-PacBlue, CD4-Qdot 605, CD8-PerCP Cy5.5, and either IL-4-PE or IL-13-PE. Clinical results from earlier experiments in our laboratory indicated low frequencies of BCG-specific IL-13 and IL-4 T cells. We therefore used antibodies with the highest BIS for both cytokines.

We found that the median frequency for BCG-specific IL-14-expressing $CD3^+$ T cells was below 0.01% (IQR, 0.00-0.09) (n=3, **Figure 5D**), while the median

frequency for BCG-specific IL-13-expressing $CD3^+$ T cells was 0.008% (IQR,0.00-0.01) (n=3, **Figure 5D**). Previous studies of BCG-induced $CD4^+$ T cell cytokine response in our laboratory have shown a general trend of greater response to BCG, in adults than infants. Therefore, we concluded the BCG-induced IL-4 and IL-13 $CD4^+$ T cell response was very low, and likely lower in infants for reliable measurement and excluded these candidate markers from our PFC study panel.

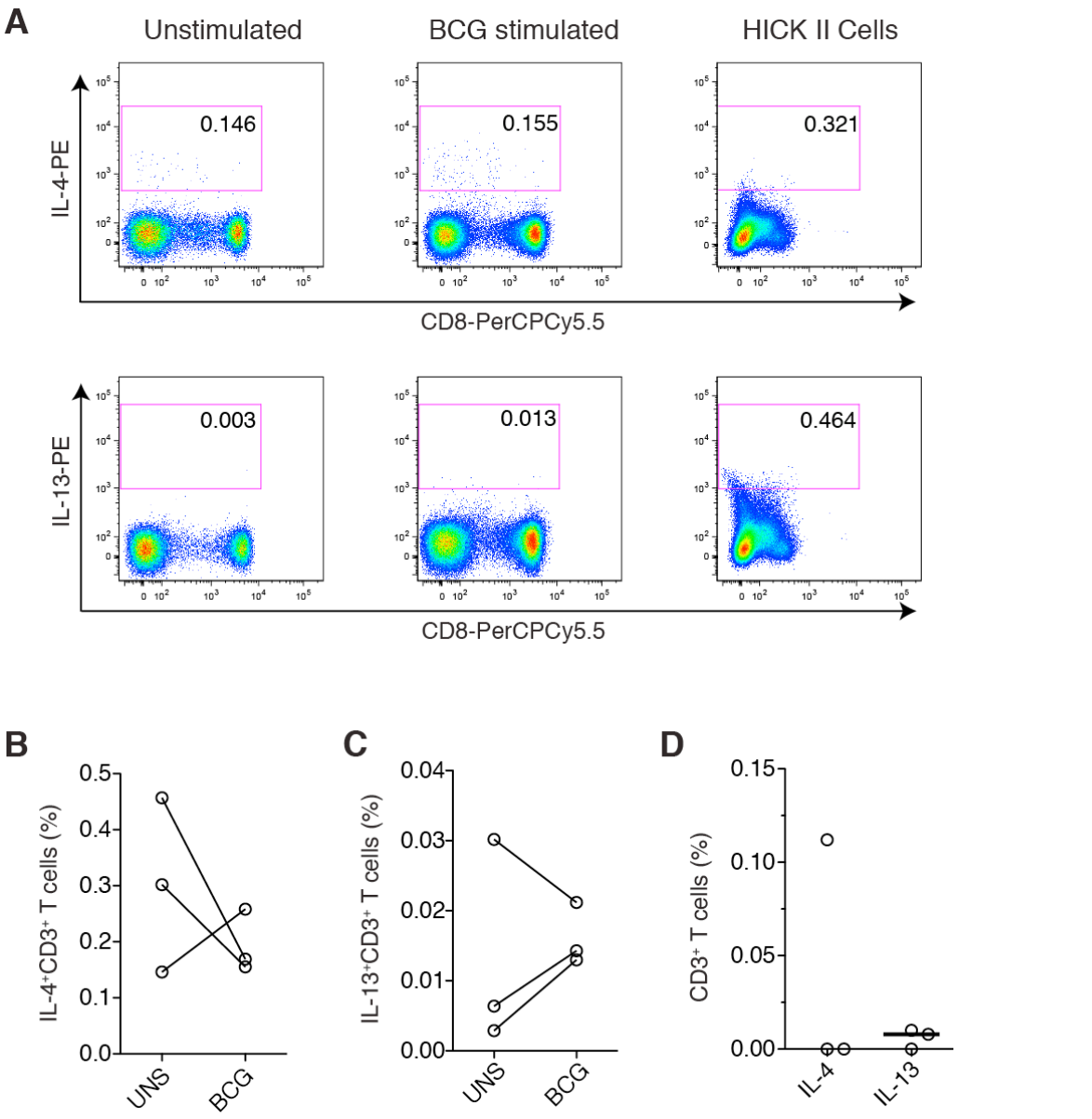

Figure 5: BCG-specific CD3$^+$ T cells producing IL-4 and IL-13 cytokines. We measured the specific responses using the short-term whole blood assay. We used blood samples from healthy adults for these experiments. Representative flow cytometry plots of IL-4 (top row) and IL-13 (bottom row) expression are shown. Frequency of IL-4 (**B**) and IL-13 (**C**) expressing CD3$^+$ T cells in unstimulated (UNS) and BCG-stimulated blood samples is shown. Scatter plot to depict frequencies of total BCG-specific (after background subtraction) IL-4 and IL-13 expressing in CD3$^+$ T cells is shown in **(D).** On the scatter plots, the horizontal lines represent the median frequencies of the specific cells.

3.6.2. CD69 expression

Having excluded IL-4 and IL-13 from our candidate T cell markers list, we were therefore left with 9 candidate T cell markers for optimizing the antibody-fluorochrome combination. Of the 9 candidate T cell markers left in our antibody-fluorochrome combination optimization list, only CD69 and perforin had not been previously measured in our laboratory, using our preferred assay system. We first titrated the CD69-PerCP-Cy5.5 antibody, chosen because we had identified a newly available CD8-Qdot 565 antibody that would replace the already optimized CD8-PerCP-Cy5.5 antibody in our laboratory, freeing the PerCP-Cy5.5 detector. For the titration experiments, we used the fixed white cells from stimulated whole blood as described in the methods section. Regardless of the antibody concentrations, CD69 showed a continuous expression by $CD3^+$ T cells (**Figure 6A**)**.** When we plotted a graph of antibody titer volume against the frequency of the BCG-induced $CD69^+CD3^+$ T cells, we showed a typical titration curve with a plateau at 0.31μl of antibody, which was then chosen for use (**Figure 6B)**. We also plotted a signal to noise ratio graph of the titration results. Signal to noise ratio assessment (**Figure 6C)** did not show definitive results (**Figure 6C**).

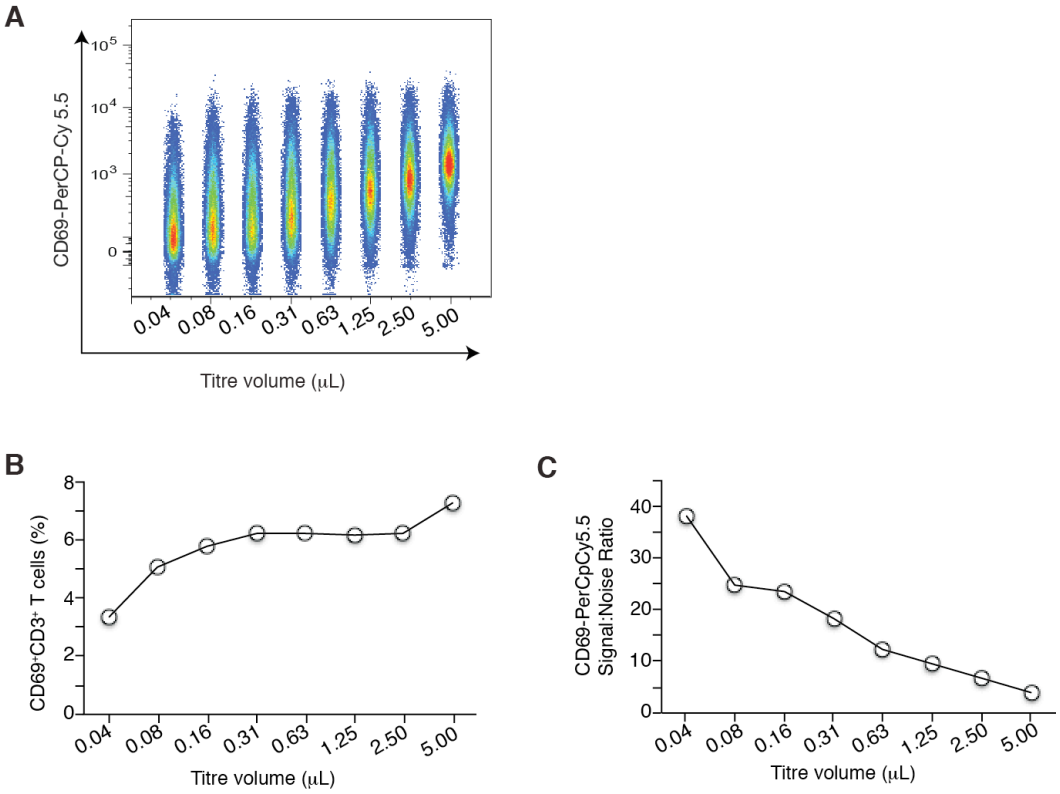

Figure 6: **CD69-PerCP-Cy5.5 antibody titration.** We used 8 different CD69-PerCP-Cy5.5 antibody concentrations for the titration using BCG stimulated whole blood from an adult volunteer. For flow analysis, singlets were first selected, then lymphocytes. Finally CD69 expression on $CD3^+$ T cells was assessed at different antibody concentration **(A)**. The optimal titration volume was assessed by plots of the frequency of $CD3^+$ T cells expressing CD69 **(B)** and the MFI of the signal ($CD69^+$) to noise ($CD69^-$) ratio **(C)**.

Having established the optimal CD69-PerCP-Cy5.5 antibody concentration, our next step was to evaluate if the early activation marker, CD69 was measurable in our assay system to identify antigen-specific $CD4^+$ and $CD8^+$ T cells. We used the frozen cells generated for these experiments. We stained the stimulated and fixed white cells from whole blood with the following antibodies: CD3-PacBlue, CD69-PerCPCy 5.5, CD4-Qdot 605 and CD8-PE-Cy7. Representative dot plots for CD69 expressing $CD4^+$ and $CD8^+$ T cells are shown in **Figure 7A**. CD69 was unregulated upon BCG stimulation for both $CD4^+$ (**Figure 7B**) and $CD8^+$ (**Figure 7C**) T cells.

The median frequency for BCG-specific CD69-expressing $CD4^+$ T cells was 3.6% (n=3, **Figure 7C**), while the median frequency for BCG-specific CD69-expressing

$CD8^+$ T cells was 2.8% (n=3, **Figure 7D**). Therefore, we concluded the BCG-induced CD69 expressing $CD4^+$ and $CD8^+$T cell responses was measureable in our assay system and therefore included the T cell marker in our final study PFC panel.

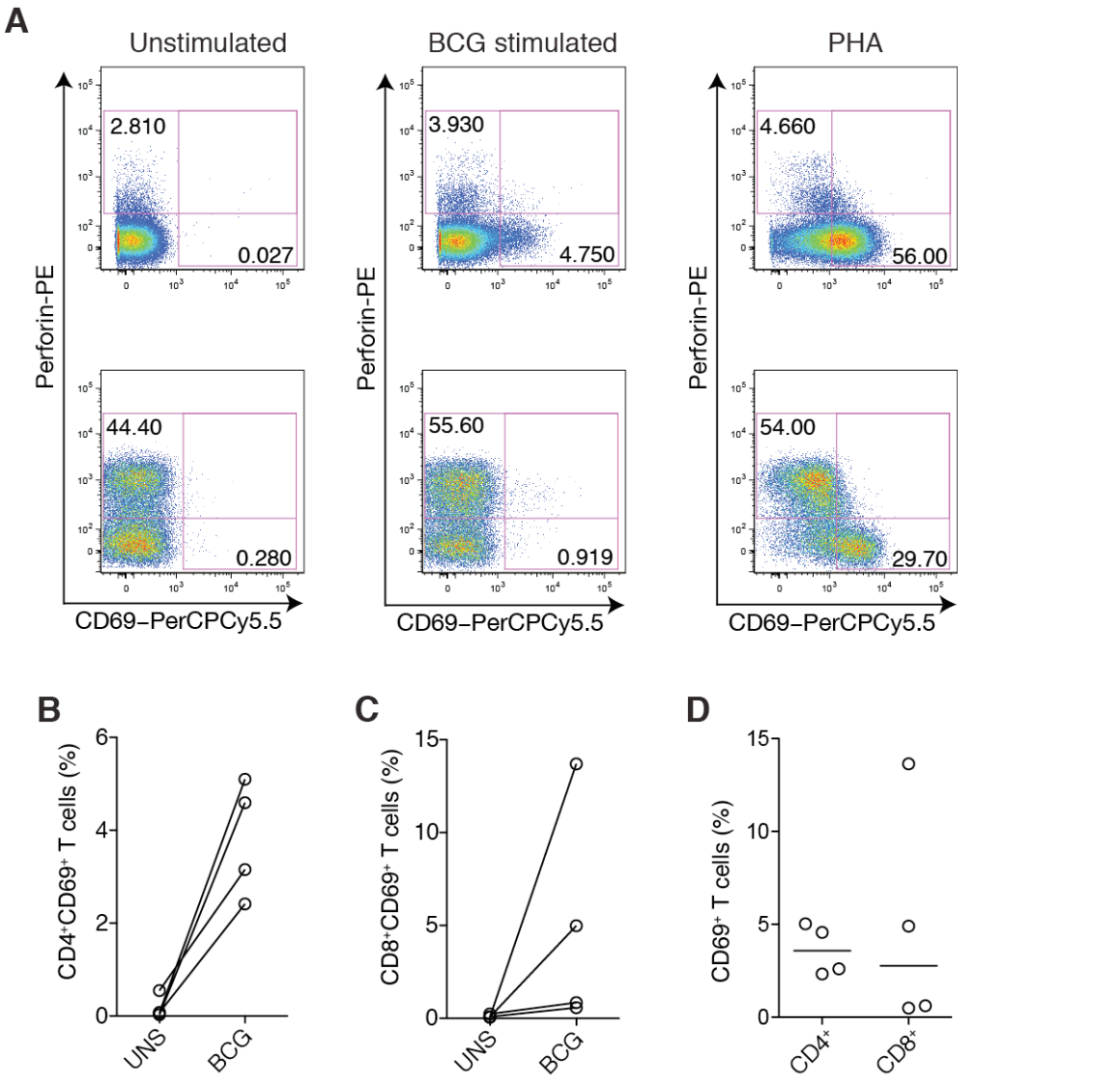

Figure 7: Expression of CD69 by BCG-specific $CD4^+$ and $CD8^+$ T cells. We measured the expression of CD69 by T cells using the short-term whole blood assay. Representative flow cytometry data of CD69 expression by $CD4^+$ (top row) and $CD8^+$ (bottom row) is shown (**A**). Frequency of CD69 expression by $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells in unstimulated (UNS) and BCG-stimulated samples are shown. **Figure D** shows the summary data of BCG-induced (after background subtraction) CD69 expressing $CD4^+$ and $CD8^+$ T cells from 4 participants. On the scatter plots, the horizontal lines represent the median frequencies of the specific cells.

3.6.3. Perforin expression

Our next aim was to evaluate whether BCG-induced perforin was measureable in our assay system. Before addressing this aim, we titrated the newly introduced PE conjugate of the D-B48 antibody clone chosen because we wanted to detect both preformed and newly formed perforin (Hersperger et al., 2008). Also, the PE channel was open on our list of antibody-fluorochrome combination, taking into account previously optimized antibodies in our laboratory.

Fixed white cells from BCG stimulated whole blood were divided into 8 aliquots and each was stained with different concentrations of Perforin-PE antibody. **Figure 8A** shows the flow cytometry plots of the different antibody titre volumes against perforin expression by $CD3^+$ T cells. When we plotted the frequency of perforin expressing cells (**Figure 8B**) as well as the signal to noise ratio of perforin expressing cells (**Figure 8C**) we observed a typical antibody titration curve. Both plots showed the curve plateaued at a concentration of 2.50μl, which was chosen for subsequent experiments.

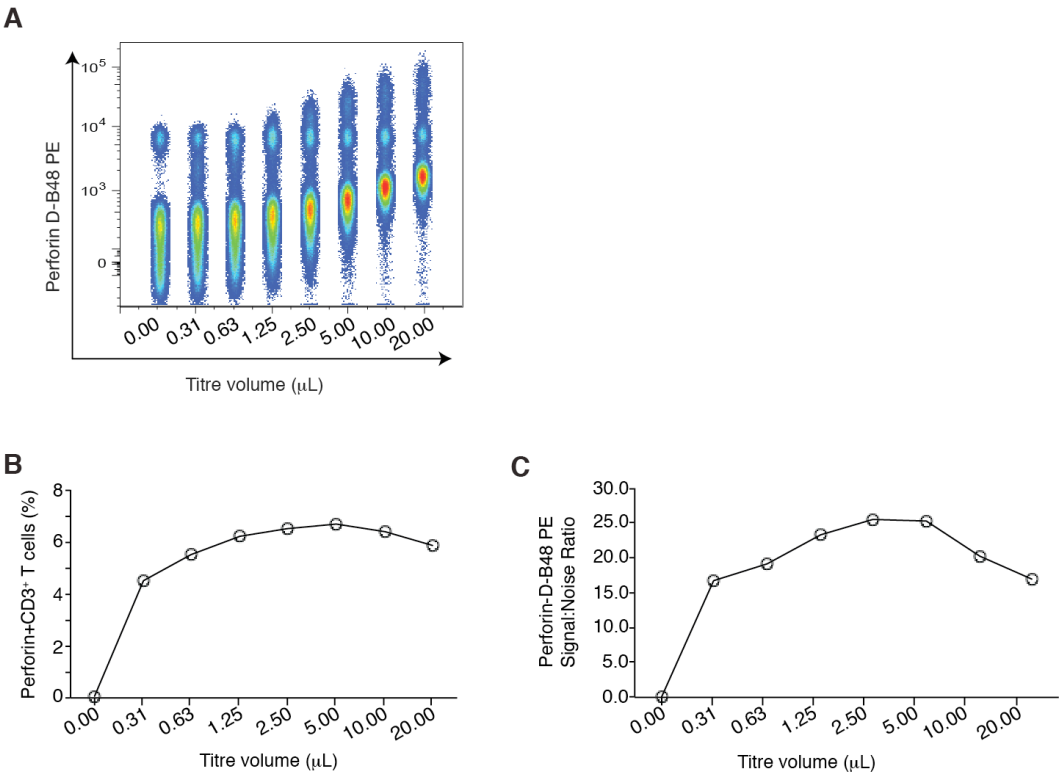

Figure 8: **Perforin-PE antibody titration.** Eight different perforin-PE antibody concentrations were tested using fixed white cells from BCG stimulated whole blood. For flow analysis, singlets were selected, then lymphocytes from which we selected $CD3^+$ T cells. Flow cytometry data of CD69 expression, measured by MFI at different concentrations are shown (**A**). We then measured frequencies (**B**) and signal to noise ratio (**C**) of perforin expression by $CD3^+$ T cells at different antibody titer volumes. The optimal titration volume was assessed by plots of the frequency of CD3 T cells expressing perforin **(B)** and the signal (perforin+) to noise (perforin-) ratio against the titration volume

After establishing the optimal concentration of the Perforin-PE antibody, we proceeded to measure BCG-induced perforin expression. We used fixed white cells from BCG stimulated whole blood samples collected from 8 healthy adult volunteers. We gated on $CD69^+$ cells to assess specific perforin expression (Murray et al., 2006). Our results showed that perforin was expressed at high frequencies following BCG than in the unstimulated sample. Although detectable in both $CD4^+$ and $CD8^+$ T cells, frequencies were higher in the latter subset (**Figure 9**). We concluded that BCG-induced perforin T cell response is measurable in our assay system. We therefore included perforin-PE in our study PFC antibody panel.

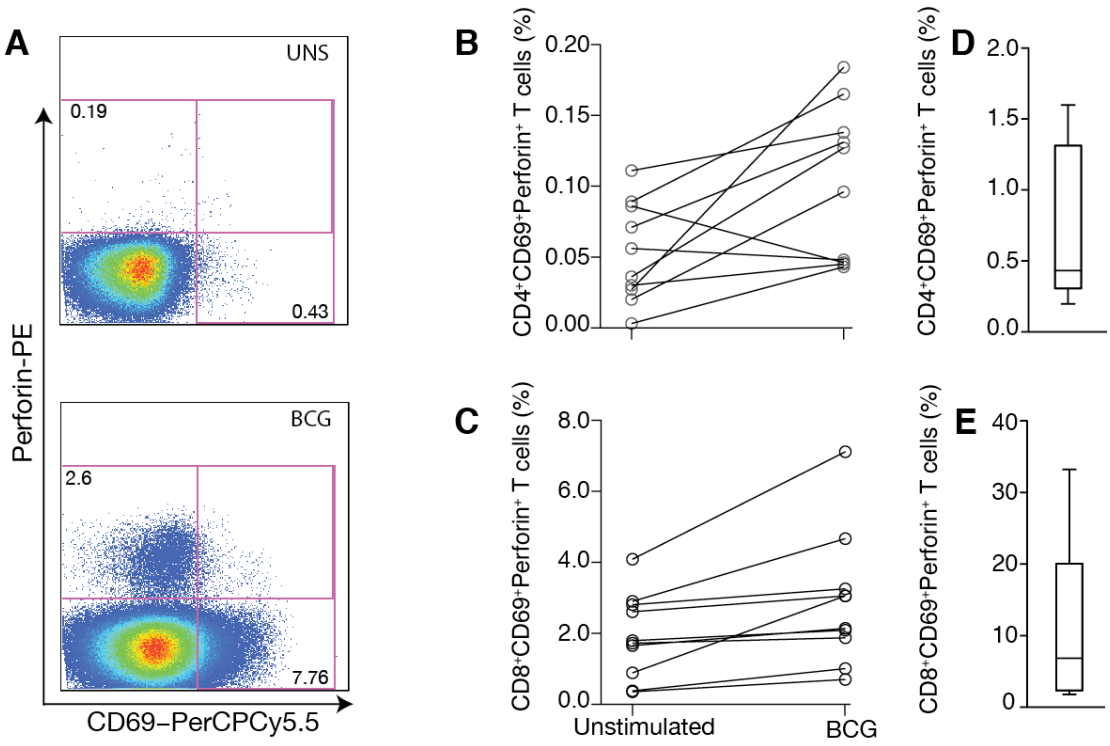

Figure 9. **BCG-specific perforin expression by CD4$^+$ and CD8$^+$ T cells.** We measured the specific responses using the short-term whole blood assay. Representative flow cytometry data of perforin expression by CD8$^+$ T cells in a negative control (unstimulated) sample (top row) or BCG stimulated sample (bottom row) (**A**). Perforin expression in CD4$^+$ (**B**) and CD8$^+$ (**C**) T cells following no stimulation or stimulation with BCG. Scatter plot to depict frequency of BCG-specific perforin expression in CD4$^+$ and CD8$^+$ T cells is shown in **D** and **E** respectively. On the scatter plots, the horizontal lines represent the median frequencies of the specific cells.

3.3.4. CD8-QDot 565 antibody titration

Our next step was to titrate the newly introduced CD8-QDot 565 antibody using the samples we had previously used to titrate other antibodies. We used a similar titration method as for other antibodies. We showed the CD8-Qdot 565 antibody staining could give distinct populations of CD8$^+$ and CD8$^-$ T cells at a titre volume of 0.5μl and above (**Figure 10A**). When we plotted the frequency of CD8-expressing CD3$^+$ T cells (**Figure 10B**) as well as the signal to noise ratio of CD8-expressing CD3$^+$ T cells (**Figure 10C**) against the antibody concentration, we chose the antibody titer volume of 0.5μl. With this antibody concentration, we could easily discriminate CD8-expressing CD3$^+$ T cells from non CD8-expressing CD3$^+$ T cells.

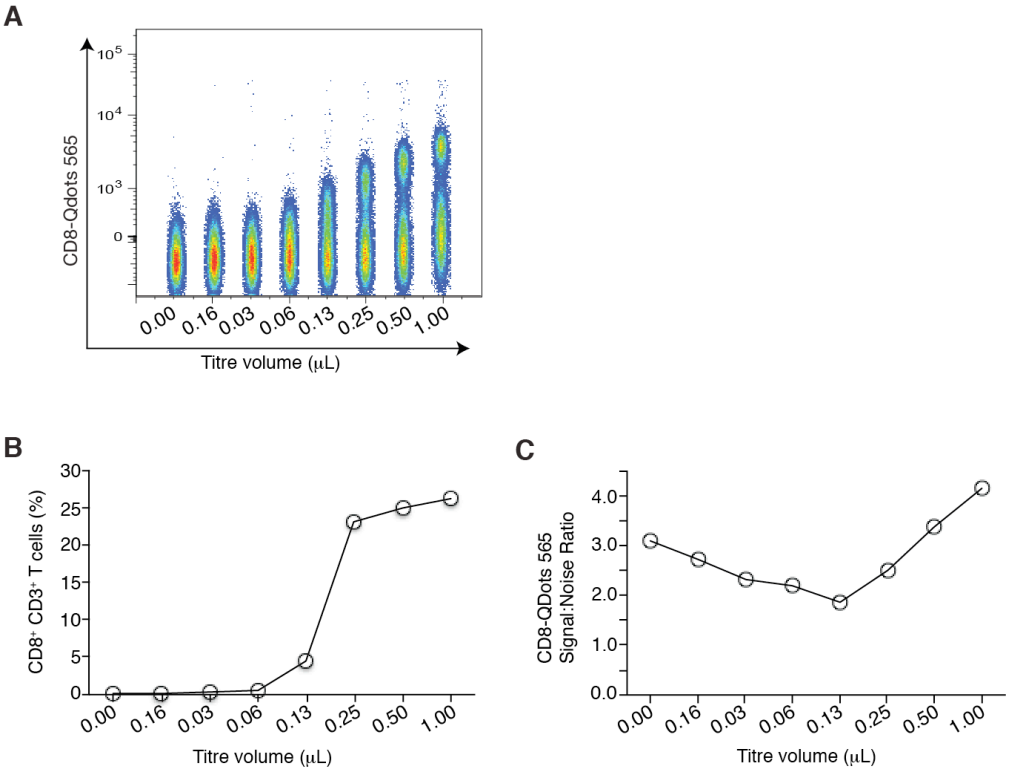

Figure 10. **CD8-Qdot 565 antibody titration.** Eight different CD8-Qdot 565 antibody concentrations were tested using fixed white cells from BCG stimulated whole blood. For flow analysis, singlets were selected, then lymphocytes from which we selected $CD3^+$ T cells. Flow cytometry plots of $CD8^+$ expression was then measured by MFI at different concentrations (**A**). We then measured frequencies (**B**) and signal to noise ratio (**C**) of $CD8^+$ expression by $CD3^+$ T cells at different antibody concentrations.

Following the successful titrations and tests of the CD69-PerCPCy5.5, Perforin-PE and CD8-Qdot 565 antibodies, we were now ready to combine these into the existing and optimized panel (CD3-Pac Blue, CD4-Qdot 605, CD8-Qdot 565, CD69-Cy5.5PerCP, TNF-α-Cy7PE, IFN-γ-Alexa 700, IL-2-FITC, IL-17-Alexa 647 and Perforin-PE) to assemble a final 9 colour PFC panel.

Before this final PFC antibody assembly step, we wanted to test if adding EDTA to our staining buffer could improve the quality of staining by antibodies conjugated to Qdot (CD4 and CD8 antibodies).

3.6.5. Effect of staining buffer on CD4-Qdot 604 and CD8-Qdot 565 brightness

Quantum dots (Qdot) are fluorescent nanocrystals that are widely used in flow cytometry analysis (Chattopadhyay et al., 2006). Antibodies conjugated to Qdot are sensitive to heavy metals such as iron, copper and zinc. These heavy metals are contained in staining buffers (Meallet-Renault et al., 2006). Previous studies have shown that adding EDTA, at a concentration of 1mM, can improve the BSI of antibodies conjugated with Qdot (Zarkowsky et al., 2011). We therefore compared the brightness of CD4-Qdot 605 and CD8-Qdot 565 if staining is done using BD Perm/wash with or without addition of 1mM EDTA. We used fixed white cells from BCG stimulated whole blood. Representative flow cytomety plots for this staining are shown (**Figure 11A**). No differences were observed in the quality of $CD4^+$ and $CD8^+$ staining with or without 1mM EDTA, when we analysed the expression levels by frequencies (**Figure 11B, C**) or by signal to noise ratios (**Figure 11D, E**).

We concluded that not adding 1mM EDTA to the BD Perm/wash staining buffer in our ICS experiments would not affect the quality of CD4-Qdot 605 and CD8-Qdot 565 antibody staining in our PFC panel. Therefore, in our subsequent ICS experiments, we did not add EDTA to BD perm/wash staining buffer.

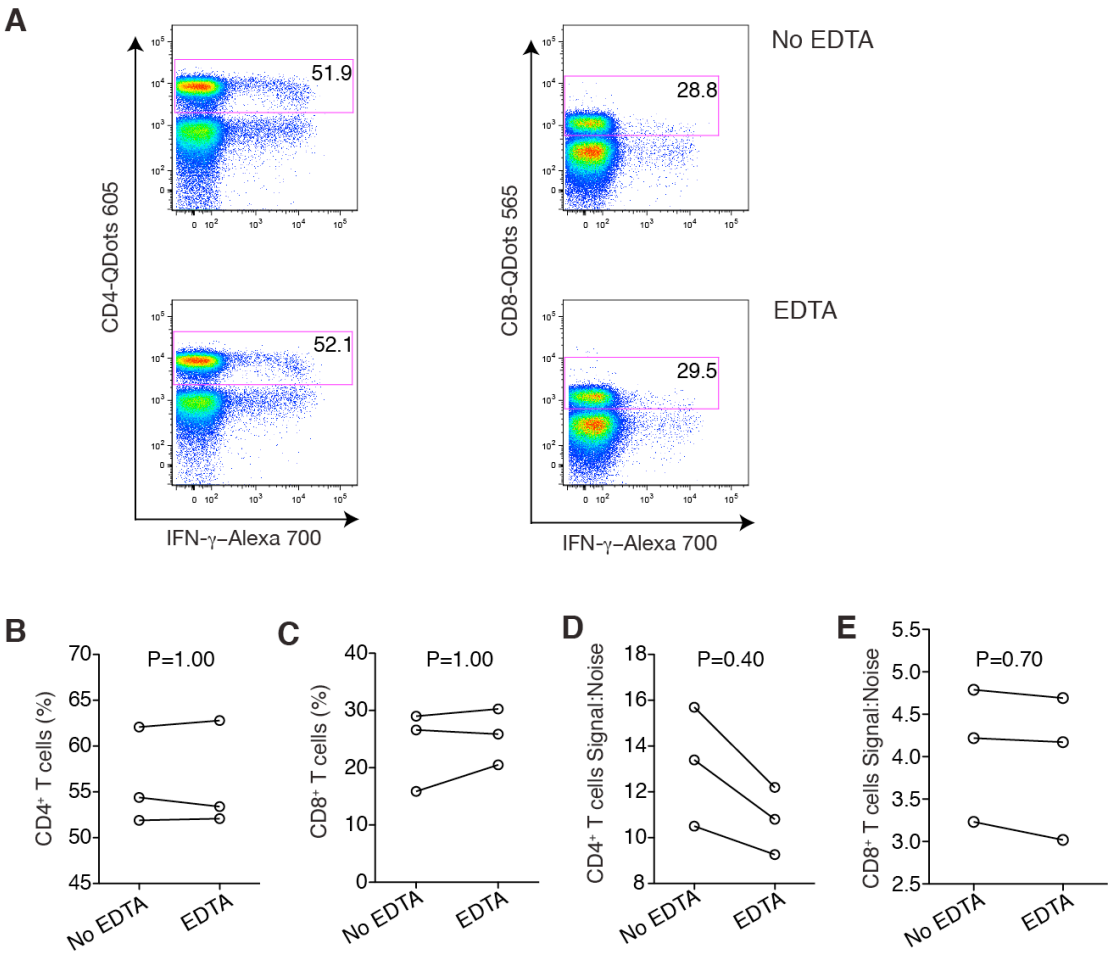

Figure 11: Effects of adding 1mM EDTA to staining buffer on Qdot-conjugated antibodies. Whole blood from three adult volunteers was stimulated with BCG for 12 hours, red blood cells lysed, white cells fixed, followed by cryopreservation. An ICS assay was performed using BD Perm/wash with or without 1mM EDTA added. Representative flow cytometry data showing expression of $CD4^+$ and $CD8^+$ T cells with EDTA (top row) or without EDTA (bottom row) are shown (**A**). Frequency of $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells with or without EDTA. Median fluorescence intensity of $CD4^+$ and $CD8^+$ T cells with or without EDTA are shown in **(D)** and **(E)** respectively.

3.6.6. Final antibody-fluorochrome combination PFC panel

Our final PFC antibody panel comprised of the following: CD3-PacBlue, CD4-QDot 605, CD8-QDot 565, CD69-Cy5.5PerCP, TNF-α-Cy7PE, IFN-γ-Alexa 700, IL-2-FITC, IL-17-Alexa 647 and Perforin-PE (**Table 5**).

Marker	Laser	Detector	Fluorochrome	Manufacturer	Cat.No
CD4	Violet	A	Qdot 605	Invitrogen	Q10008
CD8	Violet	B	Qdot 565	Invitrogen	Q10152
CD3	Violet	C	Pac Blue	BD	558117
IFN-γ	Red	B	Alexa 700	BD	544699
IL-17	Red	C	Alexa 647	eBiosciences	51-7178
TNF-α	Blue	A	Cy7PE	eBiosciences	25-7349
CD69	Blue	C	Cy5.5PerCP	Biolegend	310926
Perforin	Blue	E	PE	Diaclone	854.951.010
IL-2	Blue	F	FITC	BD	340448

Table 5: Description of the final antibody-fluorochrome combinations. For our final PFC antibody panel, all the 9 selected T cell markers and the fluorochrome combination chosen are shown. The lasers, channels (detectors) on the LSR II cytometer for assessing these markers, supplying company of the antibodies as well as the catalogue numbers are shown as well.

3.6.7. Optimisation of photomultiplier tube (PMT) voltage

Our next aim was to establish the optimal PMT voltages for all the selected channels in our 9-colour PFC panel. Optimal PMT voltages ensure that for each fluorescence parameter, the best signal to noise ratio is achieved (Maecker and Trotter, 2006). Suboptimal PMT voltage results in the stained antibodies not being on the detectable scale, or may yield unclear differentiation between negative and positive events.

To achieve the optimal PMT voltages for our PFC antibody panel, we used the FACSDIVA^{TM} 6.0 software's Cytometer Setup & Tracking (CS&T) to first define the baseline cytometer voltages for our selected channels. The BD FACSDIVA^{TM} 6.0 software's CS&T automatically determines the optimal range for the following parameters: laser delays, linear range (±2%), fluorescence detector efficiency (Qr), optical background (Br), electronic noise (SD_{EN}) and the baseline PMT voltage settings based on performance of the dim beads (**Table 6**).

The baseline PMT voltages defined for blue laser channels were as follows in: 667 for Cy5.5PerCP, 701 for both Cy7PE and 620 for PE, and 690 for FITC (**Table 6)** Baseline voltages for the red laser channels were 647 for Alexa Fluor 647 and 781 for Alexa Fluor 700 (**Table 6**). The baseline voltages for the violet laser channels were 823 for Qdot 605, 589 for Qdot 565 and 646 for Pac Blue (**Table 6**). For further PMT voltage optimization, we tested and adjusted the CS&T baseline voltages using unstained cells, followed by single stained mouse kappa compensation beads.

Laser	Detector	Parameter	PMTV	Bright Bead Robust CV	DIM Bead Robust CV	SD_{EN}	Qr	Br
Blue	FCS	FCS	614	5.31	23.78	N/A	N/A	N/A
Blue	G	SSC	394	4.21	2.47	N/A	N/A	N/A
Blue	F	FITC	690	3.48	58.87	29.4	0.0096	405
Blue	E	PE	620	3.13	71.06	20.5	0.0544	684
Blue	B	PerCP-Cy5.5	667	6.50	94.82	20.3	0.0022	136
Blue	A	PE-Cy7	701	11.46	889.56	22.7	0.0022	36
Red	C	Alexa 647	646	3.61	60.16	18.1	0.0077	90
Red	B	Alexa 700	781	4.47	46.66	79.1	0.0055	0
Violet	C	Pac Blue	646	4.94	84.46	21.2	0.0193	5935
Violet	B	Qdot 565	589	5.06	76.30	15.9	0.0328	285
Violet	A	Qdot 605	823	8.53	294.91	18.5	0.0028	491

Table 6: Baseline PMT settings. CS&T beads were placed in a falcon tube and run on a LSR II cytometer. The baseline parameters automatically generated by the CS&T system including: Baseline PMT voltages, bright bead robust CV, standard deviation of median fluorescence intensity of the bright bead, fluorescence detector efficiency (Qr), optical background (Br), and electronic Noise (SD_{EN}).

3.6.8. Choice of the final PMT voltages

Next, we adjusted the baseline PMT voltages while using unstained cells to achieve adequate positioning (within the first log decade on the x-axis scale) of the negative cell population. Thereafter, a similar approach was used to adjust the PMT voltages further while using the single stained mouse kappa compensation beads to achieve adequate positioning of the positive stained cell population (past the first decade of the x-axis but below the fifth decade) for each channel.

Using the unstained cells, we adjusted the baseline PMT voltages from 667 to 550 for Cy5.5PerCP, from 701 to 560 for Cy7PE, from 530 to 620 for PE, and from 690 to 560 for FITC to give an on scale negative cell population for the blue laser (**Figure 12A**). The voltage adjustments for the red laser channels were as

follows: from 646 to 560 for Alexa Fluor 647 and from 781 to 550 for Alexa Fluor 700 (**Figure 12B**). The voltage adjustments for the violet laser channels were as follows: From 823 to 550 for Qdot 605, from 589 to 500 for Qdot 565 and from 624 to 500 for Pac Blue (**Figure 12C**).

Finally, when we used single stained anti-mouse kappa-compensation beads (BD Biosciences), labelled with the specific fluorochrome-conjugated antibodies selected for the 9-colour panel, no adjustments were made to the voltages as the positive stained beads were in optimal fluorescence intensity for all the channels (**Figure 13A, B and C**)

From these experiments, we established the optimal PMT voltages for each and every channel selected in our final PFC antibody panel to provide maximum resolution of the positive and negative antibody staining.

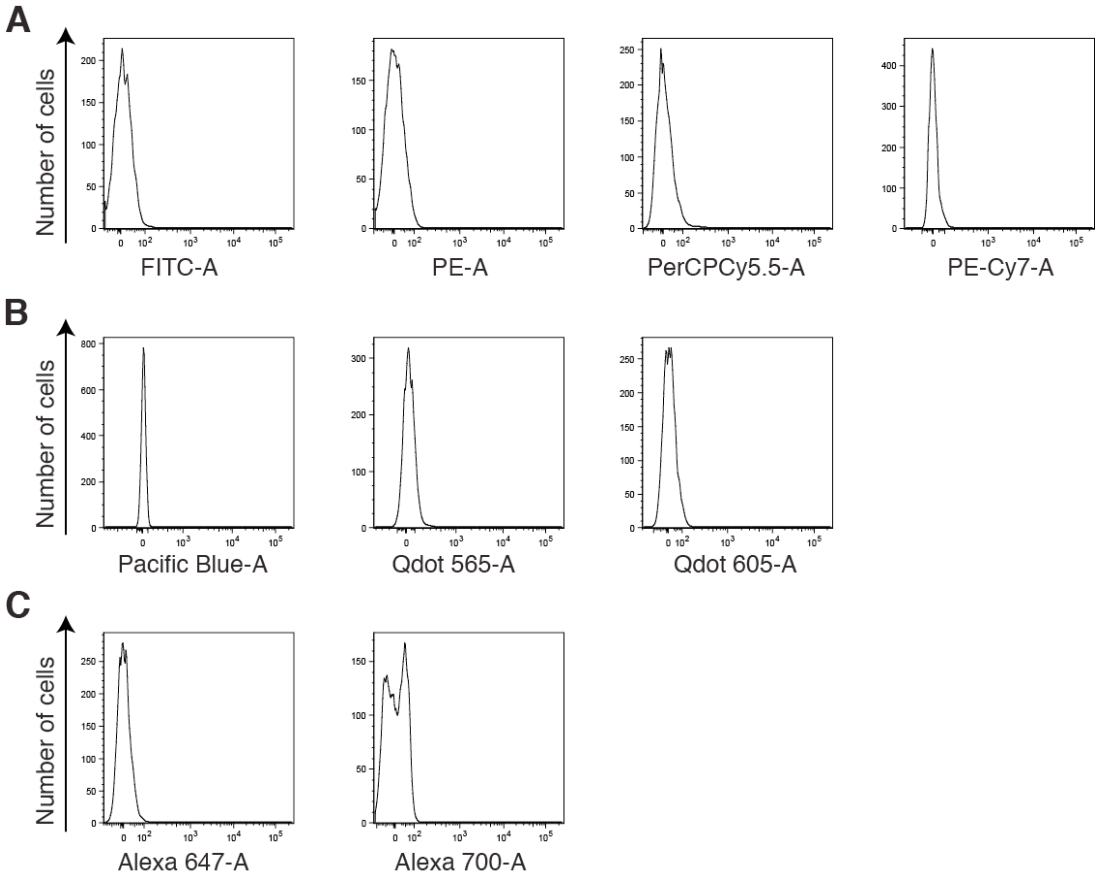

Figure 12: PMT voltage settings using unstained cells. Unstained cells were acquired on LSR II. Lymphocytes were selected by forward scatter-area (FSC-A) against side scatter-area (SSC-A). For each channel of the final 9-colour PFC panel, PMT voltages were adjusted to the established CS&T baseline settings. Then, the PMT voltages were adjusted until the unstained lymphocytes were detected within the first and second decade of the scale for the channels in the blue laser (**A**), red laser (**B**) and violet laser (**C**).

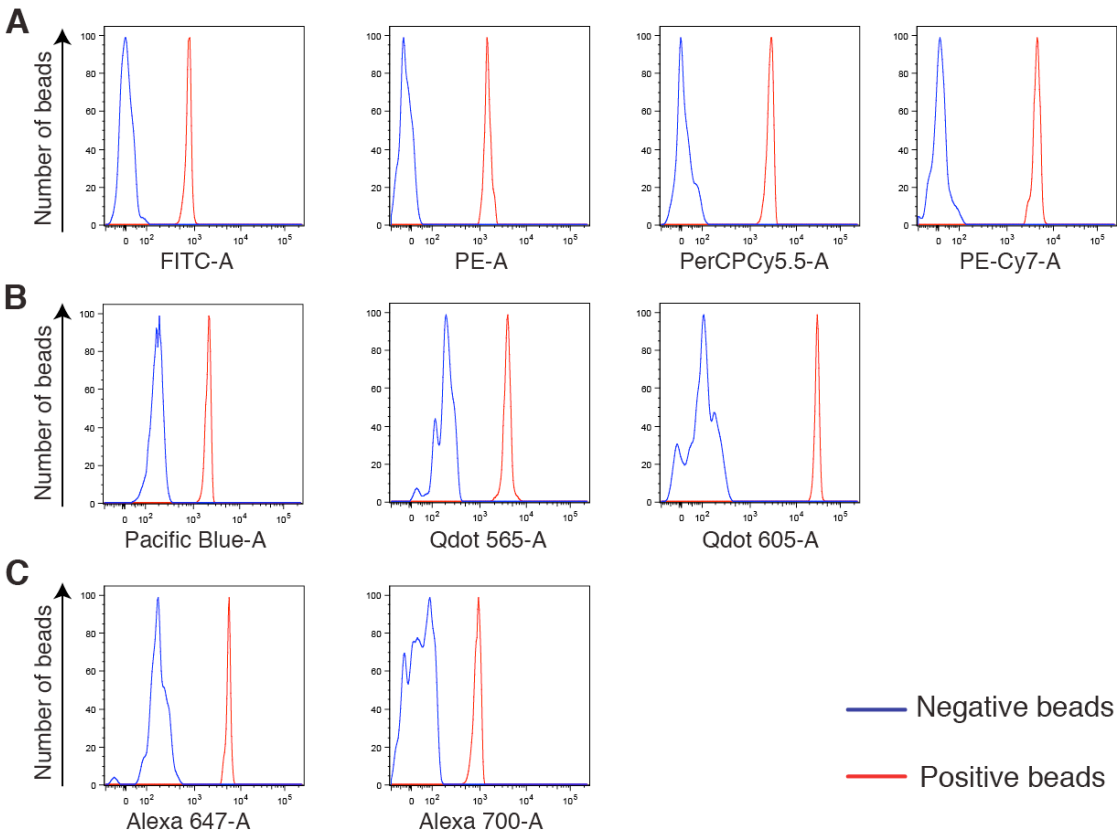

Figure 13: PMT voltage settings using single stained anti-mouse kappa compensation beads. In 9 separate 5mL falcon tubes, single drops of negative and positive anti-mouse kappa compensation beads were added. To each of the tubes, the optimal antibody staining concentrations for CD3-PacBlue, CD4-Qdot 605, CD8-QDot 565, CD69-Cy5.5PerCP, Perforin-PE, TNF-Cy7PE, IFN-γ-Alexa 700, IL-2-FITC and IL-17-Alexa 647 were added to each of the 9 tubes and acquired on LSR II. On the forward scatter-area (FSC-A) against side scatter-area (SSC-A) plot, we selected the bead population.. The final PMT voltage settings showed distint negative and positive beads population for the blue laser (**A**), red laser (**B**) and violet laser (**C**).

After we established the optimal PMT voltages for each of the 9 channels. Our next task was to test if the size of the PFC panel was optimal in measuring the BCG-induced T cell response.

3.6.9. Measurement of BCG-induced T cell responses using 5, 7 and 9 PFC antibody panels

Our next aim was to assess if the 9-colour PFC antibody panel assembled for our study, was as sensitive as smaller (5 and 7) PFC antibody panels in detecting the BCG-induced T cell response. Larger panels are more vulnerable to spectral overlap than smaller panels and this may compromise the sensitivity to measure some markers (Perfetto et al., 2004).

Fixed white cells from BCG stimulated or unstimulated whole blood derived from four healthy adult volunteers were stained with a 5, 7 or 9-colour PFC antibody panels. The 5-colour PFC antibody panel was selected based on the T cell markers that are highly induced following stimulation of whole blood (IFN-γ and TNF-α) (Maecker et al., 2004) and markers that are most highly expressed in our list of 9 candidate markers (CD3, CD4 and CD8). Two additional markers (IL-2 and IL-17) were added to the 5-colour PFC antibody panel. Finally, CD69 and perforin antibodies were added to assemble the final target 9-colur PFC antibody panel. **Table 7** shows the markers included in the 5, 7 and 9-colour panels.

Our results showed the 9-colour PFC panel was as sensitive as the 5- and 7-colour PFC panels in quantifying the frequencies of BCG-induced IFN-γ- and TNF-α-expressing $CD4^+$ T cells (**Figure 14B and C**). The Frequencies of $CD4^+$ T cells were also similar across the 3 panels (**Figure 14A**).

We were successful in assembling a sensitive 9-colour PFC antibody panel to compare BCG-induced T cell immunity in our study.

Laser	Detector	5 colour panel	7 colour panel	9 colour panel
Violet	A	CD4	CD4	CD4
Violet	B	CD8	CD8	CD8
Violet	C	CD3	CD3	CD3
Red	B	IFN-γ	IFN-γ	IFN-γ
Red	C		IL-17	IL-17
Blue	A	TNF-α	TNF-α	TNF-α
Blue	B			CD69
Blue	E			Perforin
Blue	F		IL-2	IL-2

Table 7: Summary of 5-, 7- and 9-colour antibody-fluorochrome combinations. Three different panels were used to assess if the panel size compromised the sensitivity and reliability to measure the BCG-induced T cell immunity.

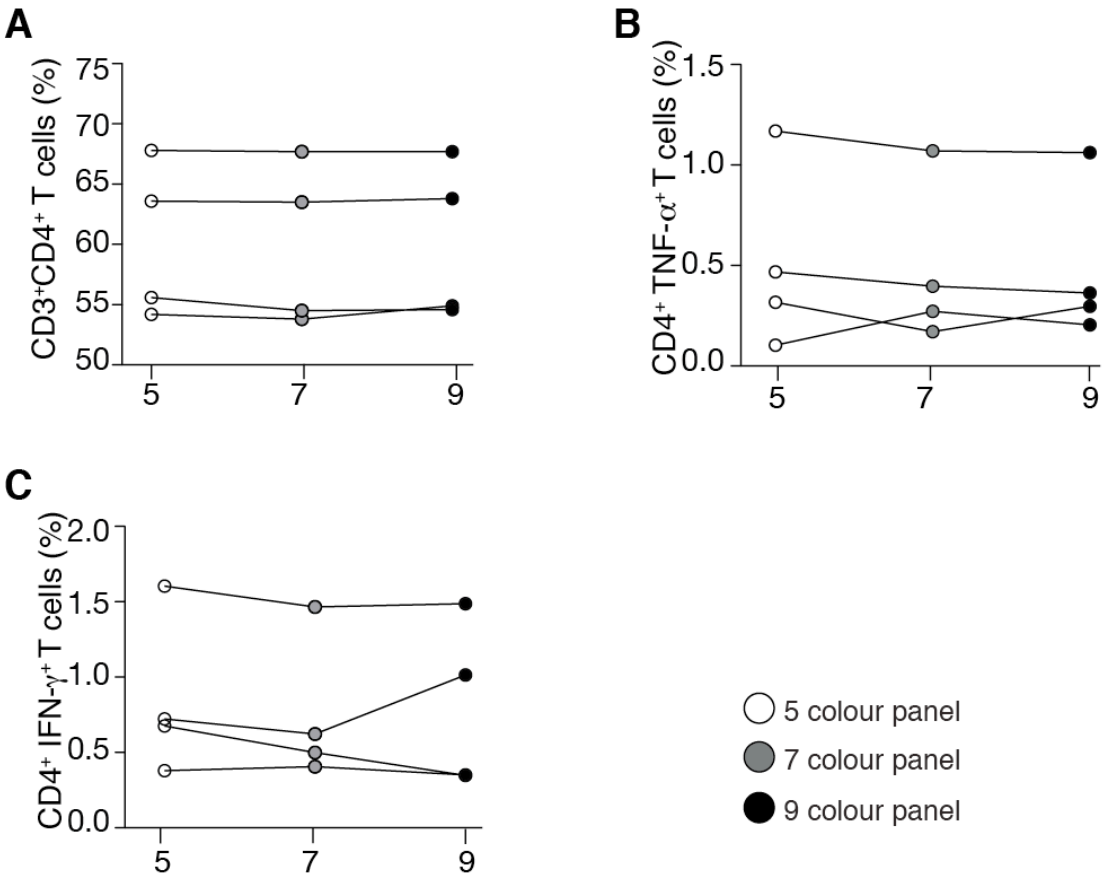

Figure 14: Frequencies of, $CD4^+$ T cells, specific $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ detected using a 5-, 7- and 9-colour PFC panels. We performed whole blood Intracellular cytokine staining on fixed white cells from BCG stimulated whole blood from 3 healthy adult volunteers. We used 5-, 7- or 9-colour PFC antibody panels to stain the cells. The graphs depict frequencies of $CD4^+$ expressing $CD3^+$ T cells (**A**), IFN-γ-expressing $CD4^+$ T cells (**B**), and TNF-α-expressing $CD4^+$ T detected with the 5-colour (open dots), 7-colour (grey dots) or a 9-colour PFC antibody panel (black dots).

We showed that our optimized 9-colour PFC antibody panel was sensitive in quantifying the BCG-induced T cell immunity. Our final step was to assess for the performance and reliability of the 9-colour panel by fluorescence minus one experiments (FMO.

3.6.10. Fluorescence minus one (FMO) analysis

Our final experiments aimed to assess unintended signals due to spectral overlap (Maecker et al., 2004), detected in each of the 9 channels selected for our final PFC antibody panel. FMO is used to address our aim; this involves

assessing signal in a specific channel when no antibody for that channel is present, while all the other antibodies are present (Perfetto et al., 2004). The signal detected in the FMO detector can be quantified and is an indication of the spectral overlap from other fluorochromes in the panel.

We used fixed white cells from BCG stimulated whole blood derived from a healthy adult. We split the cells into 10 aliquots. One aliquot was stained with a full 9-colour panel or 9 combinations of the panel minus a single antibody-fluorochrome. The FMO controls showed an acceptable range of spectral overlap of between 0.00% (IFN-γ-Alexa 700) to 0.52% (CD8-Qdot 565) for all the channels (**Table 8**). The frequencies of the different T cell markers in the PFC panel were similar when measured by different FMO controls as well as the full 9-colour PFC antibody panel. Our data indicates that the spectral overlap detected in all the nine channels was minimal and would not compromise the reliability of the panel to measure the BCG-induced T cell immunity.

We also showed low coefficients of variation (CVs) of the frequencies of the different T cell markers measured for each of the FMO controls, and for the full 9-colour PFC antibody panel (**Table 8**). The CV was calculated by dividing the standard deviation of a frequency by the mean frequency. For immunological assays a CV of up to 30% may be considered acceptable (Nomura et al., 2000). The intra-assay CV ranged from 1% for BCG-specific $CD3^+$ frequencies to 20% for BCG-specific $CD4^+$IL-17^+ and $CD4^+$TNF-α^+ frequencies (**Table 7**).

In summary, we succeeded in optimising a 9-colour PFC antibody panel that was sensitive and reliable in quantifying BCG-specific T cell responses.

	Frequency of positive events detected (%)								
FMO	**CD3**	**CD4**	**CD8**	**CD69**	**IFN-γ**	**IL-2**	**TNF-α**	**IL-17**	**Perforin**
PacBlue	0.050	43.10	25.00	19.90	3.01	0.30	1.54	0.36	36.7
Qdot 605	76.70	0.01	22.90	13.30	2.69	0.30	1.35	0.24	35.3
Qdot 565	77.30	43.70	0.52	15.80	2.92	0.30	1.53	0.29	35.8
PerCp Cy5.5	76.80	40.70	28.70	0.13	2.78	0.31	1.41	0.24	35.9
Alexa 700	77.20	40.90	25.80	16.40	0.00	0.32	0.87	0.31	35.3
FITC	76.10	40.50	25.80	16.00	3.68	0.03	1.03	0.37	34.7
PE-Cy7	76.60	40.50	25.60	16.40	2.83	0.33	0.07	0.36	35.1
Alexa 647	76.80	41.00	25.10	16.40	3.23	0.30	1.07	0.08	33.4
PE	77.40	41.10	24.90	16.10	3.26	0.30	1.00	0.44	0.06
Full Panel	74.80	39.70	24.70	16.40	2.59	0.37	1.14	0.38	33.1
CV	0.01	0.03	0.06	0.10	0.11	0.07	0.20	0.20	0.03

Table 8: Fluorescence minus one (FMO). Whole blood from an adult volunteer was stimulated with BCG then white cells fixed and harvested. Fixed white cells were stained with the optimized 9-colour panel or with FMOs (shaded boxes). The spectral overlap was assessed by comparing the expression frequencies of cells for different markers with or without single antibody-fluorochrome.

3.6.11. Gating strategy to identify BCG-Specific T cells

The aim of our next experiment was to develop a gating strategy to facilitate a focused analysis of the T cell markers we had included in our optimized 9-colour PFC antibody panel. The development of the gating strategy was guided by our main study question: is there a difference in the BCG-induced T cell immunity when BCG is administered at birth or at 6 weeks of age in Ugandan infants? We used the cells stained with the 9-colour PFC antibody panel in our FMO controls experiment to develop the gating plan.

Our gating strategy started with a time gate to select cells acquired only at the same fluorescence intensity over time. Next, we used keeper gate to exclude antibody aggregates commonly formed by Qdots. These aggregates can non-specifically label some cells leading to false positive signal. Then we proceeded to create a lymphocytes gate on a forward scatter-area (FSC-A) against side

scatter-area (SSC-A) plot. Then, doublet cells were excluded by gating on forward scatter-area (FSC-A) against forward scatter-height (FSC-H). Then, we selected conventional T cells by gating on CD3-expressing cells from which $CD4^+$ and $CD8^+$ T cells were gated. Finally, $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, IL-2, TNF-α, IL-17, CD69 and perforin were selected (**Figure 15**). The developed gating strategy was used to create an analysis template. All subsequent ICS experiments perfomed using the optimized 9-colour PFC antibody panel were loaded on the analysis template to perform batch analysis.

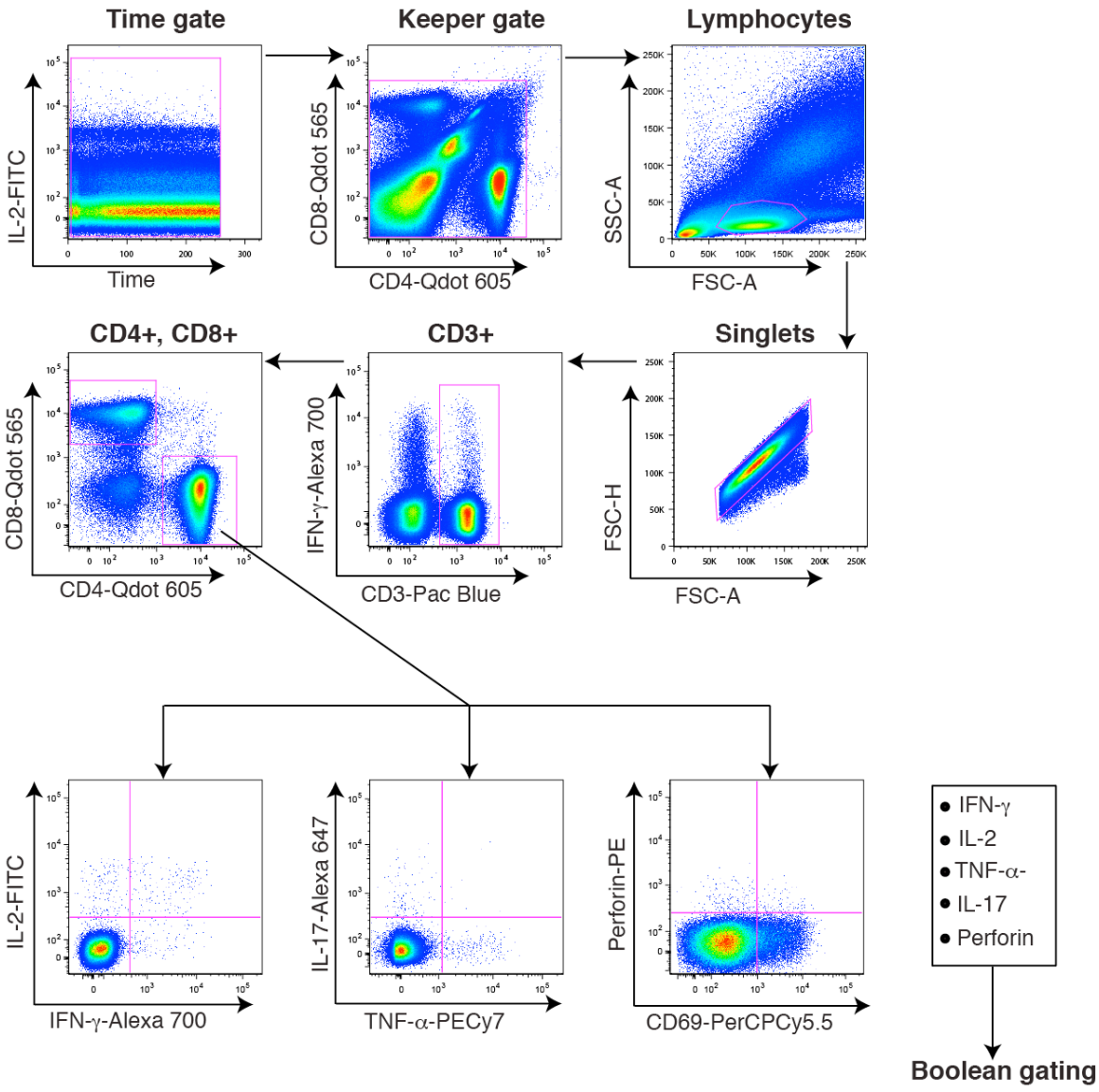

Figure 15. Gating strategy. Flow cytometry analysis of BCG-specific $CD4^+$ and $CD8^+$ T cell cytokine and perforin responses evaluated in the short-term whole blood ICS assay. We first used a time gate to select cells acquired at similar fluorescence intensity over time. Then, we proceeded to a keeper gate to exclude antibody aggregates. Next, lymphocytes were selected using a forward scatter-area (FSC-A) against side scatter-area (SSC-A) gate. Then, doublet cells were excluded by gating on forward scatter-area (FSC-A) against forward scatter-height (FSC-H). Conventional T cells were selected by gating on CD3 expressing cells, which were further divided to $CD4^+$ and $CD8^+$ T cells. Total Intracellular cytokine (IFN-γ, IL-2, TNF-α, IL-17) and perforin expression was then assessed on $CD4^+$ and $CD8^+$ T cells. Finally, Boolean gating was used to assess for cytokine and perforin co-expression

3.6.12. BCG-specific $CD4^+$ and $CD8^+$ T responses in healthy adults

Having optimized our PFC antibody panel and finalized the gating strategy, we aimed to assess whether profiles of BCG-specific T cells, measured using our PFC antibody panel, yielded patterns similar to those previously reported from our laboratory. Whole blood from 5 adult healthy volunteers was left unstimulated or stimulated with BCG or PHA, followed by ICS analysis.

We applied the gating strategy described above to analyse the frequencies of BCG-specific cytokine and perforin expression by $CD4^+$ T cells. Flow data was then exported to Microsoft Excel where we subtracted unstimulated from the stimulated responses. Thereafter, we used GraphPad Prism version 5.0a for analysis. Representative Flow cytometry data showing unstimulated, BCG and PHA-specific IFN-γ, TNF-α, IL-2 and IL-17 frequencies by $CD4^+$ T cells are shown in **Figure 16**.

The BCG-specific $CD4^+$ and $CD8^+$ T cell co-expression showed that the most dominant subset was single IFN-γ-producing cells (**Figure 17A**). Other subsets expressed at high frequencies included polyfunctional (IFN-γ^+TNF-α^+IL-2^+), double positives (IL-2^+IFN-γ^+, IL-2^+TNF-α^+ and IFN-γ^+TNF-α^+) as well as single positives (IL-2^+, IL-17^+, TNF-α^+ and perforin$^+$), mainly by $CD4^+$ T cells (**Figure 17A**). We concluded that the patterns of BCG-induced $CD4^+$ and $CD8^+$ T cell cytokines detected by using our optimized PFC antibody panel were similar to those previously reported in our laboratory (Scriba et al., 2008).

We also observed that perforin was only co-expressed with IFN-γ and not with any other cytokine (**Figure 17B**). The cytokine and perforin expression pattern we observed is similar to what has been reported in a study that assessed cytokine and cytolytic molecule expression in T cells in response to stimulation with viral peptides (Makedonas et al., 2010).

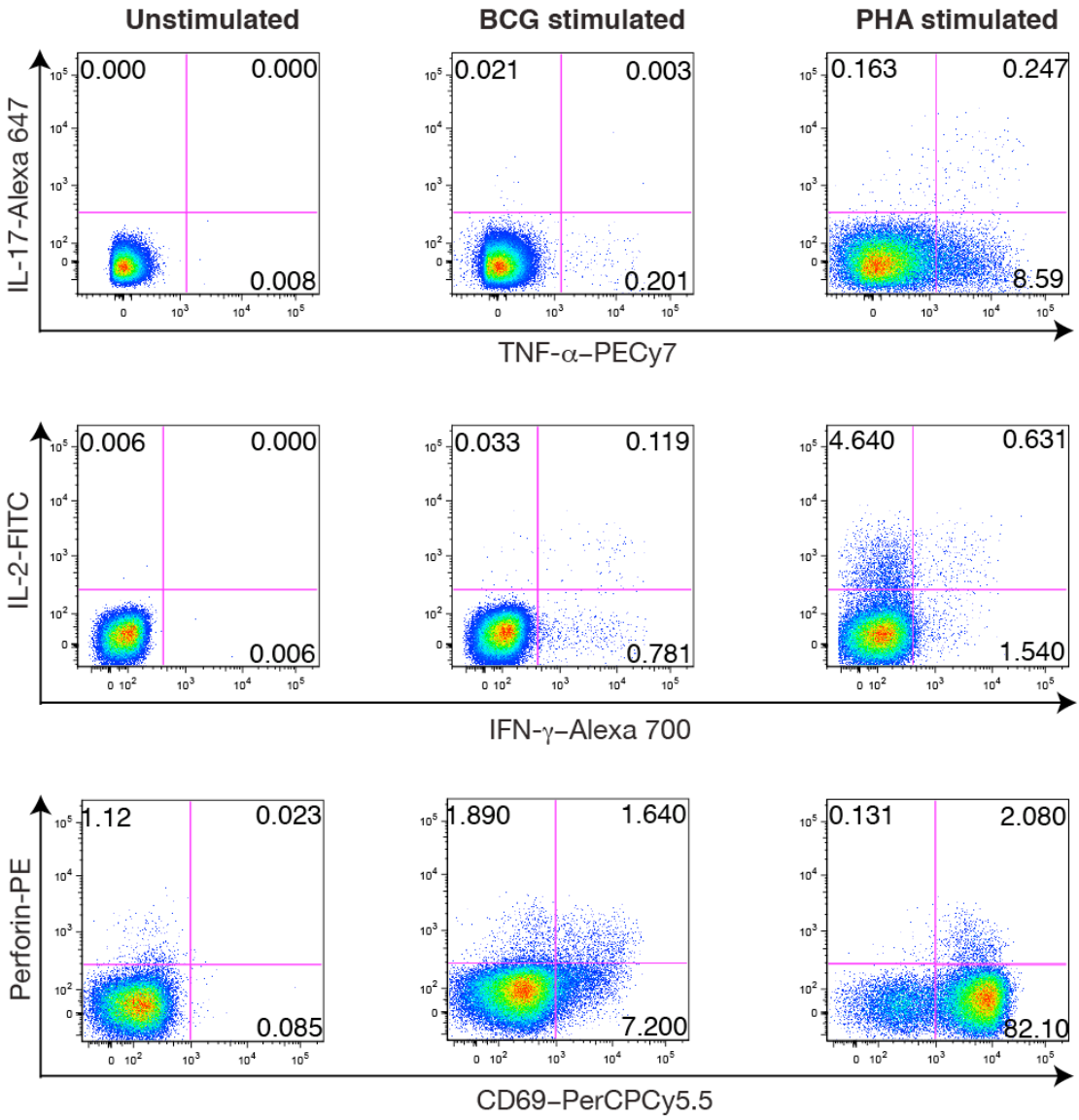

Figure 16: Frequencies of BCG specific CD69, IFN-γ, TNF-α, IL-2, IL-17 and perforin CD4$^+$ T cells. Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or PHA (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD4$^+$ T cells were stained for TNF-α and IL-17 (upper panel), IL-2 and IFN-γ (middle panel), and perforin and CD69 (lower panel).

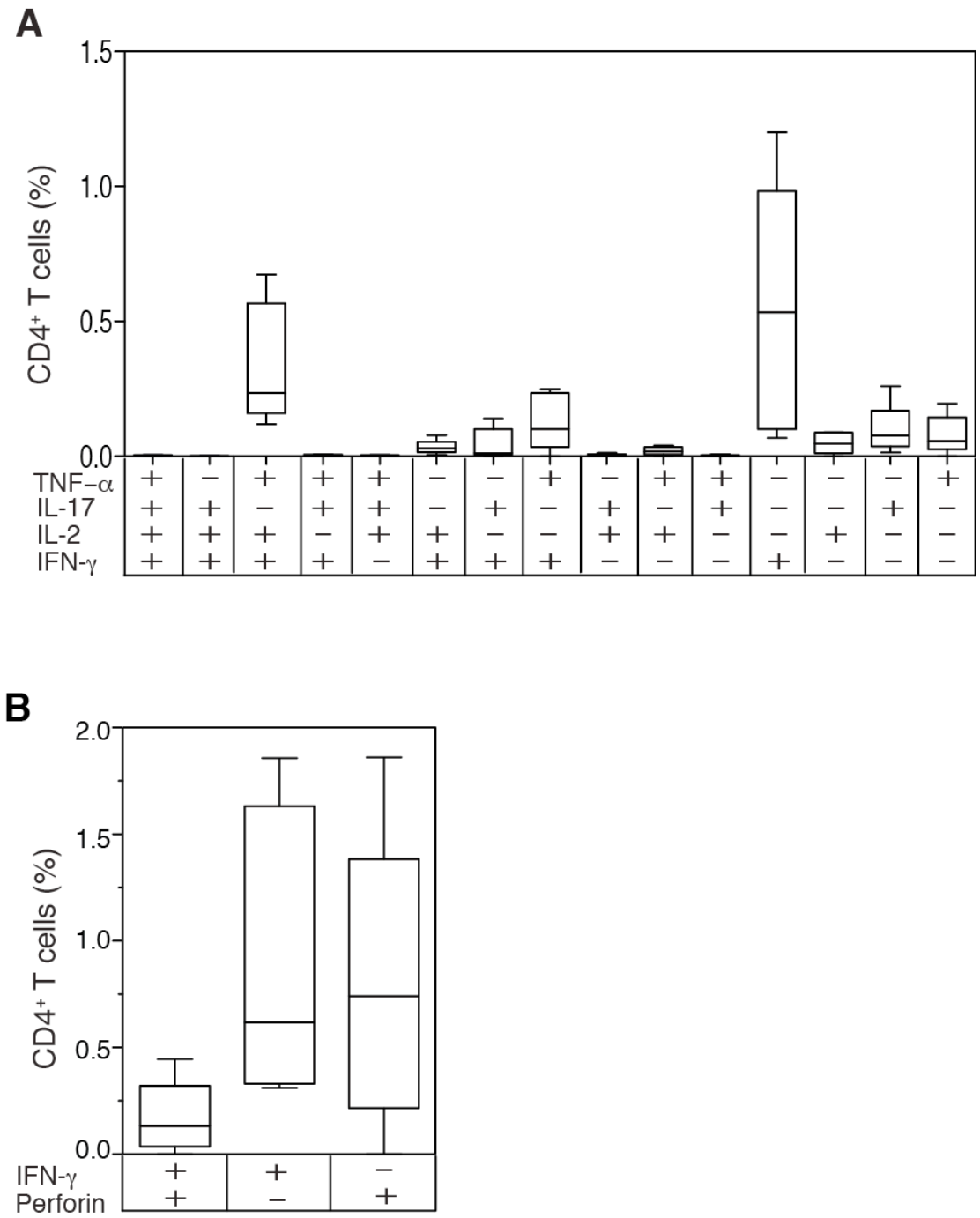

Figure 17. BCG-specific $CD4^+$ T cell cytokine subsets. Whole blood samples from 5 adult volunteers were left unstimulated or stimulated with BCG for 12 hours. White cells were harvested and subsequently stained with the 9-colour PFC antibody panel the acquired on the flow cytometer. Box and whisker plots show the frequencies of distinct subsets of specific $CD4^+$ T cells based on combinations of IFN-γ, TNF-α, IL-2, and IL-17 cytokine expression **(A)**, and $CD69^+CD4^+$ based on IFN-γ and perforin co-expression (**B**). The horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the minimum and maximum values.

We also analysed the profiles of specific $CD8^+$ T cells using a similar approach to that of $CD4^+$ T cells. The representative flow cytometry data in **Figure 18** show unstimulated, BCG- and PHA-stimulated samples. Frequencies of IFN-γ, TNF-α, IL-2 and IL-17 in $CD8^+$ T cells are also shown in (**Figure 19**). The BCG-specific $CD8^+$ T cell co-expression showed that the most dominant subset was single IFN-γ-producing cells as well as double positives (IFN-γ^+TNF-α^+) (**Figure 19A**). Perforin was only co-expressed with IFN-γ and not with any other cytokine (**Figure 19B**). We concluded that the patterns of BCG-induced $CD8^+$ T cell cytokines were similar to those previously reported (Scriba et al., 2008, Soares et al., 2013).

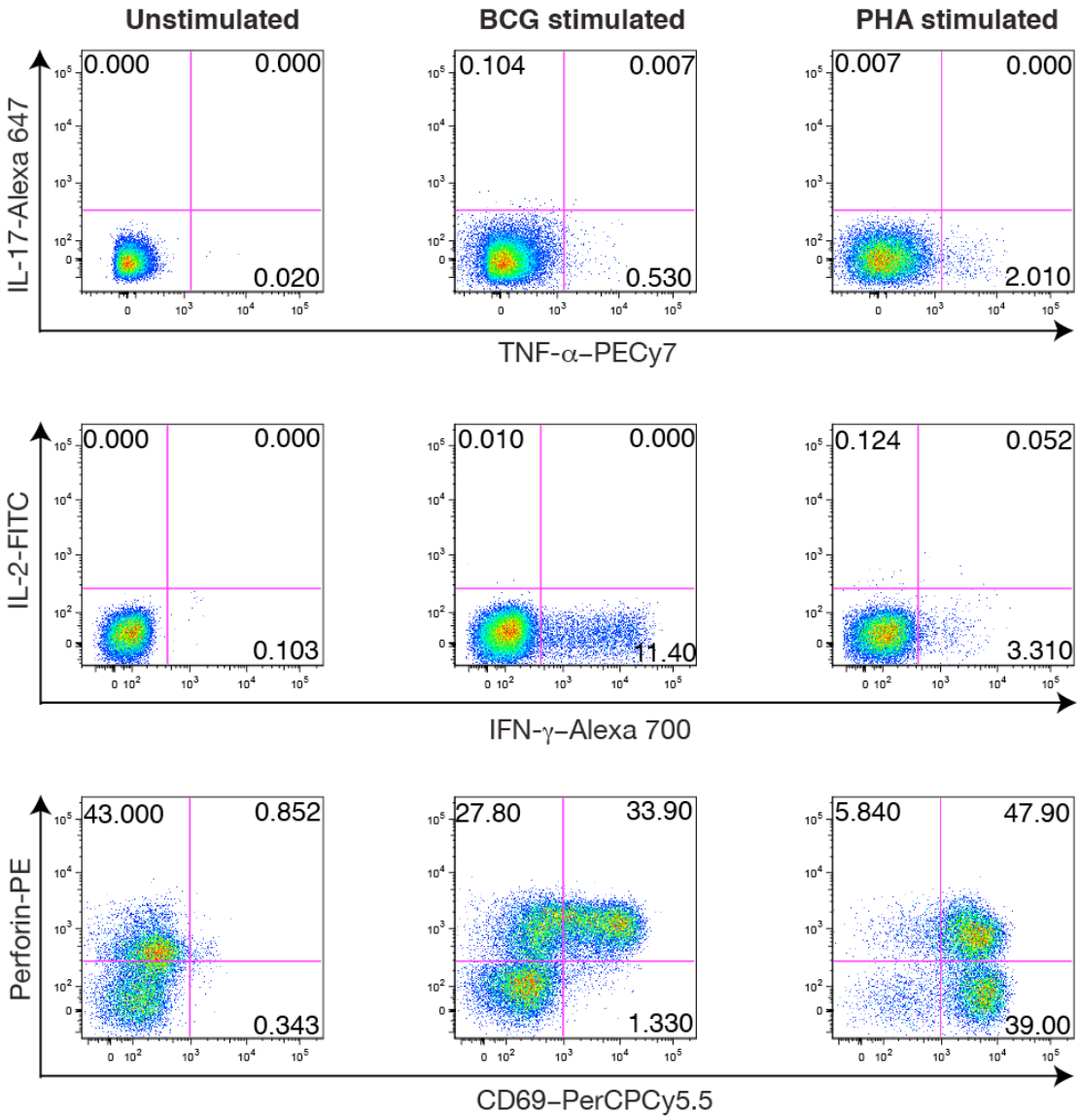

Figure 18: Frequencies of antigen specific CD69, IFN-γ, TNF-α, IL-2, IL-17 and perforin CD8$^+$ T cells. Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or PHA (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD8$^+$ T cells were stained for TNF-α and IL-17 (upper panel), IL-2 and IFN-γ (middle panel), and perforin and CD69 (lower panel).

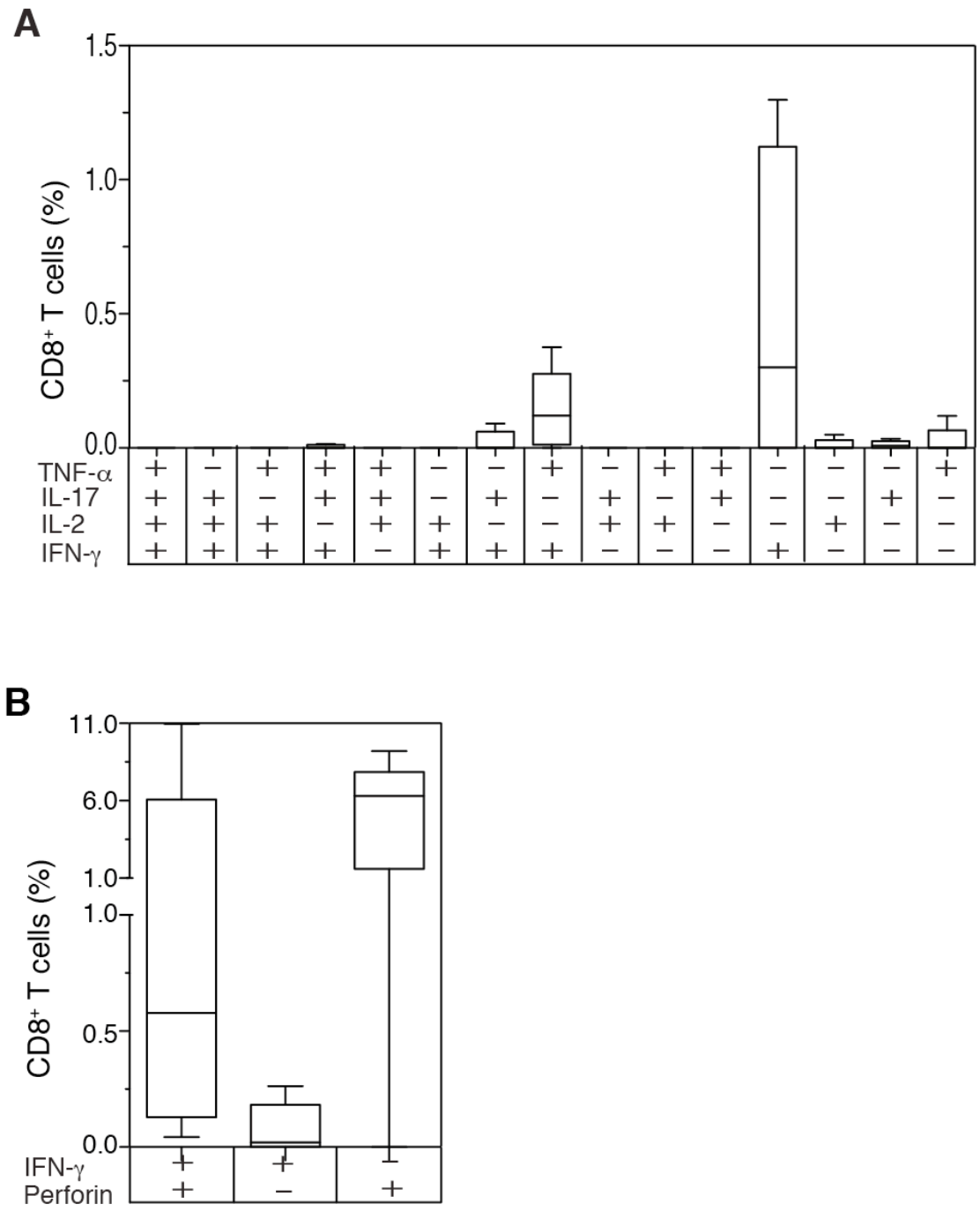

Figure 19. BCG-specific $CD8^+$ T cell perforin subsets. Whole blood samples from 5 adult volunteers were left unstimulated or stimulated with BCG for 12 hours. White cells were harvested and subsequently stained with the 9-colour panel before acquisition on the flow cytometer. Box and whisker plots show the frequencies of distinct subsets of specific $CD8^+$ T cells based on combinations of IFN-γ, TNF-α, IL-2, and IL-17 cytokine expression **(A)**, and $CD69^+CD8^+$ based on IFN-γ and perforin co-expression (**B**). The horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles.

3.6.13. BCG-specific $CD4^+$ T cell responses in healthy 10-weeks old infants

We were satisfied with the performance of the optimized 9-colour PFC antibody panel in quantifying antigen specific T cells using adult samples. Next, we wished to evaluate the performance of the PFC antibody panel in measuring the BCG-specific T cell response in infant-derived whole blood samples.

We used cryopreserved fixed white cells derived from whole blood from 5 healthy infants that was left unstimulated or stimulated with BCG or SEB, followed by ICS analysis. Representative flow cytometry plots showing unstimulated, BCG- and SEB-stimulated samples are shown in **Figure 20**. The BCG-induced $CD4^+$ T cell co-expression showed that the most dominant subsets included polyfunctional (IFN-γ^+TNF-α^+IL-2^+), double positives (IFN-γ^+TNF-α^+ and IFN-γ^+IL-2^+) as well as single positives (IFN-γ^+, IL-2^+, IL-17^+, TNF-α^+ and perforin$^+$) $CD4^+$ T cell (**Figure 21A**). We observed similar cytokine expression patterns for $CD4^+$ infants to those observed to those observed in adults. Infants showed lower BCG-induced $CD4^+$ T cell cytokine frequencies than adults. We concluded that the patterns of BCG-induced $CD4^+$ and $CD8^+$ T cell cytokines were comparable to what has been previously reported (Kagina et al., 2010, Soares et al., 2010).

We then assessed perforin expression in the infant samples. Similar to what we observed with adult samples, perforin was only co-expressed with IFN-γ and not with any other cytokine (**Figure 21B**).

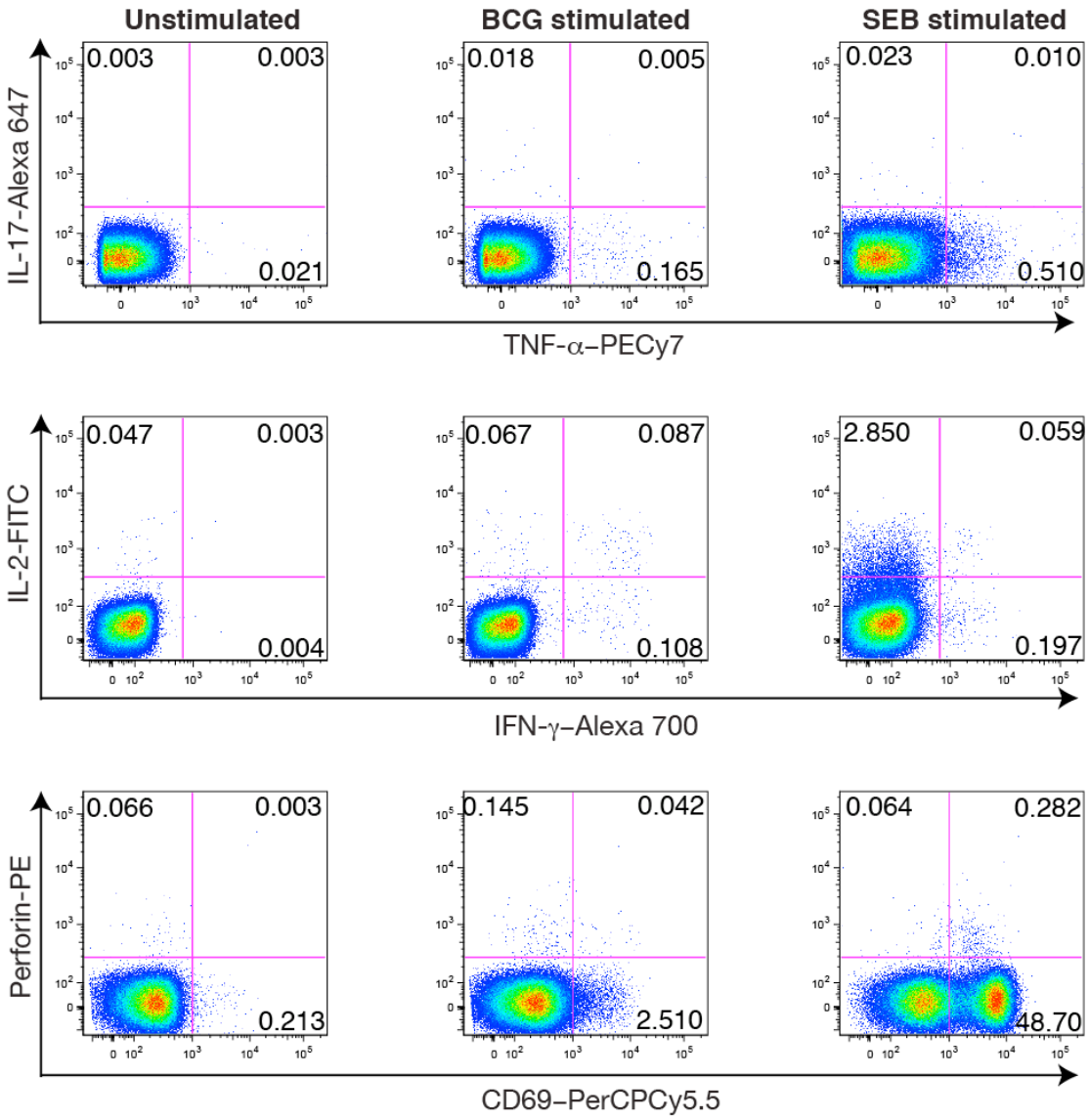

Figure 20: Intracellular cytokine expression in CD4$^+$ T cells derived from whole blood of a representative 10-week old infant, BCG vaccinated at birth. Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD8$^+$ T cells were stained intracellularly for TNF-α and IL-17 (upper panel), IL-2 and IFN-γ (middle panel), and Perforin and CD69 (lower panel).

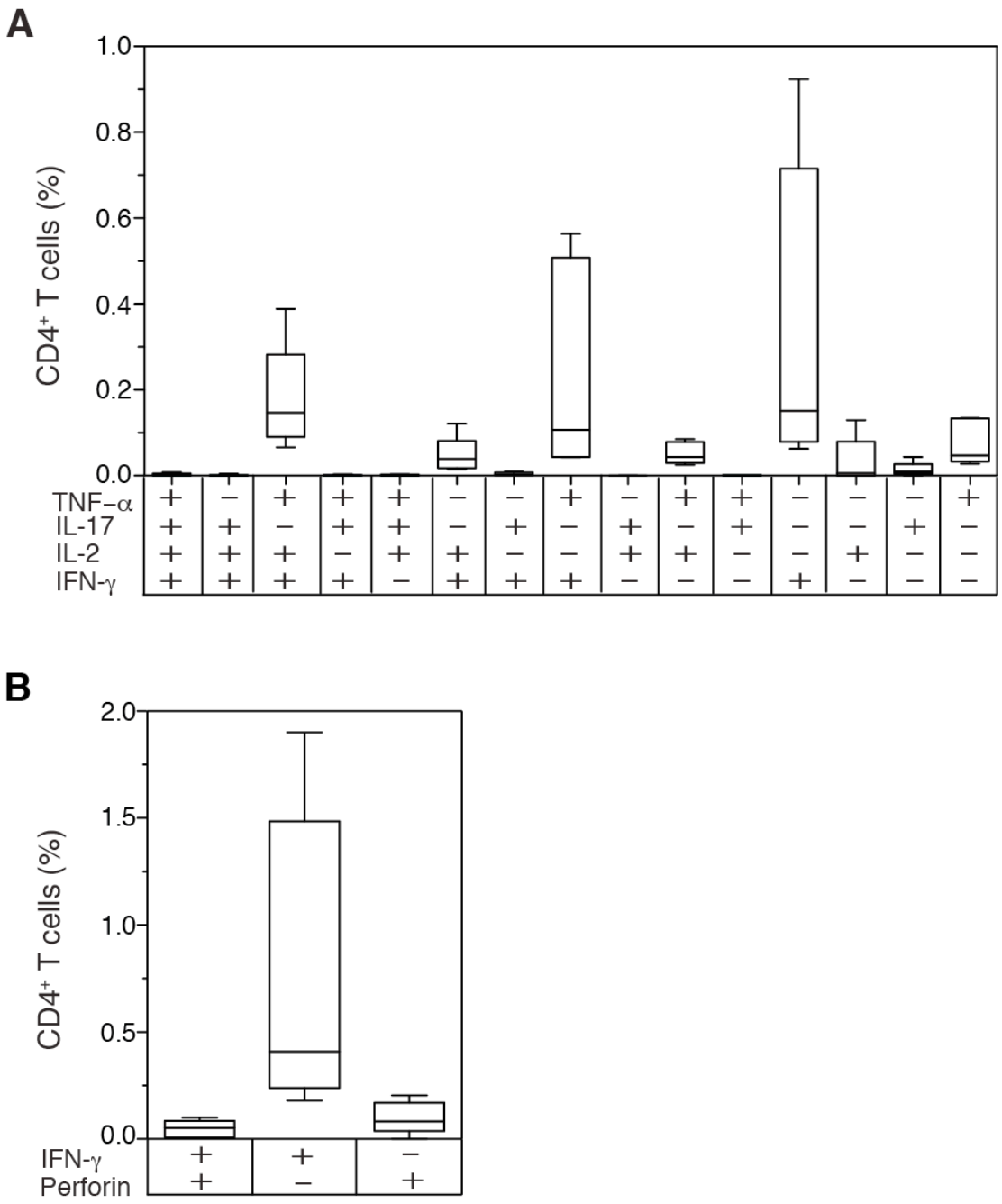

Figure 21. BCG-specific $CD4^+$ T cell cytokine subsets. Whole blood samples from five 10-weeks old infants were left unstimulated or stimulated with BCG for 12 hours. White cells were harvested and subsequently stained with the 9-colour panel before acquisition on the flow cytometer. Box and whisker plots show the frequencies of distinct subsets of specific $CD4^+$ T cells based on combinations of IFN-γ, TNF-α, IL-2, and IL-17 cytokine expression **(A)**, and $CD69^+CD4^+$ based on IFN-γ and perforin co-expression (**B**). The horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles.

Our next step was to assess BCG-specific $CD8^+$ T cell cytokine patterns in cryopreserved infant-derived whole blood samples. We used a similar analysis strategy to that used for $CD4^+$ T cells. **Figure 22** shows representative flow cytometry plots showing unstimulated, BCG and SEB-specific IFN-γ, TNF-α, IL-2 and IL-17 frequencies. The BCG-specific single IFN-γ producing $CD8^+$ T cells were the dominant subset (**Figure 23A**). Infants showed BCG-induced $CD8^+$ T cell cytokine frequencies that were similar to those seen in adults. Similar to what we observed for $CD4^+$ T cells, perforin was only co-expressed with IFN-γ and not with any other cytokine (**Figure 23B**). We concluded that the patterns of BCG-induced $CD8^+$ T cell cytokines were comparable to what has been previously reported (Kagina et al., 2010, Soares et al., 2010).

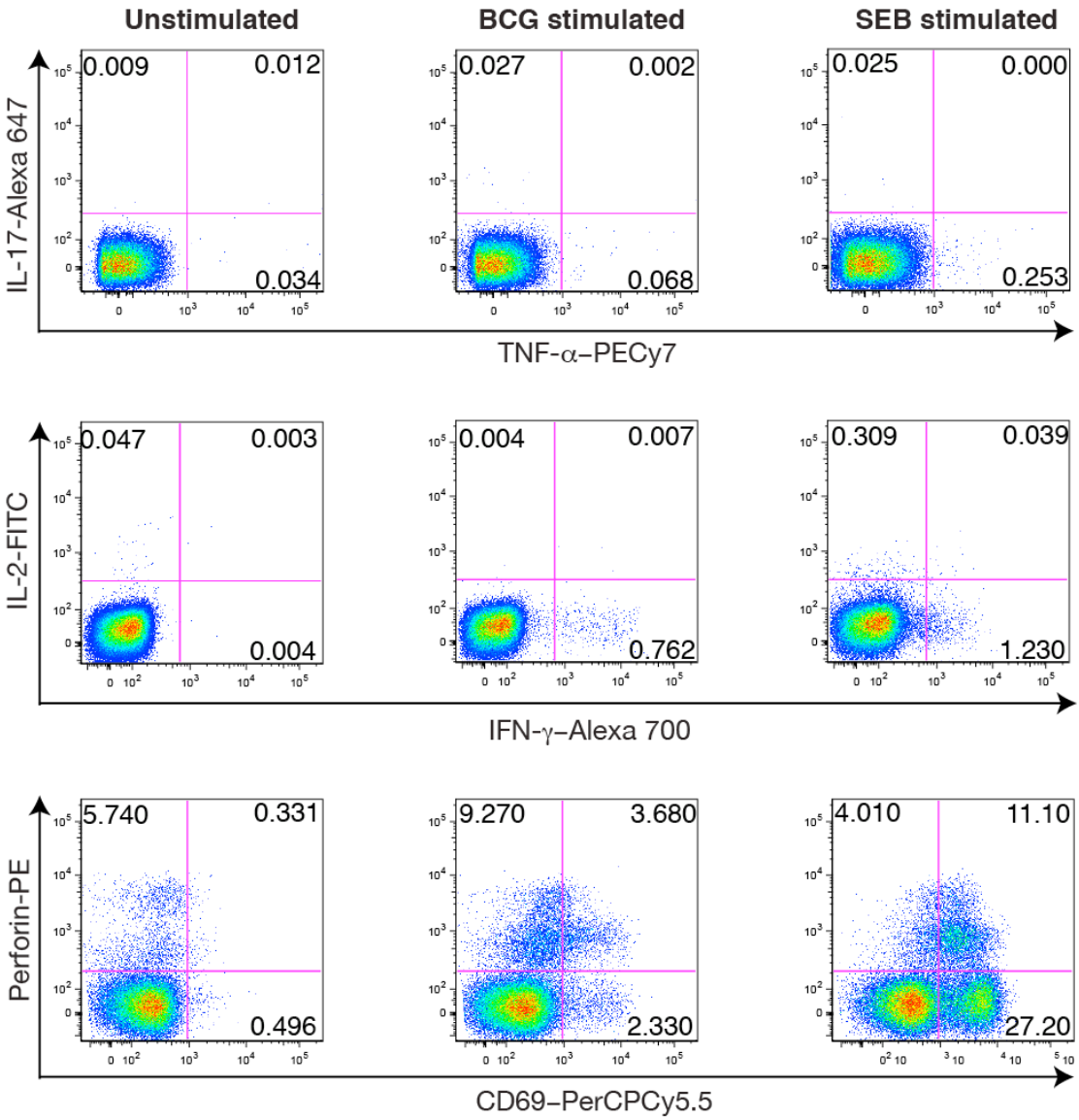

Figure 22: Intracellular cytokine expression in CD8$^+$ T cells derived from whole blood of a representative 10-week old infant, BCG vaccinated at birth. Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD8$^+$ T cells were stained intracellularly for TNF-α and IL-17 (upper panel), IL-2 and IFN-γ (middle panel), and Perforin and CD69 (lower panel).

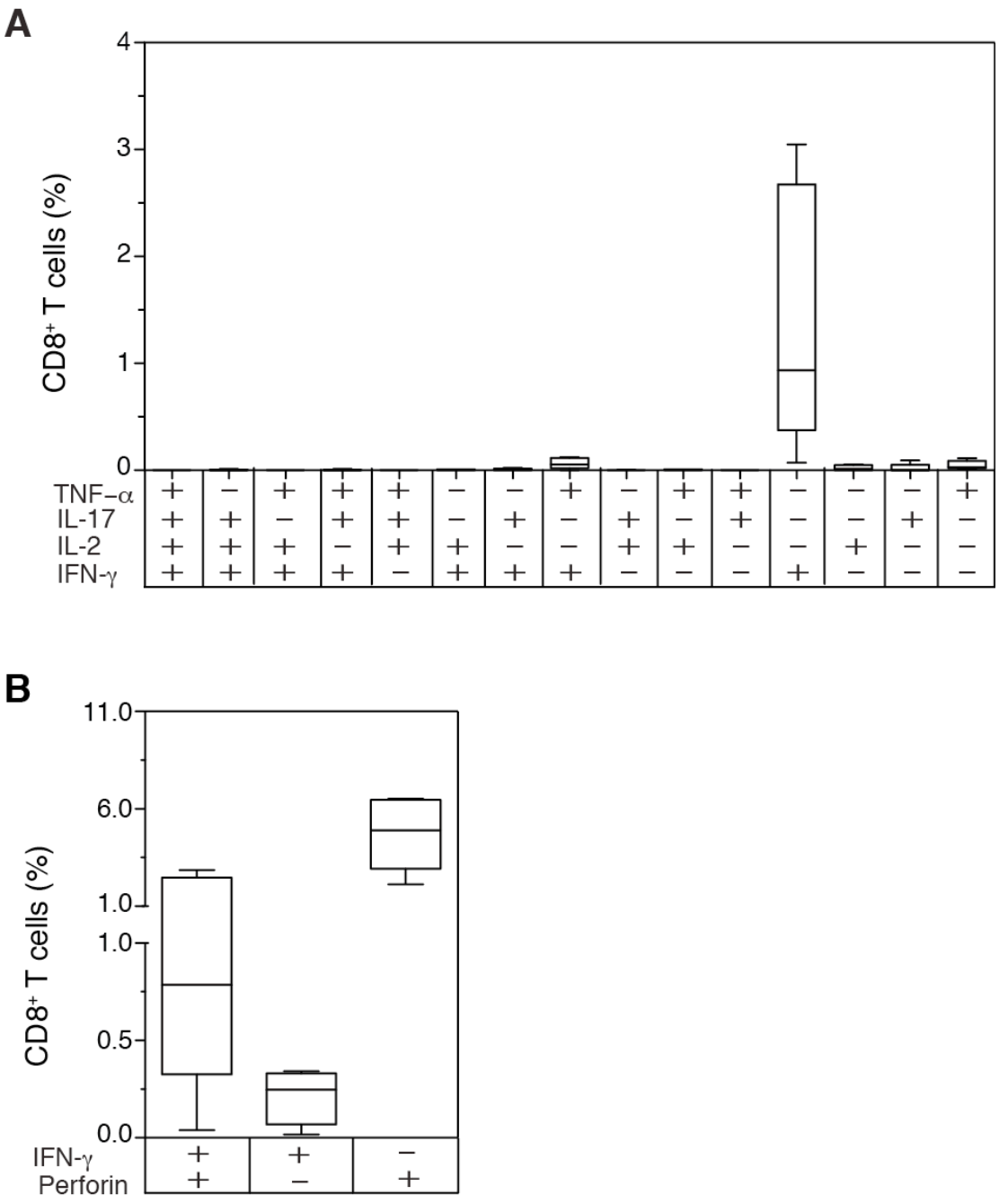

Figure 23. BCG-specific $CD8^+$ T cell cytokine subsets. Whole blood samples from five 10-weeks old infants were left unstimulated or stimulated with BCG for 12 hours. White cells were harvested and subsequently stained with the 9-colour panel before acquisition on the flow cytometer. Box and whisker plots show the frequencies of distinct subsets of specific $CD8^+$ T cells based on combinations of IFN-γ, TNF-α, IL-2, and IL-17 cytokine expression **(A)**, and $CD69^+CD8^+$ based on IFN-γ and perforin co-expression (**B**). The horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles.

3.7. Discussion

In this chapter, we successfully optimised a 9-colour PFC antibody panel to measure BCG-specific Th1 (IFN-γ, TNF-α and IL-2), Th17 (IL-17) and Tc (perforin) in stimulated whole blood cells from both adult and infants. We showed that the 9-colour panel was sensitive and reliable in quantifying BCG-specific T cell responses in our WB-ICS assay system.

Careful and meticulous development of large PFC antibody panels is important to ensure that flow cytometry data generated is reliable (Mahnke and Roederer, 2007). We used a stepwise approach to ensure optimal selection of antibody-fluorochrome combinations. We also utilized existing experience in our laboratory during the optimization process. We began by listing candidate T cell markers based on our study questions and the known functions of the selected markers in immunity against *M.tb*. In summary, we selected the final candidate markers based on the following criteria: (i) the reported immune relevance of the T cell markers to address our research questions, (ii) the capacity of the LSR II flow cytometer to measure the different parameters, (iii) the availability and performance of antibody-fluorochrome combinations, (iv) the available of PFC panels already optimized in our laboratory and, (v) the expression levels of the markers considered for inclusion in our PFC panel using the 12-hour WBA-ICS.

Individual T cells may be associated with the release of more than one cytokine and cytotoxic molecules. It is therefore important to focus on the production pattern of each active T-cell individually. Furthermore, particular T-cell subsets have been shown to associate with protection in viral and intracellular bacteria infection (Darrah et al., 2007, Forbes et al., 2008). The BCG-specific cytokine production assay described here enabled for delineation of different T cell subsets defined by production of the cytokines (IFN-γ, TNF-α, IL-2 and IL-17) and perforin by using whole blood stimulated with BCG.

Evaluation for BCG-specific perforin expression is challenging because this molecule is constitutively expressed and released upon antigenic stimulation. However perforin can also be rapidly upregulated upon antigenic stimulation (Makedonas et al., 2009). In order for us to be reliably measure BCG-specific perforin production, we used two strategies; first, we used the newly introduced anti perforin-PE B-D48 antibody clone is capable of detecting both the preformed perforin in the cytotoxic granules as well as newly formed perforin residing in the endoplasmic reticulum (Hersperger et al., 2008); second, we restricted our analysis to perforin^{+}CD69^{+} cells based on studies that have used this marker to identify activated subsets of T cells expressing specific cytolytic molecules (Murray et al., 2006).

Among the variables known to affect the results of ICS assays is the choice of the staining buffer. In our study, we observed no differences in frequencies as well as MFIs of markers conjugated on Qdot. We speculate that the BD/perm wash staining buffer we used does not contain substantial levels of heavy metals that could compromise on fluorescence of Qdot antibody conjugates.

Following successful development of a large PFC antibody panel, it is useful to test the performance of the developed PFC panel. We tested this by FMO experiments. The FMO in our optimized 9-colour PFC panel revealed minimal spectral “spill over” into any of the channels utilized. We therefore concluded that the results generated by our 9-colour panel were reliable in measuring BCG-specific T cell responses in infant samples.

Finally, we developed a gating strategy to optimize identification of true positive results and eradication of false positives. We included steps aimed at minimizing false positivity: we gated on the lymphocyte population (thus excluding monocytes that may autoflourescence) and we excluded cell doublets. As expected, the majority of samples had very low (less than 0.05%) background staining. This is may be explained by the very low constitutive cytokine producing

cells in peripheral blood given that cytokine production is dependent on continuous APC and T-cell contact (Slifka et al., 1999).

In summary, our results highlight the importance of careful and stepwise development of PFC panels to measure BCG-specific T cells by intracellular cytokine staining. We utilized this panel in Chapter 4 and modified versions of this panel in Chapter 5 and 7 to evaluate different aspects of BCG-specific immune responses in infants vaccinated at birth or at 6-weeks of age.

3.5. Contributions

Dr. F. Lutwama designed the experiments, conducted the laboratory assays and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B.M.N. Kagina, and other members of the SATVI laboratory.

Chapter 4: Comparison of frequency of specific $CD4^+$ and $CD8^+$ T cells producing cytokines and perforin following BCG vaccination at birth or at 6 weeks of age

4.1. Introduction

In this chapter, we describe the comparative frequency of BCG-specific IFN-γ, TNF-α, IL-2, IL-17 and perforin expressing $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth or 6 weeks of age. We hypothesised that infants vaccinated at birth would have lower frequencies of BCG-specific $CD4^+$ and $CD8^+$ T cells than infants vaccinated at 6 weeks of age.

We proposed to investigate $CD4^+$ and $CD8^+$ T cell immunity thought to be critical for control of *M.tb*. Furthermore, in infants, previous studies have reported that vaccination with BCG at birth mainly induces specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ (Kagina et al., 2010, Murray et al., 2006, Soares et al., 2008). Neonatal BCG vaccination also induces specific TNF-α production by both $CD4^+$ and $CD8^+$ T cells (Lalor et al., 2011, Soares et al., 2008). We therefore evaluated the frequencies of BCG-specific TNF-α expressing $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth and 6 weeks of age.

IL-2 is another Th1 cytokine induced following neonatal BCG vaccination (Smith et al., 1999, Soares et al., 2008) Antigen experienced T cell memory cells require IL-2 for maintenance and expansion. Our laboratory has shown measurable frequencies of BCG-specific IL-2, produced by conventional T cells in 10 week-old infants, vaccinated with BCG at birth (Kagina et al., 2009). We therefore investigated whether frequencies of BCG-specific IL-2 expressing $CD4^+$ and $CD8^+$ T cells in infants vaccinated with BCG at birth would be greater than those of infants vaccinated at 6 weeks of age.

In a clinical study conducted previously by our laboratory, BCG-specific IL-17 producing $CD4^+$ T cells were detected in healthy adults, previously vaccinated

with BCG at birth (Scriba et al., 2008). In our study, we were therefore interested in establishing whether frequencies of BCG-specific IL-17 $CD4^+$ and $CD8^+$ T cells would be different when the vaccine is administered at birth than at 6 weeks of age.

We also compared BCG-specific perforin-expressing $CD4^+$ and $CD8^+$ T cells when the vaccine is administered at birth and 6 weeks of age based on evidence showing that this cytotoxic molecule is induced in infants following the administration of the vaccine (Semple et al., 2011). We used CD69 antibody staining to identify BCG-activated, perforin expressing $CD4^+$ and $CD8^+$ T cells.

Our aim was to compare the frequencies of: (i) the BCG-specific total cytokine and perforin expressing $CD4^+$ and $CD8^+$ T cells in fixed whole blood from infants vaccinated at birth or at 6 weeks of age; (ii) the BCG-specific $CD4^+$ and $CD8^+$ T cells subsets expressing the different cytokine and/or perforin between the two groups of infants.

4.2. Material and methods

4.2.1. Study participants

The infants studied in this chapter were selected from the cross sectional cohort described in chapter 2.

4.2.2. Twelve-hour whole blood assay

Whole blood was stimulated with specific antigens for a total of 12 hours as described previously in chapter 2 (section 2.7.1).

4.2.3. Antibodies for intracellular cytokine staining assay

The following fluorescently conjugated monoclonal antibodies (mAbs) were used: anti-CD3 Pacific Blue (UCHT1), anti-IFN-γ Alexa Fluor 700 (B27), anti-IL-2 FITC (5344.111), all from BD Biosciences, San Jose, CA; anti-CD4 QDots605 (S3.5) and anti-CD8 QDots565 (3B5), all from Invitrogen, Eugene, OR; anti-TNF-α PE-Cy7 (Mab11), and anti-IL-17 Alexa Fluor 647 (eBio64CAP17), from eBiosciences, San Diego, CA; anti-CD69 PerCP-Cy5.5 (FN50), from Biolegend, San Diego, CA; and anti-Perforin PE (B-D48), from Diaclone, Besancon, France. All antibodies were titrated to find optimal concentrations for use as described in chapter 2 (section 3.5.5). **Table 9** shows a summary of antibody-fluorochrome combinations we used in this assay.

Marker	Fluorochrome	Description
CD3	Pac Blue	T cell marker
CD4	Qdots 605	T cell marker
CD8	Qdots 565	T cell marker
CD69	PerCpCy5.5	Early activation marker
IFN-γ	Alexa Fluor 700	Th1 cytokine
IL-2	APC	Th1 cytokine
TNF-α	PE-Cy7	Th1 cytokine
IL-17	APC	Th17 cytokine
Perforin	PE	Cytotoxic molecule

Table 9: Flow cytometry antibody-fluorochrome panel to evaluate cytokine and perforin-expressing $CD4^+$ and $CD8^+$ T cells on cryopreserved fixed cells from stimulated whole blood collected from the study participants.

4.2.4. Intracellular cytokine staining assay and flow cytometry analysis

We used a "one step" staining method as described in chapter 3 (section 3.5.4) to measure the BCG-specific T-cells cytokines and cytolytic molecule expression in infants following BCG vaccination at birth and at 6 weeks of age.

4.2.5. Data analysis

In this section, "specific" refers to either cytokine or cytotoxic expression. For each participant's sample, the frequencies of specific $CD4^+$ and $CD8^+$ T cells detected in the negative control (unstimulated) were subtracted from frequencies of the specific $CD4^+$ and $CD8^+$ T cells detected in the corresponding BCG-stimulated samples. Samples were excluded from the final analysis if the frequency of specific $CD4^+$ T cells in the positive control condition was lower than the median plus 3 times the median absolute deviations (3xMAD) of the frequencies of specific $CD4^+$ T cells of all the negative control samples. We chose to use $CD4^+$ T cells to define this criterion because the BCG-specific immune responses were predominantly observed in this T cell subset.

The Mann-Whitney U test was used to assess differences in immunological

outcomes between the two groups. Characteristics of the two groups of infants were compared using Pearson's chi-squared test for categorical variables. Prism 5.0 (GraphPad Software Inc.) was used for statistical analysis. A p-value below 0.05 was considered significant. The impact of vaccination group, sex, household income, and weight (independent variables) on the frequency of cytokine expressing T cells (outcome variable) was determined by multivariate linear regression analysis using STATA 12.1, (Stata Corporation, College Station, Texas, USA).

4.3. Results

4.3.1. Participants' characteristics

We enrolled 92 infants at 9 months of age in Kampala, Uganda. Fifty of these infants had received BCG at birth and 42 infants received the vaccine at 6 weeks of age. Participants' recruitment and clinical exclusions are summarized in **Figure 24**. The weights at 9 months of age and gender distribution in both groups of infants were comparable (**Table 10**). The birth weight for infants vaccinated at 6 weeks of age were not available as they were mostly born at home. We have therefore not included this variable. Infants vaccinated with BCG at birth were more likely to be from a higher social economic background than infants vaccinated at 6 weeks of age (**Table 10**).

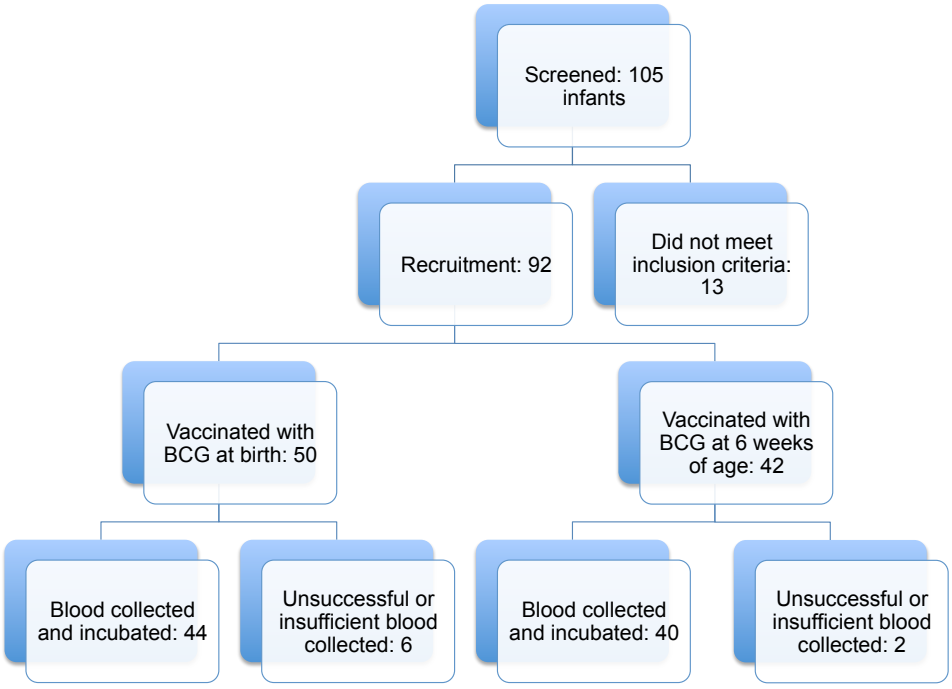

Figure 24. Recruitment, enrollment and exclusion of infants in the study. Initially infants interested in taking part in the study were screened and eligible infants were enrolled into the study. Blood was then drawn from the infants on the day of enrollment.

Variable	Vaccinated at birth: (n=44)	Vaccinated at 6 weeks of age: (n=40)	P-value
Sex n (%)			
Female	18 (41)	20 (53)	0.29[a]
Male	26 (59)	18 (47)	
Income n (%)			
Below 125 USD	12 (27)	25 (64)	0.001[a]
Above 125 USD	32 (73)	14 (36)	
Season of BCG administration n(%)			
Dry	28 (64)	24 (60)	0.73[a]
Rainy	16 (36)	16 (40)	
Weight at 9 months (Kg) (Median, IQR)	9.0 (8, 9.55)	8.5 (8, 9.45)	0.26[b]

Table 10. Demographic characteristics of the study participants at 9 months of age. Gender, income, season of BCG administration and weight of the infants vaccinated with BCG at birth or at 6 weeks of age are shown. Differences in the characteristics were statistically evaluated: [a]Chi Square and [b]Mann–Whitney U tests were used for the analysis.

4.3.2. Gating strategy

We used the gating strategy developed during the optimisation process described in chapter 3 (section 3.6.11). We used the strategy to analyse the BCG-specific cytokine and perforin expressing $CD4^+$ and $CD8^+$ T cells. **Figure 25** shows representative flow cytometry dotplots to identify the specific $CD4^+$ and $CD8^+$ T cells from a 9-month old infant vaccinated with BCG at 6 weeks of age.

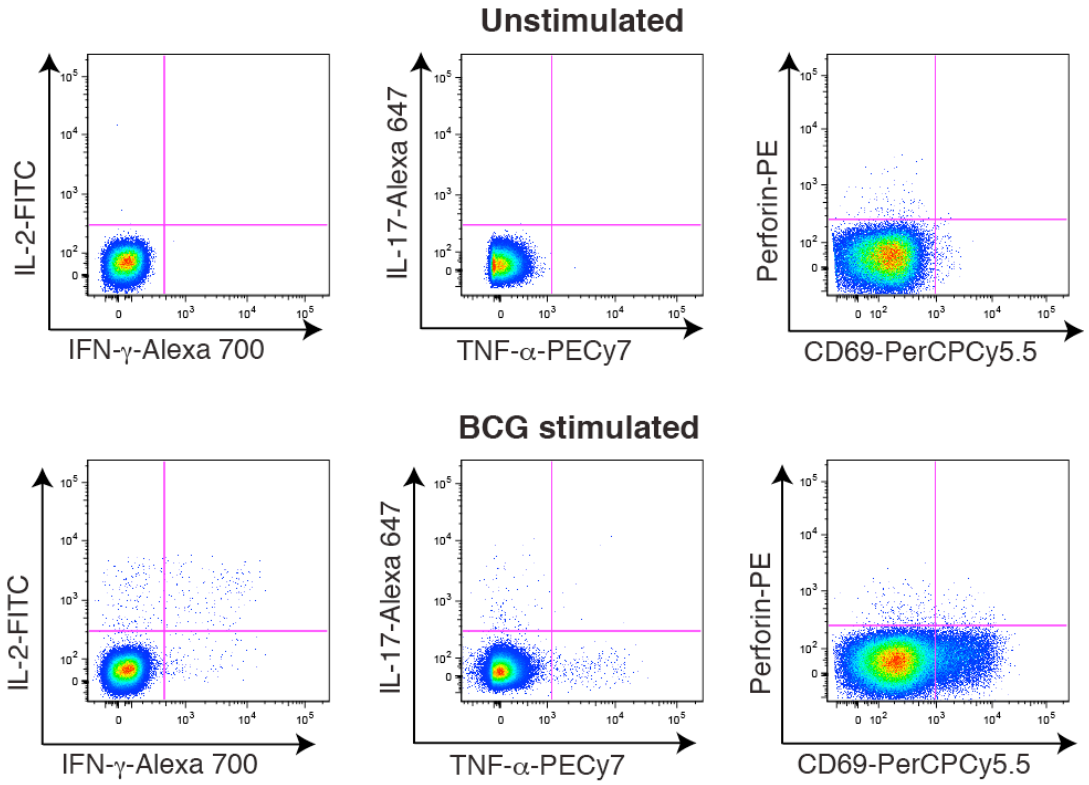

Figure 25: Frequencies of BCG specific CD69, IFN-γ, TNF-α, IL-2, IL-17 and perforin CD4$^+$ T cells. Whole blood was either left unstimulated, or was stimulated with BCG for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. Representative flow cytometry plots of cytokine and perforin expression in an unstimulated (top row) and BCG stimulated (bottom row) sample from an infant vaccinated with BCG at birth are shown.

4.3.3. Participants' exclusion from the analyses

We used the exclusion criteria described in the data analysis section to ensure only successfully processed samples were included in the final analyses. **Table 11** shows the participants excluded form the analysis.

Reason for exclusion	44 infants vaccinated at birth (all samples processed)	40 infants vaccinated at 6 weeks (all samples processed)
	Number of infants excluded from the WB-ICS final analyses	
PHA responses < median + 3MAD of all negative controls combined	0	6

Table 11: Exclusions from the final WB ICS analysis. Infant samples with a PHA CD4 T cell total cytokine response of less than the median plus 3MAD of the frequencies of specific $CD4^+$ T cells of all the negative control samples were excluded from the analysis.

4.3.4 Greater frequencies of BCG-specific total IFN-γ expressing $CD4^+$ T cells in birth vaccinated infants, compared with infants vaccinated at 6 weeks of age

Our aim was to compare the frequency of BCG-specific cytokine and perforin expressing $CD4^+$ T cells in infants vaccinated with BCG at birth or at 6 weeks of age. Our hypothesis was that birth vaccinated infants would have lower frequencies of BCG-specific $CD4^+$ T cells than infants vaccinated at 6 weeks of age. The great majority of infants vaccinated with BCG at either time point had a detectable specific IFN-γ, TNF-α, IL-2, IL-17 and perforin $CD4^+$ T cell response (**Figure 26A, B**). Frequency of BCG-specific $CD4^+$ T cells expressing either IL-2, IL-17, TNF-α or perforin were comparable in the 2 vaccination groups (**Figure 26A, B**). However infants vaccinated with BCG at birth had greater frequencies of $CD4^+$ T cells expressing IFN-γ, compared with infants vaccinated at 6 weeks of age (**Figure 26A**).

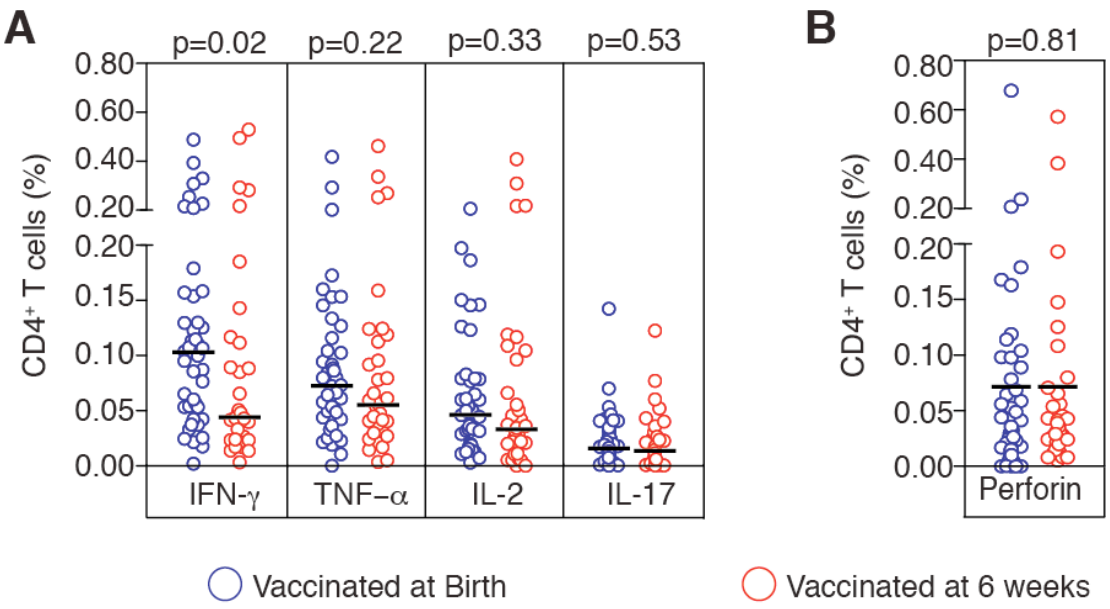

Figure 26. Frequencies of specific CD4$^+$ T cells producing cytokines and perforin. We measured the specific responses using the short-term whole blood assay. Scatter plots depict frequencies of total IFN-γ, TNF-α, IL-2, and IL-17 cytokine expressing CD4$^+$ T cells (**A**), and total perforin expressing CD69$^+$CD4$^+$ T cells (**B**). On the scatter plots, the horizontal lines represent the median frequencies of the specific cells. The results shown are corrected for unstimulated responses. The *Mann–Whitney* U test was used to compare differences in frequencies of cytokine and perforin expressing CD4$^+$ T cells between infants vaccinated at birth (blue circles) or 6 weeks of age (red circles). P values less than 0.05 were considered significant.

4.3.5. Greater frequencies of BCG-specific CD4$^+$ T cells expressing IFN-γ, with or without perforin, in infants vaccinated at birth compared with those vaccinated at 6 weeks of age

The observed difference in BCG-specific CD4$^+$ T cells expressing IFN-γ between the two groups prompted us to further assess the profile of the vaccine-induced immune responses. Therefore, we compared the profile of BCG-specific CD4$^+$ T cell expressing IFN-γ, TNF-α, IL-2 and IL-17 alone or in different combinations, between the two groups of infants. In both groups, we did not observe co-expression of IL-I7 with any of the cytokines evaluated (**Figure 27A and B),** therefore, we did not compare these outcomes between the two groups: the frequencies were too low for reliable comparison. The frequencies of BCG-specific polyfunctional (IL-2$^+$IFN-γ$^+$TNF-α$^+$), double positive (IL-2$^+$IFN-γ$^+$, IL-

2^+TNF-α^+ and IFN-γ^+TNF-α^+) as well as single positive (IL-2^+, TNF-α^+ or IL-17^+) $CD4^+$ T cell subsets were not different between the two groups (**Figure 27A**). However, the birth vaccinated group had greater frequencies of BCG-specific IFN-γ single-positive $CD4^+$ T cells, than the 6 weeks of age vaccinated group (**Figure 27A**). We also evaluated the co-expression of the different specific cytokines with perforin. Only IFN-γ was co-expressed with perforin in both groups of infants. We showed that frequencies of specific IFN-γ^+perforin$^+$ double-positive $CD4^+$ T cells were greater in the birth than 6 weeks of age vaccinated infants (**Figure 27C**).

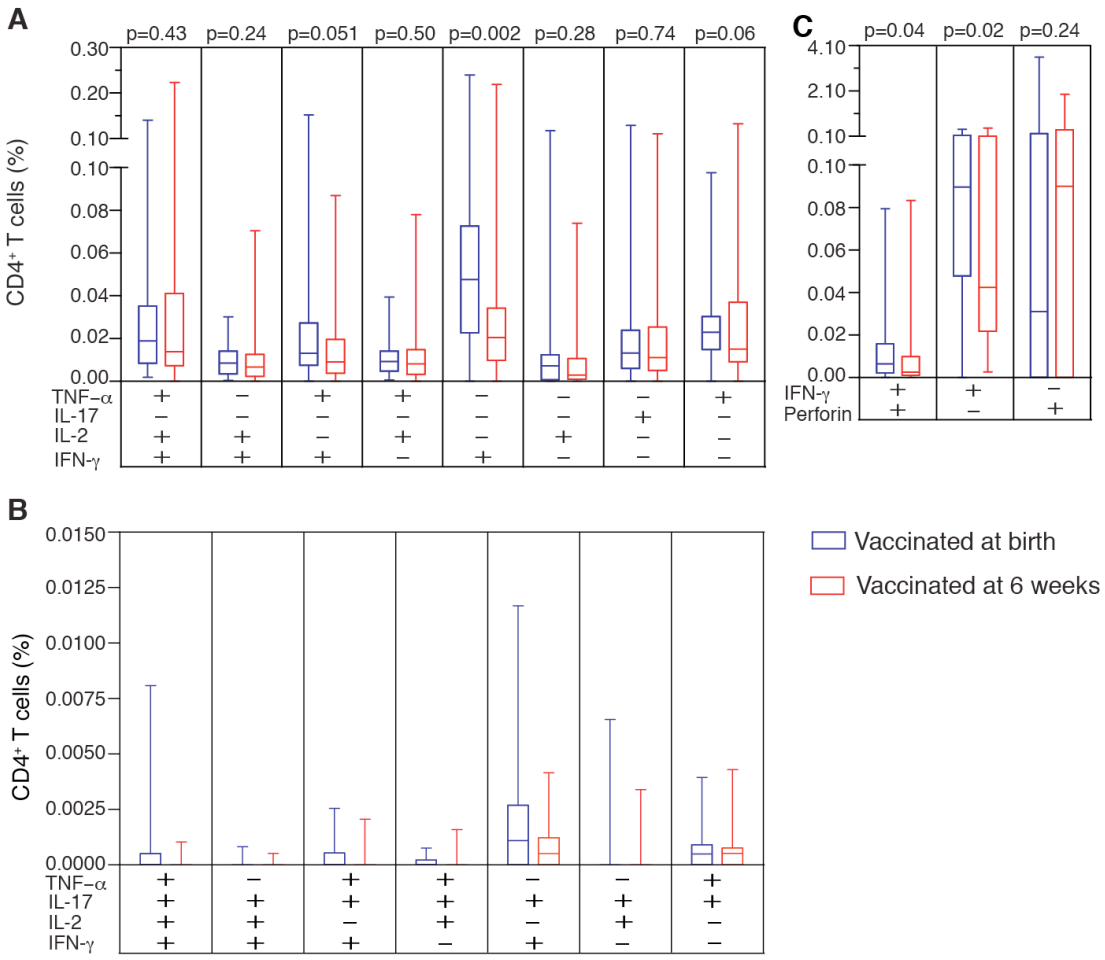

Figure 27. BCG-specific CD4$^+$ T cell cytokine subsets. We measured specific responses using the short-term whole blood assay. Box and whisker plots show frequencies of the distinct subsets of specific CD4$^+$ T cells based on combinations of expression for: IFN-γ, TNF-α, IL-2, and IL-17 (**A and B**) as well as IFN-γ and perforin (**C)** only. Co-expression of all cytokines was assessed on CD4 T cells while that of IFN-γ and perforin was on CD69$^+$CD4$^+$. Whiskers represent the maximum and minimum value; the box represents the interquartile range, while the line in the box represents the median. Values are shown after subtraction of the unstimulated responses in the negative controls from the BCG-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in frequencies of CD4$^+$ T cell subsets between infants vaccinated at birth (blue bars) or 6 weeks of age (red bars). P values less than 0.05 were considered significant.

4.3.6. Greater frequencies of BCG-specific total IFN-γ expressing $CD8^+$ T cells in infants vaccinated at birth compared with those vaccinated at 6 weeks

Next, we compared the frequency of BCG-specific IFN-γ, TNF-α, IL-2, IL-17 and perforin expressing $CD8^+$ T cells in infants vaccinated at birth or 6 weeks of age. Our hypothesis was that birth vaccinated infants would show lower frequencies of BCG-specific $CD8^+$ T cells than infants vaccinated at 6 weeks of age. We performed an identical analysis as that performed for $CD4^+$ T cells. In both groups, BCG-specific $CD8^+$ T cell responses were dominated by IFN-γ and perforin expression (**Figure 28A, B**). As for $CD4^+$ T cells, the frequencies of BCG-specific TNF-α, IL-2, IL-17 and perforin expressing $CD8^+$ T cells in both groups were not different, while infants vaccinated with BCG at birth had a greater frequency of specific $CD8^+$ T cells expressing IFN-γ compared with infants vaccinated at 6 weeks of age (**Figure 28A**).

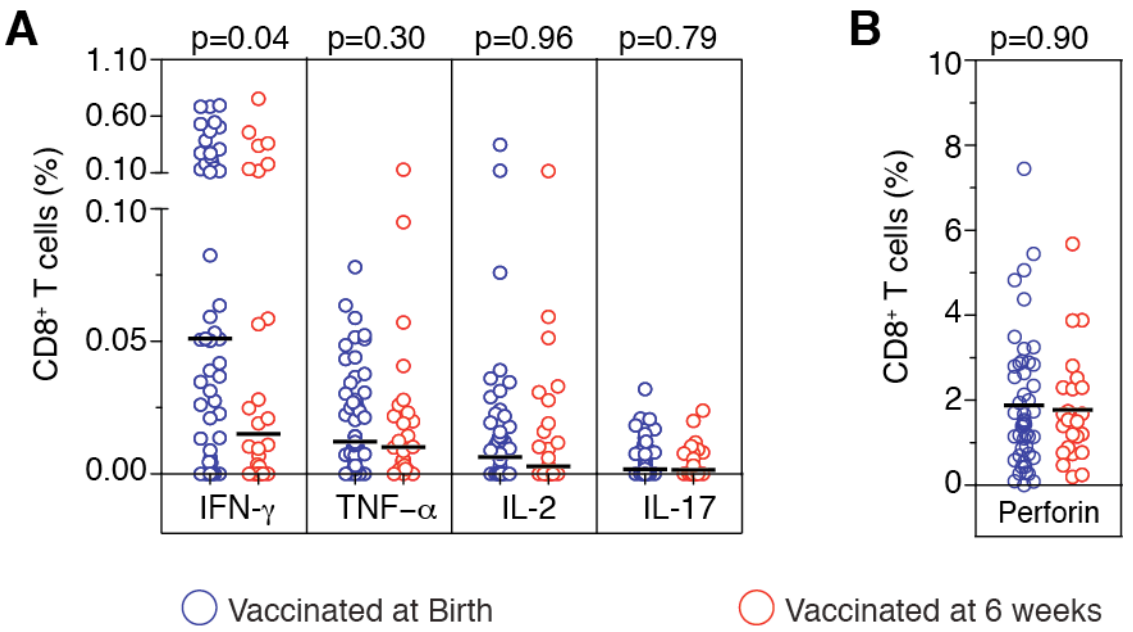

Figure 28. BCG-specific CD8$^+$ T cell cytokine and perforin responses. Scatter plots depict frequencies of total IFN-γ, TNF-α, IL-2, and IL-17 cytokine expressing CD8$^+$ T cells (**A**), and perforin expressing CD69$^+$CD8$^+$ T cells (**B**). The horizontal lines shown in the scatter plots represent the median frequencies of the specific cells. Values are shown after subtraction of the unstimulated responses (negative controls) from the BCG-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in frequencies of cytokine and perforin expressing CD8$^+$ T cells between the infants vaccinated at birth (blue bars) or 6 weeks (red bars) vaccinated infants. P values less than 0.05 were considered significant.

4.3.7. Greater frequencies of BCG-specific CD8$^+$ T cells expressing IFN-γ, with or without perforin, in infants vaccinated at birth compared with those vaccinated at 6 weeks of age

Next, we proceeded to compare the profile of BCG-specific CD8$^+$ T cell subsets. As for CD4$^+$ T cells, single expressing IFN-γ CD8$^+$ T cell were the dominant subset, while IL-17 expressing cells were the least dorminant subset in both infant groups (**Figure 29A**). In both groups, we did not observe co-expression of IL-17 with any of the cytokines evaluated (**Figure 29A and B**), therefore, we did not compare these outcomes between the two groups: the frequencies were too low for reliable comparison. The frequencies of BCG-specific CD8$^+$ T cells

expressing only TNF-α or IL-2 were also not different between the two groups. Consistent with what we observed for $CD4^+$ T cells, the birth vaccinated group had greater frequencies of specific $CD8^+$ T cells expressing only IFN-γ compared with infants vaccinated at 6 weeks of age (**Figure 29A**).

Further analysis of BCG-specific $CD8^+$ T cell subsets showed that perforin was either expressed alone or co-expressed with IFN-γ, and not any other cytokine (**Figure 29C**). Also similar to $CD4^+$ T cells, the birth vaccinated group had greater frequencies of specific $CD8^+$ T cells that expressed IFN-γ alone, or in combination with perforin (**Figure. 29C**).

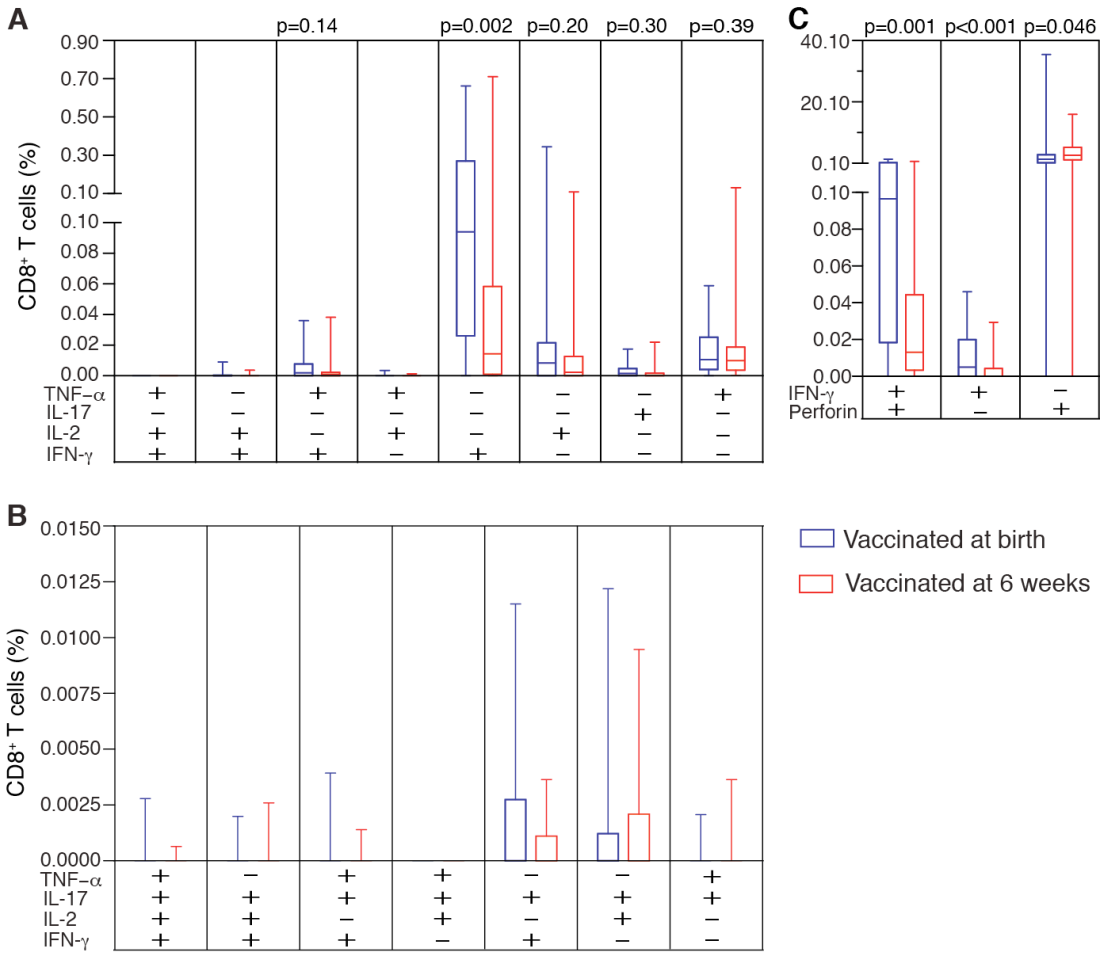

Figure 29. BCG-specific CD8$^+$ T cell cytokine subsets. Box and whisker plots show the frequencies of the distinct subsets of specific CD8$^+$ T cells based on combinations of expression for: IFN-γ, TNF-α, IL-2, and IL-17 (**A and B**) as well as IFN-γ and perforin (**C)** only. Co-expression of all cytokines was assessed on CD8$^+$ T cells while that of IFN-γ and perforin was on CD69$^+$CD8$^+$cells. Whiskers represent the maximum and minimum value; the box represents the interquartile range, while the line in the box represents the median. Values are shown after subtraction of the unstimulated responses in the negative controls from the BCG-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in frequencies of CD8$^+$ T cell subsets between infants vaccinated at birth (blue bars) or 6 weeks of age (red bars). P values less than 0.05 were considered significant.

4.3.8. No difference in the frequency of TB10.4-specific $CD4^+$ T cells expressing cytokines or perforin in the two groups of infants

We also measured TB10.4-specific T cell responses in the two groups of infants. We chose to evaluate TB10.4 specific responses because the protein is expressed by BCG and is an immunodominant antigen (Skjot et al., 2000). Some novel TB vaccines designed to boost the BCG-induced immunity have incorporated TB10.4 protein (Abel et al., 2010). We compared the frequencies of TB10.4-specific cytokine and perforin expressing $CD4^+$ T cell in infants vaccinated with BCG at birth or at 6 weeks of age. The great majority of infants vaccinated with BCG at either time point had a detectable specific IFN-γ, TNF-α, IL-2, IL-17 and perforin $CD4^+$ T cell response (**Figure 30A, B**). The frequencies of BCG-specific IFN-γ, TNF-α, IL-2, IL-17 and perforin expressing $CD4^+$ T cells in both groups were not different (**Figure 30A, B**).

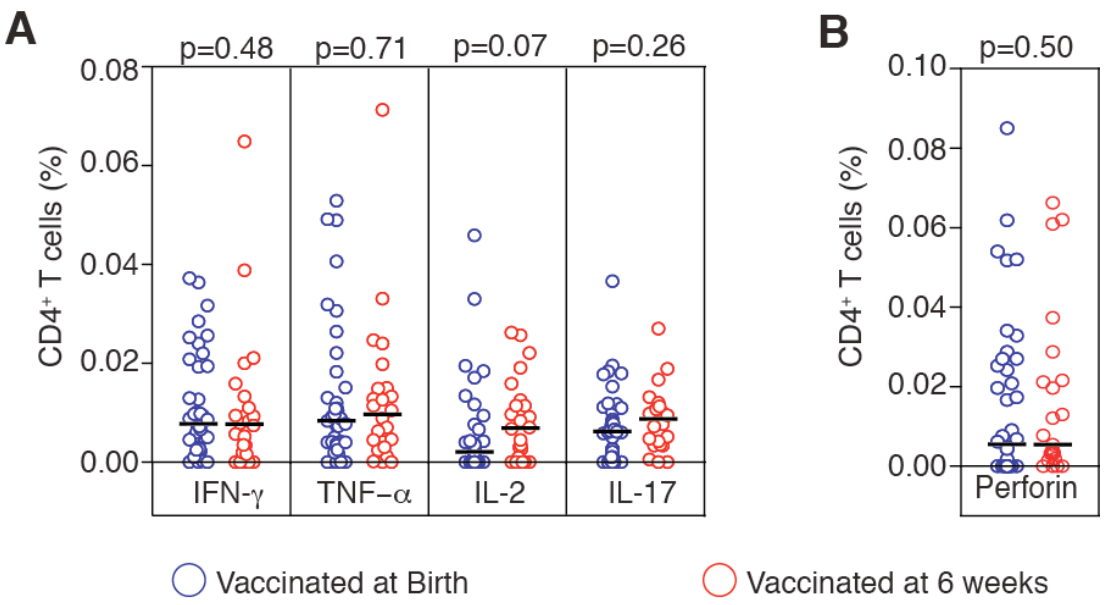

Figure 30. TB10.4-specific CD4$^+$ T cell cytokine and perforin response. Scatter plots depict frequencies of total IFN-γ, TNF-α, IL-2, and IL-17 cytokine expressing CD4$^+$ T cells (**A**), and perforin expressing CD69$^+$CD4$^+$ T cells (**B**). The horizontal lines represent the median frequencies. The blue and red circles represent infants vaccinated at birth or 6 weeks of age BCG vaccinated infants respectively. Values are shown after subtraction of the unstimulated responses in the negative controls from the TB10.4-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in frequencies of cytokine and perforin expressing CD4$^+$ T cells between infants vaccinated at birth (blue circles) or 6 weeks of age (red circles). P values less than 0.05 were considered significant.

4.3.9. No difference in the frequencies of TB10.4-specific CD8$^+$ T cell expressing cytokines or/and perforin in the two groups of infants

Next, we compared frequencies of TB10.4-specific IFN-γ, TNF-α, IL-2, IL-17 and perforin expressing CD8$^+$ T cells in infants vaccinated with BCG in the two groups. In both groups, TB10.4-specific CD8$^+$ T cell responses were dominated by perforin expression (**Figure 31A, B**). Similar to CD4$^+$ T cells, frequencies of BCG-specific IL-2, IL-17, IFN-γ, TNF-α and perforin expressing CD4$^+$ T cells in both groups were not different (**Figure 31A, B**).

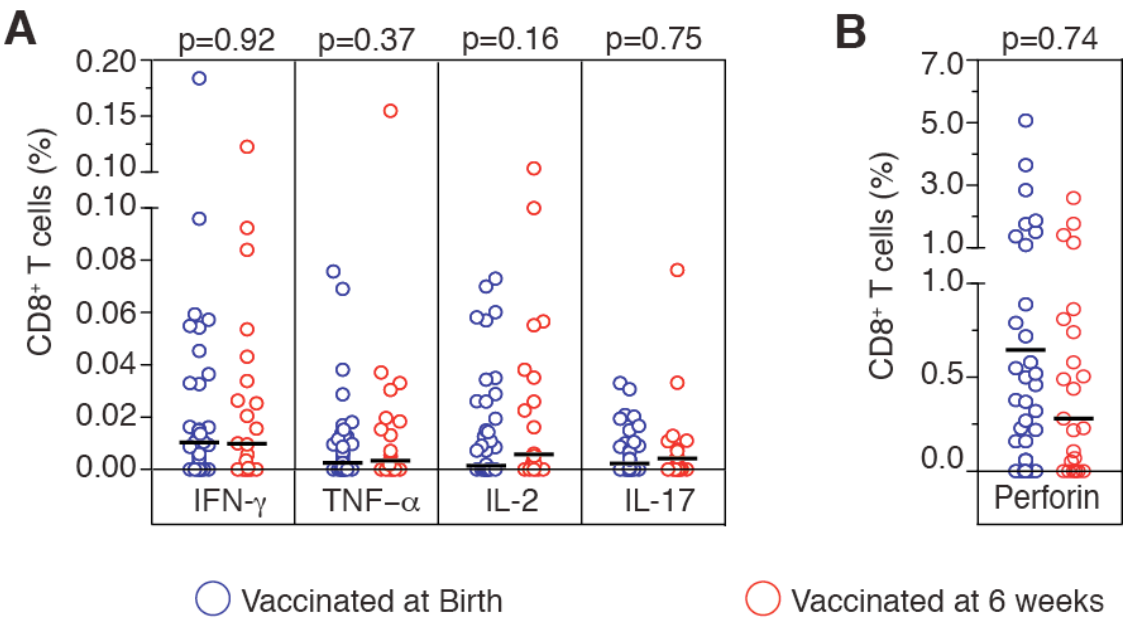

Figure 31. TB10.4-specific CD8$^+$ T cell cytokine and perforin response. Scatter plots depict frequencies of total IFN-γ, TNF-α, IL-2, and IL-17 cytokine expressing CD8$^+$ T cells (**A**), and perforin expressing CD69$^+$CD8$^+$ T cells (**B**). The horizontal lines represent the median frequencies. The blue and red circles represent infants vaccinated at birth or 6 weeks of age BCG. Values are shown after subtraction of the unstimulated responses in the negative controls from the TB10.4-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in frequencies of cytokine and perforin expressing CD8$^+$ T cells between infants vaccinated at birth (blue circles) or 6 weeks of age (red circles). P values less than 0.05 were considered significant.

4.3.10. No difference in frequencies of BCG-specific total IFN-γ expressing CD4$^+$ and CD8$^+$ T cells in infants from low income category, compared with infants from high income category

Next, we evaluated if the observed greater frequencies of IFN-γ expressing CD4$^+$ and CD8$^+$ T cell in infants vaccinated at birth compared to 6 weeks could be explained by household income. We observed that a higher proportion of infants vaccinated at 6 weeks of age were from families of lower social economic status than infants vaccinated at birth (**Table 10**). We compared the frequency of BCG-specific IFN-γ expressing T cells in infants from low (below 125 USD) and high (above 125 USD) household income categories. We observed no differences in frequencies of BCG-specific IFN-γ expressing CD4$^+$ and CD8$^+$ (**Figure 32A and**

B) T cells were observed between the high income and the low income groups. Nevertheless, frequencies of IFN-γ expressing $CD4^+$ T cell and $CD8^+$ T cell were significantly associated with household income (**Table 12 and 13**).

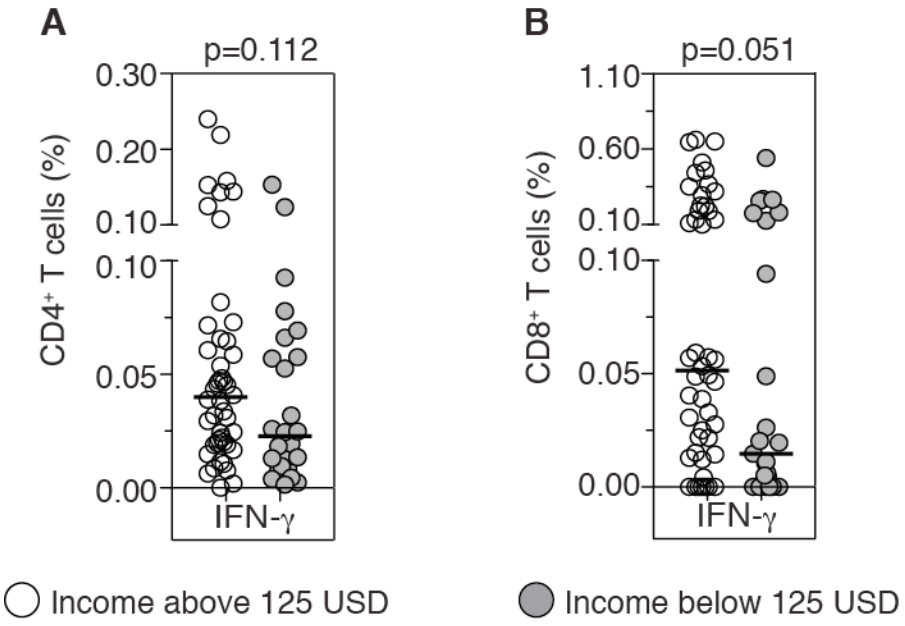

Figure 32. BCG-specific IFN-γ expressing $CD4^+$ and $CD8^+$ T cells by income category.. Scatter plots depict frequencies of total IFN-γ expressing $CD4^+$ T cells (**A**) and $CD8^+$ T cells (**B**). On the scatter plots, the horizontal lines represent the median frequencies of the specific cells. The results shown are corrected for unstimulated responses. The *Mann–Whitney* U test was used to compare the difference in frequencies of IFN-γ expressing $CD4^+$ T cells and $CD8^+$ T cells between infants in above 125 USD income category (open circles) and infants in the below 125 USD income category (closed circles). P values less than 0.05 were considered significant.

LogCD4$^+$ IFNγ^+	Coef	s.e	P-value	[95% Conf. Interval]	
Univariate analysis					
Vaccination group	0.3639	0.0969	0.000	0.1710236	0.5567765
Sex	0.0845	0.1020	0.410	-0.1185748	0.2876241
Income category	0.2615	0.1017	0.012	0.0591906	0.4637975
Weight	0.0695	0.0479	0.151	-0.0258983	0.1648879
Multivariate analysis					
Vaccination group	0.3117	0.1022	0.003	0.1082647	0.5150414
Income category	0.1554	0.1028	0.135	-0.0493226	0.3600253
R^2 = 0.17; adjusted R^2= 0.15					

Table 12: Univariate and multivariate linear regression analyses considering LogCD4$^+$ IFNγ^+ as outcome. The independent variables were vaccination group, sex, household income, and weight. In the multivariate analysis, the variables considered were the vaccination group and household income.

LogCD8$^+$IFNγ^+	Coef	s.e	P-value	[95% Conf. Interval]	
Univariate analysis					
Vaccination group	0.6611	0.1267	0.000	0.4089	0.9133
Sex	0.0540	0.1502	0.720	-0.2451	0.3532
Income category	0.3194	0.1429	0.028	0.0348	0.6040
Weight	0.0477	0.0696	0.496	-0.0912	0.1866
Multivariate analysis					
Vaccination group	0.6254	0.1351	0.000	0.3564	0.8944
Income category	0.1053	0.1358	0.440	-0.1648	0.3755
R^2 = 0.26; adjusted R^2= 0.24					

Table 13: Univariate and multivariate linear regression analyses considering LogCD8$^+$ IFNγ^+ as outcome. The independent variables were vaccination group, sex, household income, and weight. In the multivariate analysis, the variables considered were the vaccination group and household income.

4.4. Discussion

In this chapter, we describe a comparison of BCG-induced cytokine and perforin-expressing T cell responses between infants vaccinated at birth and at 6 weeks of age. We showed that age of vaccination impacted the induced immune response, measured at 9 months of age: infants vaccinated at birth had greater frequencies of BCG-specific total IFN-γ expressing $CD4^+$ and $CD8^+$ T cells than infants vaccinated at 6 weeks of age. Furthermore, infants vaccinated at birth showed greater frequencies of BCG-specific single (IFN-γ^+) and double (IFN-γ^+perforin$^+$) producing $CD4^+$ and $CD8^+$ T cells than infants vaccinated at 6 weeks of age. Finally, the frequencies of TB10.4-specific $CD4^+$ and $CD8^+$ T cell responses were not different in both groups of infants.

We measured the specific IFN-γ-expressing cells based on the proposed important roles for this molecule in control of mycobacterial infection (Bogunovic et al., 2012, Newport et al., 1996, Ottenhoff et al., 2000). In our study, greater frequencies of BCG-specific $CD4^+$ and $CD8^+$ T cells expressing either IFN-γ and co-expressing IFN-γ and perforin in infants vaccinated at birth, compared to 6 weeks, could be a reflection of more effective vaccine-take in the former group. However, we cannot speculate about clinical relevance of this observation in human infants, in terms of protection against TB disease (Kagina et al., 2010); BCG-induced correlates of protection are not known.

The proposed role for perforin in mediating immunity against *M.tb* involves perforation of cell membranes of infected cells to permit entry of cytolytic granzymes that directly kill *M.tb* or the infected cells (Thiery et al., 2011). Supporting the role of perforin in TB immunity is a study by Rahman *et al.* that showed non-human primates vaccinated with a novel TB vaccine (rBCG/rAD35), and subsequently challenged with *M.tb*, had greater frequencies of mycobacteria-specific perforin expressing T cells that associated with better protection (Rahman et al., 2012). In this study, we observed increased frequencies of BCG-specific IFN-γ^+perforin$^+$-expressing T cells when the vaccine

was administered at birth compared to 6 weeks of age. However, to the best of our knowledge, the association between vaccine-specific IFN-γ^+perforin$^+$-expressing T cells and the risk of TB disease has not been demonstrated in a clinical setting.

We also evaluated immune responses to TB10.4, an immunodominant antigen present in both BCG and *M.tb* (Skjot et al., 2000). We showed that infants in both groups had detectable TB10.4-specific $CD4^+$ and $CD8^+$ T cell responses. As expected, the frequencies of TB10.4-specific T cells were lower than those observed following stimulation of whole blood with the whole viable BCG. Nevertheless, no differences in the TB10.4-specific $CD4^+$ and $CD8^+$ T cells between birth and 6 weeks of age BCG vaccinated infants. We speculate that boosting TB10.4-specific T cell responses induced by BCG vaccination in infants at birth or at 6 weeks of age, with novel TB vaccines containing the TB10.4 antigen may result in similar frequencies of these specific immune responses.

What are the possible explanations for the differences observed in the BCG-induced T cell immunity between the two groups of infants? First, we observed that a higher proportion of infants vaccinated at 6 weeks of age were from families of lower social economic status than infants vaccinated at birth. Lower social economic status may result in poor health seeking behavior (including anti-helminthes treatment), or poor nutrition of infants leading to suboptimal immune responses (Rodriguez et al., 2005). We did not show a difference in the frequencies of BCG-specific IFN-γ expressing $CD4^+$ and $CD8^+$ T cell responses in infants from a higher income, compared with those from the lower income categories. Nevertheless, we observed a significant association between frequencies of IFN-γ expressing $CD4^+$ T cell and household income. We speculate that the lower social economic status may have indirectly and negatively impacted the specific T cell response observed in the group of infants vaccinated at 6 weeks of age.

Our results could also be explained by co-administration of BCG with DPT-Alum (Sartono et al., 2010). We speculate that alum may potentially result in reduced Th1 responses through two mechanisms; first, alum induces predominantly induces Th2 immune responses (Bungener et al., 2008), which may attenuate the BCG-induced Th1 responses (Abbas et al., 1996, Marrack et al., 2009); second alum inhibits production of the Th1 promoting cytokine IL-12 by dendritic cells (Mori et al., 2012). Therefore, co-administration of DPT-alum with BCG in infants vaccinated at 6 weeks of age may have resulted in attenuated IFN-γ $CD4^+$ and $CD8^+$ T cell responses relative to infants vaccinated at birth.

The observed differential specific T cell response, when BCG is given at birth versus at 6 weeks of age prompts further follow up studies that may give more insight into clinical relevance, primarily focusing on the influence of the results to the risk of *M.tb* infection and/or TB disease.

4.5. Contributions

Dr. F. Lutwama designed the experiments, conducted the laboratory assays and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B.M.N. Kagina, Dr. C.L. Day and Dr T.J Scriba.

Chapter 5: Comparison of proportions of specific T-cell memory and maturation phenotypes following BCG vaccination at birth and at 6 weeks of age

5.1. Introduction

In this chapter, we describe the comparative proportions of memory phenotypes and maturational characteristics of BCG-specific $CD4^+$ and $CD8^+$ T cells. We designed follow up studies based on the findings that administration of BCG at birth induces greater frequencies of BCG-specific single IFN-γ^+ as well as bifunctional IFN-γ^+perforin$^+$-expressing T cells than BCG administered at 6 weeks of age.

We hypothesised that greater frequencies of BCG-specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ positively associate with greater proportions of effector memory T cells (T_{EM}). Our aim therefore was: (i) to compare the proportions of memory T cell subsets between the two groups of infants; (ii) to compare the maturational phenotype of BCG-specific memory T cells between the two groups of infants.

To address our aim, we used a combination of CD45RA, CCR7 and CD27 to characterise the specific IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells in both groups of infants. Different subsets of memory T cells can be defined based on the expression of the surface markers, CD45RA and CCR7 (Sallusto et al., 1999). In addition, memory T cells can be further dissected into different subsets based on expression of CD27 (Adekambi et al., 2012, Fritsch et al., 2005, Tena-Coki et al., 2010). These markers are discussed in detail in chapter 1 (section 1.9). Studies in our laboratory have shown presence of specific memory T cells in the peripheral blood of 10-week old infants who had received BCG at birth (Kagina et al., 2009). In these studies, an effector memory phenotype was observed in BCG-specific memory T cells expressing IFN-γ (Kagina et al., 2009, Soares et al., 2008).

We used IFN-γ expressing $CD4^+$ and $CD8^+$ T cells to define antigen specific $CD4^+$ and $CD8^+$ T cells. Furthermore, we used CD69 antibody to identify early, activated and antigen-specific $CD4^+$ and $CD8^+$ T cells (Testi et al., 1994). In our preliminary experiments during optimisation, we found that $CD4^+CD69^+$ T cells not expressing IFN-γ produced IL-2, IL-17 or TNF-α . We were therefore able to also characterise antigen-specific and non-IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells in the two groups of infants.

5.2. Material and methods

5.2.1. Study participants

The participants studied in this chapter were 9 month-old infants selected from the cross sectional cohort described in chapter 2.

5.2.2. Twelve-hour whole blood assay

Whole blood was stimulated with specific antigens for a total of 12 hours as described previously in chapter 2 (section 2.7.1).

5.2.3. Antibodies for intracellular cytokine staining assay

The following fluorescently conjugated monoclonal Abs were used: anti-CD3 Pacific Blue (UCHT1), anti-CD8 Horizon V500 (RPA-T8), anti-IFN-γ Alexa Fluor 700 (B27), anti-CD45RA FITC (HI100), anti-CD27 APC (L128) and anti-CCR7 PE (560765), all from BD Biosciences, San Jose, CA; anti-CD4 QDot605 (S3.5) from Invitrogen, Eugene, OR; anti-CD69 PerCP-Cy5.5 (FN50), from Biolegend, San Diego, CA. All antibodies were titrated to find optimal concentrations for use. **Table 14** below summarises the flow cytometry panel used in this study.

Marker	Fluorochrome	Description
CD3	Pac Blue	T cell marker
CD4	Qdots 605	T cell marker
CD8	Horizon V500	T cell marker
CD45RA	FITC	Naïve T cell marker
CCR7	PE	Homing marker
CD27	APC	Maturation marker
CD69	PerCpCy5.5	Early activation marker
IFN-γ	Alexa Fluor 700	Th1 cytokine

Table 14: Flow cytometry panel for assessing the memory phenotypes of the specific $CD4^+$ and $CD8^+$ T cell. Shown are the markers included in the panel, the fluorochromes to which they are conjugated, as well as the description of the markers.

5.2.4. Intracellular cytokine staining assay and flow cytometry analysis

In our optimization experiments, we observed that CCR7 staining was optimal at 37°C. However, the rest of the antibodies in our panel stained optimally at 4°C. We therefore used a "two step" staining method to assess memory phenotypes of BCG-specific T cells. In this method, we stained for the surface marker, CCR7 followed by the rest of the surface markers and intracellular cytokines.

Fixed, cryopreserved white cells from the stimulated whole blood were thawed, washed in phosphate buffered saline (PBS, BioWhittaker), permeabilised in Perm/Wash Buffer (BD Biosciences) and stained with CCR7 mAbs for 30 minutes at 37°C. Stained cells were then washed in 1mL BD Perm/Wash buffer and stained with mAbs to intracellular cytokines and the rest of the surface markers for 1 hour at 4°C. Cells were then washed and acquired on a LSRII flow cytometer (BD Biosciences). For quality assurance, compensation was done with positive and negative anti-mouse Ig kappa-beads (BD Biosciences) labeled with the respective fluorochrome-conjugated antibodies. Cytometer Setting and Tracking (CST) beads (BD Biosciences) were used for daily settings. After

acquisition, data were compensated and analysed using FlowJo software (v9.4.11; Treestar). Flow data were then exported to Pestle v1.7 (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and Spice (v5.1) for further analysis (Roederer et al., 2011).

5.2.5. Data analysis

For each study participant, frequencies of IFN-γ-expressing $CD4^+$ T cells detected in the negative control sample were subtracted from frequencies detected in the corresponding BCG-stimulated samples to determine the frequency of BCG-specific cells. Similar analysis was performed on $CD8^+$ T cells. Background subtraction was not performed for memory phenotype analysis. Samples were excluded from the final analysis if the frequency of total PHA-induced IFN-γ-expressing $CD4^+$ T cells was lower than the median plus 3 time median absolute deviations (3xMAD) of the specific $CD4^+$ T cells of all the negative control samples. In addition, participants were excluded if: (1) frequency of BCG-specific IFN-γ-expressing $CD4^+$ T cells was less than 0.01% after subtraction of the response in the unstimulated condition; (2) the fold change in the frequency of IFN-γ^+ or IFN-γ^- $CD69^+$ expressing $CD4^+$ T cells in the BCG stimulated to the unstimulated sample was less than two and (3) number of BCG-specific IFN-γ^+ or IFN-γ^- $CD69^+$ expressing $CD4^+$ T cells was less than 20 after subtracting the non-specific cells in the unstimulated sample.

The Mann-Whitney U test was used to assess differences in the proportions of specific T cell memory and maturation phenotypes between the two groups. A p-value of less than 0.05 was considered statistically significant. The Prism 5.0 (GraphPad Software Inc.) was used for statistical analyses.

5.3. Results

5.3.1. Participants excluded from the analysis

We used the exclusion criteria described in the data analysis section. **Table 15, 16 and 17** below show the reasons and number of participants excluded in the analysis.

Reason for exclusion	44 infants vaccinated at birth (all samples processed)	34 infants vaccinated at 6 weeks (all samples processed)
Frequency of specific IFN-γ-expressing $CD4^+$ T cells < 0.01%	0	0
Fold change in the frequency of IFN-γ^+ $CD4^+$ T cells in BCG compared to unstimulated sample < 2	7	9
Number of IFN-γ-expressing $CD4^+$ T cells after correcting for the unstimulated number of cell < 20	1	2

Table 15: Participants excluded from the final memory phenotype analysis of BCG-specific IFN-γ-expressing $CD4^+$ T cells.

	44 infants vaccinated at birth (all samples processed)	34 infants vaccinated at 6 weeks (all samples processed)
Reason for exclusion		
Frequency of specific IFN-γ-expressing $CD8^+$ T cells < 0.01%	0	0
Fold change in the frequency of IFN-γ^+ $CD8^+$ T cells in BCG compared to unstimulated sample < 2	20	9
Number of IFN-γ-expressing $CD8^+$ T cells after correcting for the unstimulated number of cell < 20	0	0

Table 16: Participants excluded from the final memory phenotype analysis of BCG-specific IFN-γ-expressing $CD8^+$ T cells

	44 infants vaccinated at birth (all samples processed)	34 infants vaccinated at 6 weeks (all samples processed)
Reasons for exclusion		
Frequency of specific IFN-γ^- $CD69^+$ CD4 T cells < 0.01%	0	0
Fold change in the frequency of IFN-γ^- $CD69^+$CD4 T cells in BCG compared to unstimulated sample < 2	20	25
Number of IFN-γ^- $CD69^+$CD4 T cells after correcting for the unstimulated number of cell < 20	0	0

Table 17: Participants excluded from the final memory phenotype analysis of BCG-specific $CD69^+$ and non-IFN-γ-expressing $CD4^+$ T cells

5.3.2. Gating strategy

We modified the gating strategy developed during the optimisation process described in **Chapter 3** to analyse the profile of CCR7, CD45RA and CD27

expression by BCG-specific IFN-γ-expressing and non-IFN-γ-expressing $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells. **Figure 33** shows the modified gating strategy and representative flow cytometry dotplots from an infant vaccinated with BCG at 6 weeks of age.

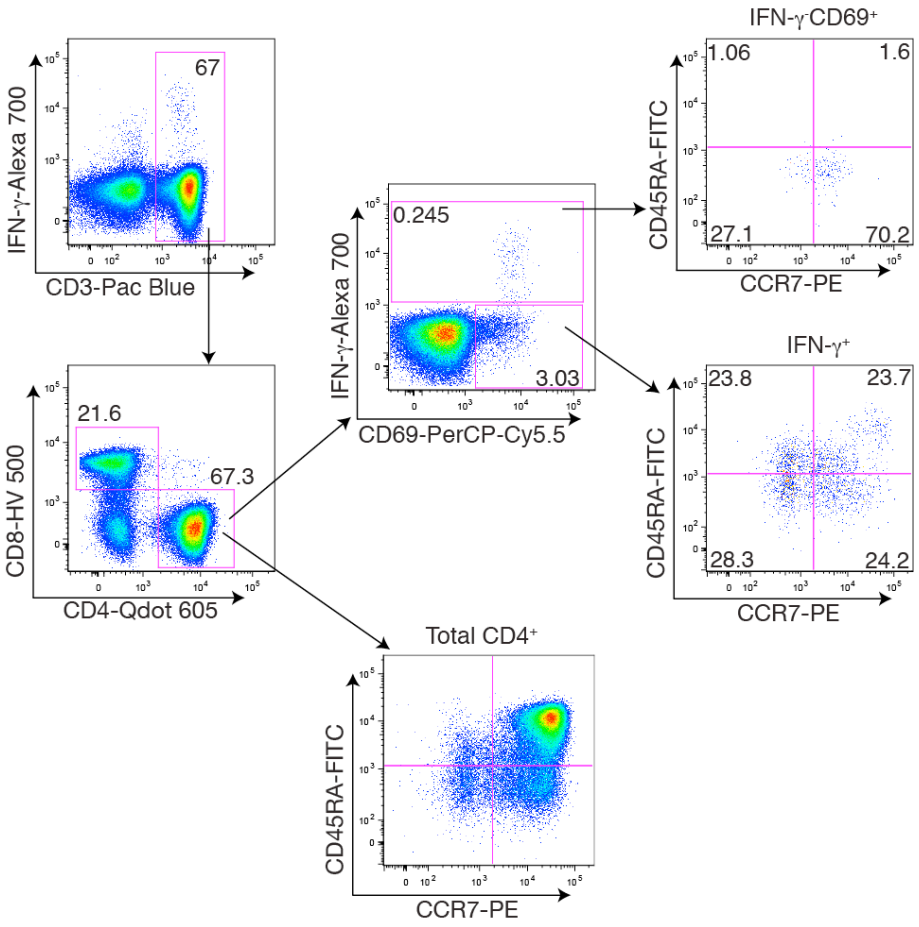

Figure 33. Gating strategy to analyse the memory phenotype of the specific IFN-γ–expressing $CD4^+$ T cells. To analyse expression of CD45RA, CCR7 and CD27 memory markers on the specific T cells, we selected $CD3^+$ T cells and divided this population into $CD4^+$ and $CD8^+$ T cells. Then, cells expressing IFN-γ or CD69 were selected from the $CD4^+$ T cells. Finally, CD45RA and CCR7 and CD27 expression were assessed within the specific IFN-γ- and non-expresssing cells. A similar analysis strategy was used for $CD8^+$ T cells.

5.3.3. No difference in BCG-specific $CD4^+$ and $CD8^+$ T cell memory phenotypes between the two groups

We aimed to characterise the profile of memory phenotypes of BCG-specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ. We hypothesized that birth vaccinated infants would show a greater proportion of specific IFN-γ^+ $CD4^+$ and $CD8^+$ T cells expressing an effector memory phenotype, compared to those vaccinated at 6 weeks of age.

In both groups of infants, the majority of the BCG-specific IFN-γ-expressing $CD4^+$ T cells showed a dominant central memory (T_{CM}) phenotype (**Figure 34A**). The second most dominant memory phenotype characterising the BCG-specific IFN-γ-expressing $CD4^+$ T cells was T_{EM}, while the proportions of the other phenotypes of T_{Naive} and T_{EMRA} were least represented.

There were no differences in proportions of any of the memory phenotypes characterising the BCG-specific IFN-γ-expressing $CD4^+$ T cells between the two groups of infants. (**Figure 34A**). We used a similar strategy to analyse the proportions of T cell memory phenotypes expressed by BCG-specific IFN-γ $CD8^+$ T cells. BCG-specific IFN-γ-expressing $CD8^+$ T cells mainly showed a T_{EM} and T_{EMRA} phenotypes in both groups of infants, and again the two groups were not different (**Figure 34B**).

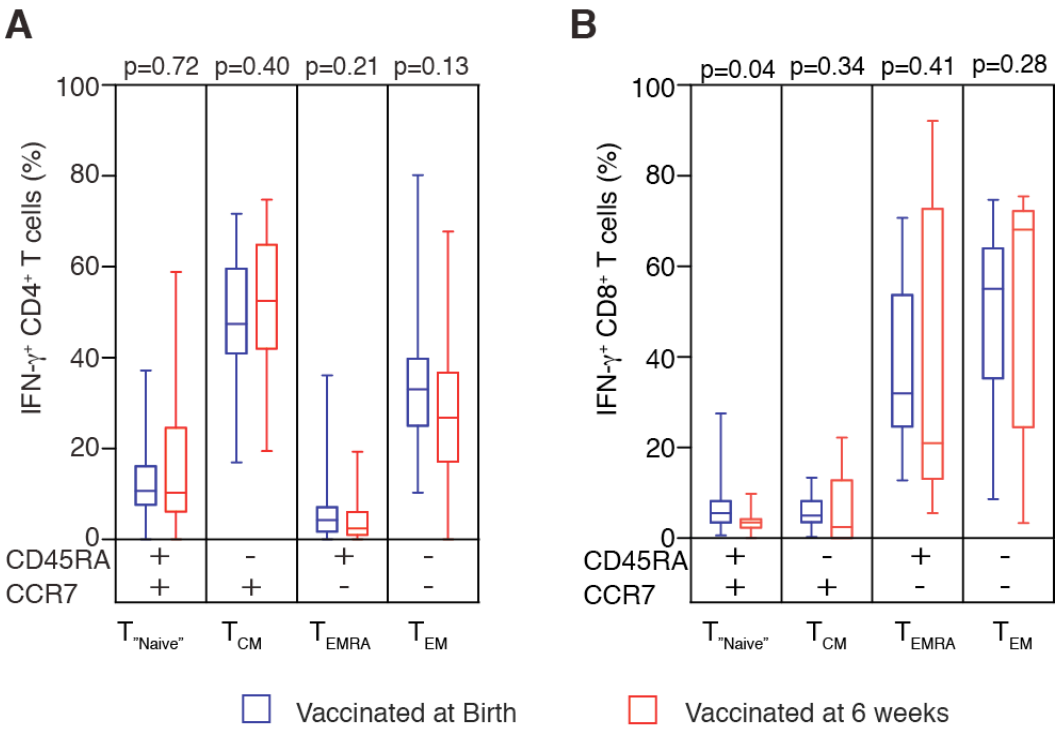

Figure 34. Memory phenotypes of BCG-specific IFN-γ-expressing CD4$^+$ and CD8$^+$ T cells. Box and whisker plots show the proportions of BCG-specific IFN-γ-expressing CD4$^+$ T cells (**A**), and BCG-specific IFN-γ-expressing CD8$^+$ T cells (**B**). In both graphs, expression of the CD45RA and CCR7 memory markers, either singly or in combination are shown. Whiskers represent the minimum and maximum value; the box represents the interquartile range, while the line in the box represents the median. Values are shown without subtraction of the unstimulated responses in the negative controls from the BCG-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in proportions of specific T cell memory phenotypes between the birth (blue bars) and 6 weeks of age (red bars) vaccinated infants. P values less than 0.05 were considered statistically significant.

5.3.4. No difference in BCG-specific IFN-γ-non-expressing CD4$^+$ T cell memory phenotype between the two groups.

In our studies reported in **Chapter 4,** we did not observe a difference in the frequencies of TNF-α-, IL-2- and IL-17-expressing BCG-specific CD4$^+$ T cells in the two groups of infants. The BCG-activated but non-IFN-γ producing T cells represented TNF-α-, IL-2- and IL-17-expressing BCG-specific CD4$^+$ T cells (We used CD69 in place of TNF-α-, IL-2- and IL-17 because we were limited by the number of parameters that we could include in our flow cytometry panel). We therefore hypothesised that similar proportions of memory T cell phenotypes

would be observed between the two groups of infants by the BCG-activated but non-IFN-γ producing cells. To test this hypothesis, we assessed the expression of memory markers in the activated non-IFN-γ producing $CD4^+$ T cells (IFN-γ^- $CD69^+$). We observed no difference in the proportions of $T_{Naive,}$ $T_{CM,}$ T_{EM} and T_{EMRA} phenotypes of activated and non-IFN-γ producing $CD4^+$ T cells between the two groups of infants (**Figure 35A**). Non-IFN-γ producing $CD8^+$ T cells were infrequent and therefore not analysed for memory phenotype. When we analysed memory phenotypes of the activated non-IFN-γ producing $CD4^+$ T cells in the unstimulated samples, greater proportions of $T_{CM,}$ and $T_{EMR,A}$ $CD4^+$ T cells were observed in infants vaccinated at birth than at 6 weeks of age (**Figure 35B**). The phenotype of non-specific (IFN-γ^- $CD69^+$) and apparently activated $CD4^+$ T cells showed that the birth vaccinated infants had a greater proportion of T_{CM} and T_{EMRA} phenotypes than the 6 weeks vaccinated infants (**Figure 35B**).

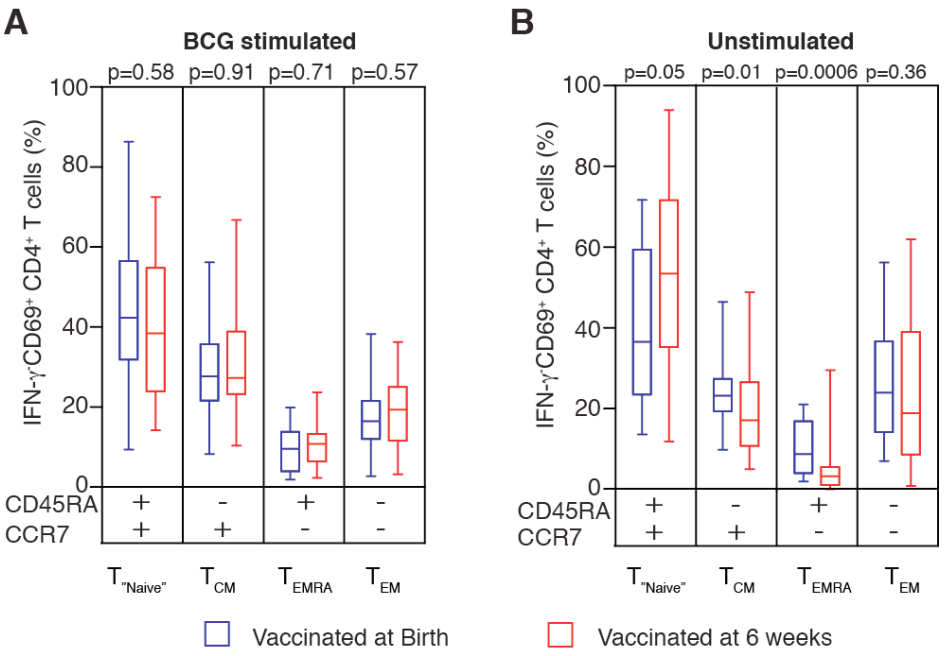

Figure 35. Memory phenotype of BCG-specific and non-specific CD4$^+$ T cells. BCG-specific and activated IFN-γ$^-$CD4$^+$ T cells were selected based on CD69 expression as detailed in the gating strategy figure **(A)**. BCG-specific IFN-γ$^-$CD69$^+$ CD4$^+$ T cells (**A**) and non-specific IFN-γ$^-$CD69$^+$ CD4$^+$ T cells (**B**) expressing different combinations of CD45RA and CCR7 either singly, or in combination are shown. Whiskers represent the minimum and maximum value; the box represents the interquartile range, while the line in the box represents the median. Values are shown without subtraction of the unstimulated responses in the negative controls from the BCG-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in proportions of specific T cell memory phenotypes between the birth (blue bars) and 6 weeks of age (red bars) vaccinated infants. P values less than 0.05 were considered statistically significant.

5.3.5. No difference in BCG-specific CD4$^+$ and CD8$^+$ T cells maturation phenotype between the two groups

We also evaluated the expression of the maturation marker, CD27 by the BCG-specific IFN-γ producing CD4$^+$ and CD8$^+$ T cells. We hypothesised that greater frequencies of IFN-γ-expressing T cells in the birth vaccinated infants may be explained by presence of more differentiated specific T cells than in infants vaccinated at 6 weeks of age. Representative flow cytometry plots of CD27 expression by CD4$^+$ and CD8$^+$ T cells are shown in **Figures 36 A** and **B** respectively. We observed no difference in BCG-specific IFN-γ-expressing CD4$^+$

and CD8$^+$ T cells expressing CD27 between the two groups of infants (**Figures 36C, D**).

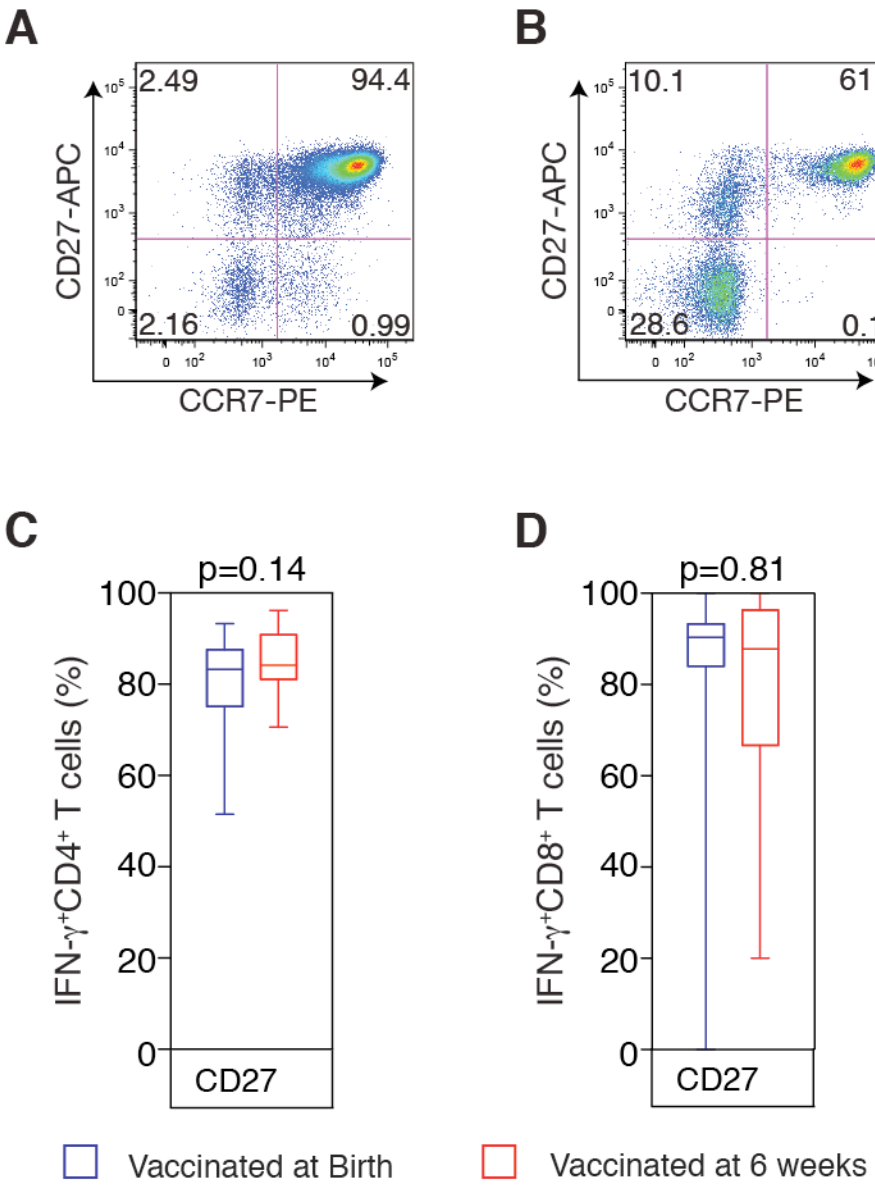

Figure 36. CD27 expression by BCG-specific CD4$^+$ and CD8$^+$ IFN-γ-expressing T cells. Box and whiskers plots show the proportions of BCG-specific IFN-γ-expressing CD4$^+$ T cells (**A**) and CD8$^+$ T cells (**B**) expressing CD27. Whiskers represent the minimum and maximum value; the box represents the interquartile range, while the line in the box represents the median. Values are shown without subtraction of the unstimulated responses in the negative controls from the BCG-stimulated samples. The *Mann–Whitney* U test was used to compare the difference in proportions of specific T cells expressing CD27 between the birth (blue bars) and 6 weeks of age (red bars) vaccinated infants. P values less than 0.05 were considered significant

5.4. Discussion

In this chapter, we compared memory and maturational phenotypes of BCG-specific IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells in infants either vaccinated at birth or at 6 weeks of age. Our results showed BCG-specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ were not different with regard to expression of memory and maturational phenotypes in both groups of infants. We also showed that, in both groups of infants, BCG-specific IFN-γ-expressing $CD4^+$ T cells were characterised by a dominant central memory (T_{CM}) phenotype, while the non-IFN-γ-expressing $CD4^+$ T cells were mostly 'naïve" like (CD45RA+CCR7+) and T_{CM}. Finally, BCG-specific IFN-γ expressing $CD8^+$ T cells were characterised by T_{EM} and T_{EMRA} phenotypes.

In the presence of a persistent antigen such chronic viral infections, effector memory cells, characterised by production of cytokines such as IFN-γ dominate the specific immune response (Harari et al., 2004). In line with these reports by Harari *et al.*, previous studies in our laboratory have shown that BCG-specific responses in infants are dominated by IFN-γ producing $CD4^+$ and $CD8^+$ T cells (Kagina et al., 2009, Soares et al., 2008). This is contrary to the dominant T_{CM} phenotypes of the specific IFN-γ-expressing $CD4^+$ T cells we observed in both groups of infants in our study. The discrepancy in the memory phenotypes from the previous studies may be explained by the difference in the antibody clone used in these studies from ours. Nevertheless, our findings are in agreement with a study by Soares *et al.* that showed a dominant T_{CM} phenotype by BCG-specific $CD4^+$ T cells in 27 week old infants vaccinated with BCG at birth (Soares et al., 2013). Our results suggest that development of the T_{CM} phenotype specific to BCG may not be influenced by timing of BCG. It is rational to propose that in prime-boost vaccination strategies, boosting with a novel TB vaccine may not be sensitive to whether BCG was given at birth or 6 weeks of age.

Our findings that BCG-specific IFN-γ producing $CD4^+$ T cells predominantly expressed a T_{CM} phenotype also differ from the BCG-specific T_{EM} reported in

mice (Dudani et al., 2002, Henao-Tamayo et al., 2010, Kaveh et al., 2011). BCG causes a chronic infection in mice resulting in persistent immune activation (Dudani et al., 2002, Mittrucker et al., 2007). This persistence of antigen results in induction of BCG-specific effector memory $CD4^+$ and $CD8^+$ T cells (Dudani et al., 2002, Henao-Tamayo et al., 2010). However, the T_{EM} phenotype is short-lived and may underlie the limited duration of protection conferred by BCG against TB (Orme, 2010). The T_{CM} phenotype observed in 9-month old infants in our study may indicate that BCG does not persist in the human. In line with this argument, a recent study from our laboratory shows that BCG-specific T cell expressed high levels of BCL-2 and low levels of the activation marker, CD38 following the contraction phase of the BCG immune response (Soares et al., 2013).

We also evaluated memory phenotypes of non-IFN-γ expressing $CD4^+$ T cells. We had observed that the activated and non-IFN-γ expressing $CD4^+$ T cells comprised BCG-specific T cells expressing IL-2, IL-17 and TNF-α. We observed that non-IFN-γ-expressing $CD4^+$ T cells in both groups of infants expressed a dominant T_{Naive} phenotype. Such 'naive' like BCG-specific memory T cells have been previously described in our group (Kagina et al., 2009, Soares et al., 2013, Tena-Coki et al., 2010, Dintwe et al., 2013). Functionally, the 'naive' like specific cells described in this study do not fit the definition of classical antigen inexperienced cells which would not produce effector cytokines (Sallusto et al., 1999). Detailed studies in our laboratory are interrogating this population of naive' like mycobacteria-specific T cells with respect to relevance in mycobacteria immunity. Regardless of the immune function of the naïve-like BCG-specific T cells, our results show no difference in the naive' like memory phenotype $CD4^+$ T cells, irrespective of administration of BCG at birth or 6 weeks of age.

We also characterised the memory phenotypes of the IFN-γ-expressing $CD8^+$ T cells. Both groups showed dominant T_{EM} and T_{EMRA} phenotypes. Our results are

consistent with those of a previous study that reported T_{EM} and T_{EMRA} as the dominant phenotypes of PPD-specific $CD8^+$ T cells in healthy children (5-11 years) (Caccamo et al., 2006, Tena-Coki et al., 2010). This may be explained by the presence of T_{EM} and T_{EMRA} memory subsets in circulation in the absence of TB disease, in which case the cells would be sequestered to the site of infection (Dieli et al., 1999). We observed similar proportions in all $CD8^+$ T cell memory phenotype subsets in the two groups of infants.

The loss of CD27 expression has been associated with repeated antigenic stimulation of the T cells with concomitant T cell senescence (De Jong et al., 1992). We showed that in both groups of infants, a high proportion of BCG-specific IFN-γ expressing $CD4^+$ and $CD8^+$ T cells expressed CD27, although at similar proportions. Our results are in agreement with a previous study reporting high proportion of CD27 expression on specific $CD4^+$ and $CD8^+$ T cells from BCG vaccinated infants (Tena-Coki et al., 2010).

In summary, our data indicate that BCG vaccination at birth or at 6 weeks of age results in similar memory phenotypes expressed by both $CD4^+$ and $C84^+$ T cells. Future studies should assess if the predominant $CD4^+$ T cell central memory phenotype we report in these 9 month-old infants is associated with long term persistence of BCG-specific memory T cells.

5.5. Contributions

Dr. F. Lutwama designed the experiments, conducted the laboratory assays and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B.M.N. Kagina, Dr. C.L. Day and Dr T.J Scriba.

Chapter 6: Comparison of BCG-specific soluble cytokine plasma levels following BCG vaccination at birth and at 6 weeks of age

6.1. Introduction

In this chapter, we describe the comparative plasma cytokine levels in infants vaccinated with BCG at birth or 6 weeks of age. In the previous two chapters, we showed that vaccination of infants with BCG at birth induces greater frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, than vaccination at 6 weeks of age. We also showed that greater proportions of T_{EM} could not explain the greater frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ in infants vaccinated at birth than at 6 weeks of age. We were therefore interested in exploring other potential immunological reasons that may underlie the observed differences in frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ.

Upon BCG-vaccination, APCs, mainly monocytes and dendritic cells, phagocytose BCG, kill and process the mycobacteria into small peptides (Inaba et al., 2000, Turley et al., 2000). The BCG derived peptides are presented to MHC-II or MHC-I restricted T cells. When the APCs process and present BCG derived peptides to cognate T cells, the cytokine milieu may influence the BCG-induced T cell response. Therefore, we reasoned that the differences observed in the frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ between the two groups of infants, could be explained by levels of soluble plasma cytokines, a surrogate marker for the cytokine milieu.

Cytokine milieu characterised by the presence of high Th2 soluble cytokines; IL-4, IL-5 and IL-13 may attenuate mycobacteria-induced Th1 immune responses (Rook, 2007). For example, IL-4 may attenuate anti-mycobacterial immunity by driving arginase production leading to alternative macrophage activation (Gordon, 2003). Alternative activation of macrophages leads to decreased IL-12 production, an important Th1 cells differentiation factor that is critical in the IFN-γ production by CD4 and CD8 T cells.

During the induction of BCG-induced T cell immunity, presence of high levels of the immunoregulatory cytokine, IL-10 has been shown to attenuate the BCG-induced Th1 (IFN-γ, IL-2) immune response (Pitt et al., 2012). The immunoregulatory mechanism of IL-10 involves inhibition of production of the Th1 promoting differentiation factor, IL-12. Furthermore, IL-10 has been shown to inhibit IFN-γ induced effector mechanisms by driving alternative activation of macrophages (Schreiber et al., 2009).

On the other hand, presence of high levels of Th1 cytokines, for example, IL-2 and IFN-γ, during the induction of BCG-induced T cell immunity may favor increased production of IFN-γ by specific $CD4^+$ and $CD8^+$ T cells (Wenner et al., 1996). Conversely, it has been shown that high IFN-γ levels may attenuate Th2 differentiation during the priming of the naive T cells (Seder and Paul, 1994).

It is therefore plausible that the cytokine milieu may play a key role in skewing the BCG-specific T cell responses. Our aim was to compare soluble levels of BCG-specific Th1 (IFN-γ, IL-2), Th2 (IL-4, IL-5 and IL-13) and IL-10 cytokines in infants either vaccinated with BCG either at birth or at 6 weeks of age. Our hypothesis was that infants vaccinated at 6 weeks of age would show greater levels of soluble Th2 and IL-10 cytokine than infants vaccinated at birth. We used the bead-based multiplex assay to measure the soluble cytokines levels in plasma collected after 7hrs of whole blood stimulation with BCG.

6.2. Material and methods

6.2.1. Study participants

The infants studied in this chapter were selected from the cross sectional cohort described in chapter 2.

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

Whole blood was stimulated with specific antigens for a total of 7 hours as described previously in chapter 2 (section 2.7.1). Plasma was removed and stored at -80^{o}C for measurement of soluble cytokine levels.

6.2.3. Multiplex soluble cytokine assay

Plasma collected after 7 hours of incubation during the short-term whole blood assay was used to measure of levels of soluble IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-13 with the MILLIPLEX® MAP assay (Millipore, Billerica, MA), following the manufacturers' instructions. In brief, a filter 96-well plate was blocked with assay buffer then aspirated using a vacuum manifold. The provided standards, controls, and samples were added to the appropriate wells. Plasma from the unstimulated samples as well as from BCG- and PHA–stimulated samples was added undiluted into single wells. Then, mixed beads were added to all wells containing the plasma samples, and incubated at room temperatures in the dark for one hour. Next, the plates were washed twice, detection antibody cocktail added, and further incubated for 30 minutes. Thereafter, streptavidin-phycoerythrin solution was added. The plates were then washed twice, sheath fluid added, and resuspended on a shaker. Fluorescence was detected using a Luminex 100 IS machine (xMAP technology, Luminex Corporation). Data were acquired using the Bio-Plex Manager™ Software. The standard curve for each cytokine ranged from 3.2 to 10 000pg/ml.

6.2.4. Data analysis

Values falling outside range of the standard curve were excluded from the analysis. For the samples, cytokine values below the lowest level of detection were assigned a value of 2.2 pg/mL before background subtraction.

For each participant, the response detected in the negative control (unstimulated) was subtracted from the response detected in the corresponding BCG-stimulated sample. Samples were excluded from the final analysis if: (1) response detected in the positive control was less than the median plus 3xMAD of the response detected in all the negative control samples; (2) response detected in the positive control condition was lower than that in the corresponding negative control sample; (3) response detected in the positive control was less than the lower limit of detection (3.2 pg/mL) for the assay.

The Mann-Whitney U test was used to assess differences in cytokine levels between the two groups of infants. A p-value of less than 0.05 was considered significant. Spearman-rank correlation was used to assess associations between frequencies of $CD4^+$ and $CD8^+$ T cell cytokine producing cells and levels of soluble cytokines in plasma. Prism 5.0 (GraphPad Software Inc.) was used for statistical analyses.

6.3. Results

6.3.1 Participants excluded in the analysis of the luminex assay

Table 18 shows the number of infants excluded from the luminex assay results as well as reasons for exclusion. In total, we excluded 12 and 11 infants vaccinated at birth and 6 weeks of age respectively.

Reason for exclusion	44 infants vaccinated at birth (all samples processed)	40 infants vaccinated at 6 weeks (all samples processed)
PHA response < median + 3MAD of all negative controls samples	4	5
PHA response < negative control response	0	2
PHA response < 3.2 pg/ml	4	2

Table 18: Participants excluded from the final multiplex assay analysis

6.3.2. BCG-specific soluble cytokine levels in infants vaccinated at birth or at 6 weeks of age

We also evaluated levels of IFN-γ, IL-2 (Th1), IL-4, IL-5, IL-13 (Th2) and IL-10 cytokines in plasma. Our hypothesis was that this would reflect a cytokine milieu that would impact the BCG-induced T cell response, e.g., presence of high levels of Th2 cytokines (IL-4, IL-5 and IL-13) as well as IL-10 might attenuate this immunity (Rook 2007;Schreiber 2009). We reasoned that the differential Th2 and IL-10 cytokine soluble cytokine levels would possibly explain why we had observed greater frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, in birth than 6 weeks of age vaccinated infants in our earlier experiments. The Th2 cytokines IL-4, IL-5 and IL-13 were all either undetectable or detected at very low levels in both groups of infants and were not different (**Figures 37A, B and C)**. Relative to Th2 cytokines, IFN-γ and IL-2 were detected at higher levels but were also not different in both groups of infants (**Figures 37D and E)**. In part, our hypothesis was confirmed by IL-10 results: we observed that infants vaccinated

at 6 weeks of age had greater levels of soluble IL-10 than infants vaccinated at birth (**Figure 37E**). However, birth vaccinated infants had higher levels of IL-10 in the unstimulated controls, compared with infants vaccinated at 6 weeks of age (**Figures 38A**). Moreover, we observed no difference in the levels of IL-10 in BCG-stimulated samples (without background subtraction) in the two groups of infants (**Figure 38B**). We concluded that the greater BCG-specific levels observed for in infants vaccinated at 6 weeks compared to those vaccinated at birth were influenced by the lower unstimulated IL-10 levels in the former. (IL-10 was not measured with the WB-ICS, as preliminary experiments showed that T cell-specific expression was too low for valid analysis.)

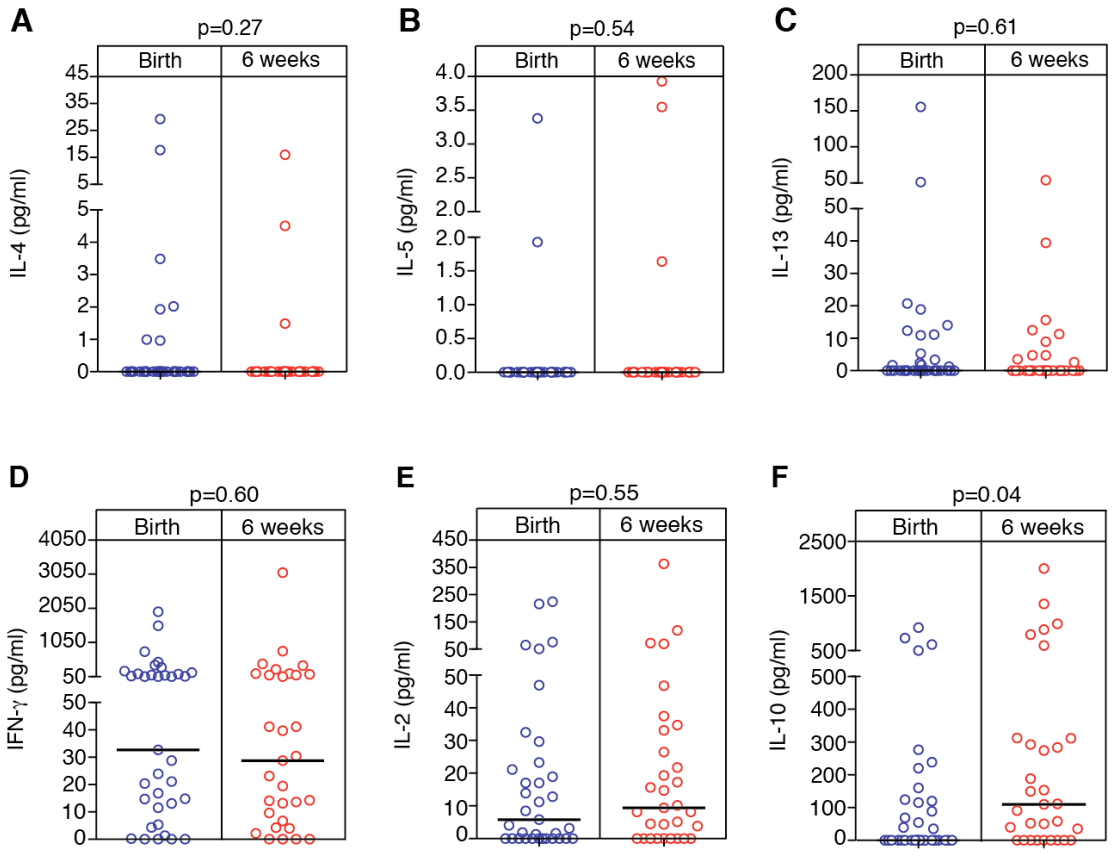

Figure 37. BCG-specific soluble cytokine levels in plasma. Plasma was taken from whole blood left unstimulated or stimulated with BCG for 7 hours. Soluble cytokine levels were measured by luminex assay. The scatter plots show the following cytokines: IL-4 (**A**), IL-5 (**B**), IL-13 (**C**), IFN-γ (**D**), IL-2 (**E**) and IL-10 (**F**). On the scatter plots, the horizontal lines represent the median cytokine levels. The results shown are corrected for unstimulated responses. The *Mann–Whitney* U test was used to compare the difference in cytokine levels between the birth (blue circles) and 6 weeks of age (red circles) vaccinated infants. P values less than 0.05 was considered significant.

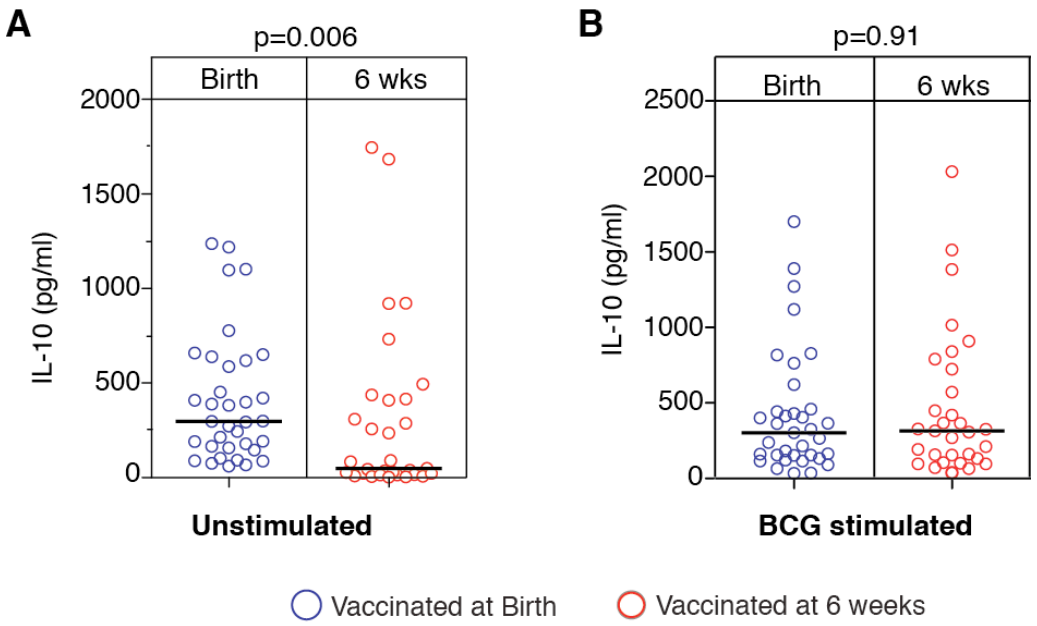

Figure 38. Unstimulated and BCG stimulated IL-10 soluble cytokine levels. Plasma was taken from whole blood left unstimulated or stimulated with BCG for 7 hours. Soluble cytokine levels were measured by luminex assay. The scatter plots show soluble levels of IL-10 detected in the (**A**) unstimulated and (**B**) BCG-stimulated samples (without correction for levels in unstimulated samples). Horizontal lines represent the median soluble cytokine levels. The *Mann–Whitney* U test was used to compare cytokine levels between the birth (blue circles) and 6 weeks of age (red circles) vaccinated infants. P values less than 0.05 was considered significant.

6.3.3. Correlation of specific IL-10 levels with frequencies of specific IFN-γ expressing $CD4^+$ and $CD8^+$ T cells

Our next analysis was aimed at evaluating whether the soluble IL-10 levels were associated with decreased frequencies of specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells. We therefore performed correlation analysis of soluble IL-10 levels with the frequencies of BCG-induced $CD4^+$ and $CD8^+$ T cells expressing IFN-γ. In both groups of infants, there was no significant correlation between levels of IL-10 cytokine and the frequency of IFN-γ expressing $CD4^+$ T cells (**Figure 39A**). However, a significant but weak positive correlation was observed between levels of IL-10 cytokine and the frequency of IFN-γ expressing $CD8^+$ T cells, only in infants vaccinated at 6 weeks of age (**Figure 39B**).

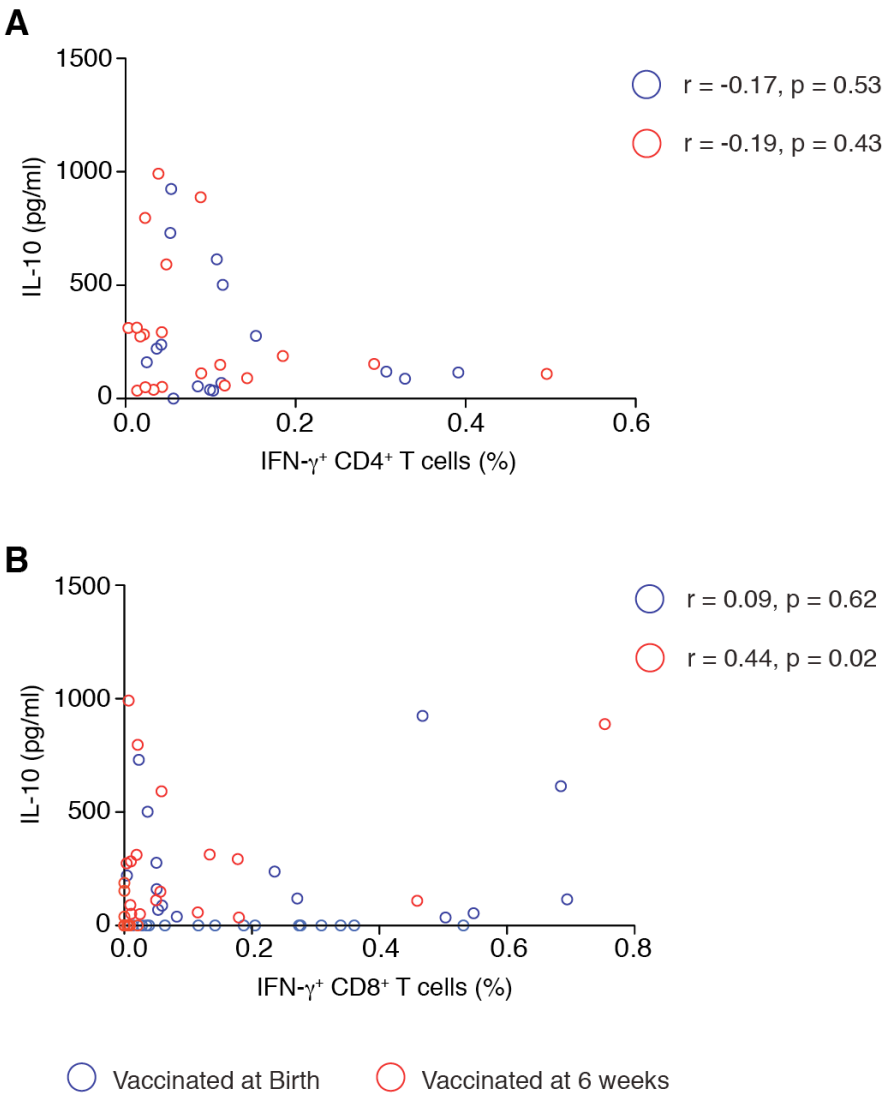

Figure 39. Correlation analysis of solube IL-10 levels and frequencies of IFN-γ⁻ expressing T cells. Scatter plots show the correlation analysis between soluble levels of BCG-specific IL-10 and frequency of BCG-specific IFN-γ-expressing $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells. The blue and red circles represent the birth (blue circles) and 6 weeks of age (red circles) BCG vaccinated infants respectively. Correlation analysis was performed by Spearman test. P values less than 0.05 were considered statistically significant.

6.4. Discussion

In this chapter we compared BCG-induced and non-specific soluble cytokine levels in infants vaccinated at birth or at 6 weeks of age. We showed greater non-specific IL-10 levels in infants vaccinated at birth, compared with those vaccinated at 6 weeks of age. We also showed that in infants vaccinated with BCG at 6 weeks of age, BCG-induced IL-10 levels positively correlated with the frequencies of BCG-specific IFN-γ-expressing $CD8^+$ T cells. Other soluble cytokines were all detected at very low levels and were not different between the two groups of infants.

We measured IL-10 cytokine levels based on studies that have shown that this cytokine may attenuate Th1 (IFN-γ) T cell responses (Liu et al., 2011). The attenuation of IFN-γ by IL-10 was shown in an experimental study using *M.tb*-susceptible CBA/J mice where antibody blockade of IL-10R during BCG vaccination resulted in an enhanced BCG-specific IFN-γ response and protection against subsequent *M.tb* challenge (Pitt et al., 2012). However, our results do not suggest that IL-10 is inhibiting BCG-specific Th1 responses, at least when measured at 9 months of age: infants vaccinated with BCG at birth had greater levels of non-specific IL-10 production and greater frequencies of BCG-specific IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells compared to infants vaccinated with BCG at 6 weeks of age. Our finding of elevated pro-inflammatory (IFN-γ) and regulatory (IL-10) cytokines is analogous to studies that have reported increased soluble levels of these two cytokines in patients with Immune reconstitution inflammatory syndrome (IRIS) (Skolimowska et al., 2012, Tadokera et al., 2011). The probable explanation for this unexpected paradoxical observation is that induction of a pro-inflammatory response may be accompanied by induction of regulatory responses that may mediate inhibition of the potentially destructive inflammation.

We assessed Th2 (IL-4) soluble levels based on previous studies that have reported a reduced mycobacterial-specific Th1 response in the presence of high

levels of Th2 (IL-4) cytokines (Hernandez-Pando et al., 1996). The soluble Th2 cytokine levels we detected in this study were comparable to those reported by Soares 2008 et al, where the authors used a similar assay system to measure IL-4 cytokine in plasma from 10-week old infants vaccinated with BCG-vaccinated at birth (Soares et al., 2008). In our study, both groups of infants had too low production of IL-4 as well as IL-5 and IL-13.

Our study had several limitations. We assessed the levels of soluble plasma cytokine at 9 months of age. However, priming of BCG-specific T cell immune responses occurred at the time of vaccination; at birth or at 6 weeks of age. Our study setting limited us from accessing participants at these time points to measure soluble cytokine levels. Second, we measured cytokine levels in blood in unstimulated samples, or following in vitro stimulation with BCG. However, upon administration of BCG, the priming of T cell responses is known to occur in lymph nodes. We acknowledge that our interpretation of how the cytokine milieu may affect the BCG-induced T cell response is speculative, since plasma cytokine levels may not necessarily reflect the levels of cytokines in lymph nodes.

In summary, infants vaccinated at 6 weeks of age had lower baseline IL-10 cytokine levels. We speculate that the greater non-specific IL-10 levels observed in infants vaccinated at birth compared to those vaccinated at 6 weeks of age may “potentially” result from the differences in social economic status observed in the two groups: Infants vaccinated at 6 week of age were from a lower social economic category compared to those vaccinated at birth.

6.5. Contributions

Dr. F. Lutwama designed the experiments, conducted the laboratory assays and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B.M.N. Kagina, Dr. C.L. Day and Dr T.J Scriba.

Chapter 7: Comparison of the capacity of BCG-specific T-cells to proliferate and produce cytokines in infants following BCG vaccination at birth or at 6 weeks of age

7.1. Introduction

Findings from the previous chapters showed that using a short-term ICS assay: (1) birth vaccinated infants had greater BCG-specific IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells than infants vaccinated at 6 weeks of age, and, (2) in both groups of infants, the BCG-specific IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells displayed a dominant T_{CM} phenotype. In this chapter, we followed up on these earlier observations to establish whether the proliferative capacity of BCG-specific T cells would be greater in infants vaccinated at birth than at 6 weeks of age. Our hypothesis was that BCG vaccination at birth would induce BCG-specific $CD4^+$ and $CD8^+$ T cells with greater proliferative and cytokine-expression capacity than vaccination at 6 weeks of age. We used a 6-day whole blood proliferation assay to measure the BCG-specific $CD4^+$ and $CD8^+$ T cell proliferative and cytokine producing capacity in the two groups of infants.

The ability of T cells to proliferate upon antigenic stimulation is an important function of antigen-specific T cell memory. For example, in human viral (small pox) models, long-term vaccinees had detectable specific T cell proliferative responses to vaccinia after two decades in the absence of exposure to small pox (Combadiere et al., 2004). Specific memory T cells are thought to be important in mediating long-term protective immunity (Wherry et al., 2003). Investigators in our laboratory have previously optimized a sensitive whole blood proliferation assay that measures Ki67 expression by specific T cells upon in vitro culture with specific antigen, including BCG (Soares et al., 2010). Ki67 is expressed during the active phases of cell division; high levels of Ki67 expression is therefore a good biomarker of proliferating cells (Gerdes et al., 1984).

Advantages of measuring T cell responses with such a long-term proliferation assay includes: First, increased sensitivity to measure memory T cell responses because long-term culture allows clonal expansion of specific cells; second, the assay enables measurement of proliferative capacity of antigen-specific T cells as well as production of cytokines, another important function of T cells; Third, long-term assays are robust and less sensitive to time between blood draw and incubation of whole blood compared with short term assays (Hanekom et al., 2008).

Therefore, in this study, we aimed to compare the capacity of BCG-specific T cells to proliferate and express Th1 (IL-2, IFN-γ and TNF-α) and IL-17 cytokines in infants who were vaccinated at birth or at 6 weeks of age. In our previous experiments using the short term assay (Chapter two), we reported that infants vaccinated with BCG at birth showed greater frequencies of BCG-specific IFN-γ expressing $CD4^+$ and $CD8^+$ T cell compared to infants vaccinated at 6 weeks of age. We therefore hypothesized that infants vaccinated with BCG at birth would show greater BCG-specific proliferative capacity, associated with increased production of effector molecules than infants vaccinated at 6 weeks of age.

7.2. Material and methods

7.2.1. Study participants

The infants studied in this chapter were selected from the cross sectional cohort described in chapter 2.

7.2.2. Six-day whole blood proliferation assay

Whole blood was diluted in with RPMI media and stimulated with specific antigens for a total of 6 days as described in chapter 2 (section 2.7.2).

7.2.2. Antibodies

The following fluorescently conjugated mAbs were used in flow cytometry experiments: anti-CD3 Pacific Blue (UCHT1), anti-CD8 PerCP-Cy5.5 (SK-1), anti-IFN-γ Alexa Fluor 700 (B27), anti-IL-2 FITC (5344.111), anti-Ki67 PE (B56), all from BD Biosciences, San Jose, CA, all from Invitrogen, Eugene, OR; anti-TNF-α PE-Cy7 (Mab11), and anti-IL-17 Alexa Fluor 647 (eBio64CAP17), both from eBiosciences, San Diego, CA. All antibodies were titrated to establish the optimal concentration for use. **Table 19** summarises the flow cytometry panel used in this study.

Marker	Fluorochrome	Description
CD3	Pac Blue	T cell marker
CD8	PerCpCy5.5	Cytolytic T cell marker
Ki67	PE	Proliferation marker
IFN-γ	Alexa Fluor 700	Th1 cytokine
IL-2	APC	Th1 cytokine
TNF-α	PE-Cy7	Th1 cytokine
IL-17	APC	Th17 cytokine

Table 19: Flow cytometry antibody-fluorochrome panel to evaluate the capacity of $CD4^+$ and $CD8^+$ T cells to proliferate and produce cytokines in cryopreserved fixed cells from stimulated whole blood collected from the study participants.

7.2.3. Intracellular cytokine staining assay and flow cytometry analysis

We used a "one step" staining method as described in chapter 3 (section 3.5.4) to assess the capacity of BCG-specific T-cells to proliferate and produce cytokines in infants following BCG vaccination at birth and at 6 weeks of age.

7.2.4. Data analysis

For each assay and participant, the response detected in the negative control (unstimulated) was subtracted from the response detected in the stimulated sample. Samples were excluded from the final analysis if the response detected in the positive control condition was lower than the median plus 3 median absolute deviations (3MAD) of the negative control samples from all infants. For cytokine expression, in addition to the above criterion, samples were excluded if: (1) the frequency of BCG-specific Ki67 expressing $CD4^+$ T cells was less than 0.01%; (2) the number of BCG-specific Ki67 expressing $CD4^+$ T cells was less than 20 after correcting for the non-specific cells in the respective unstimulated sample. The Mann-Whitney U test was used to compare differences in the immunological outcomes between the two groups. A p-value of less than 0.05

was considered significant. Prism 5.0 (GraphPad Software Inc.) was used for statistical analyses.

7.3 Results

7.3.1. Gating strategy

We used a modified gating strategy from that described in chapter 3 (section 3.6.11) to measure BCG-specific proliferative and cytokine production capacity. $CD4^+$ T cells were gated as $CD3^+CD8^-$ because PMA and ionomycin stimulation down-regulates CD4 expression on T cells. The flow cytometric representative data from an infant vaccinated with BCG at birth are shown in **Figure 40.**

7.3.2. Participants excluded from analysis based on quality control criteria

We used the exclusion criteria described in the data analysis to ensure reliability of the data reported. **Table 20** shows the summary of participants excluded form the 6-day proliferation assay analysis.

Reason for exclusion	Vaccinated at birth Excluded (n)	Vaccinated at 6 weeks Excluded (n)
BCG-specific frequency < 0.01%	0	5
BCG events count < 20	3	13

Table 20: Participants exclusion from the 6-day whole blood proliferation and cytokine expression analysis.

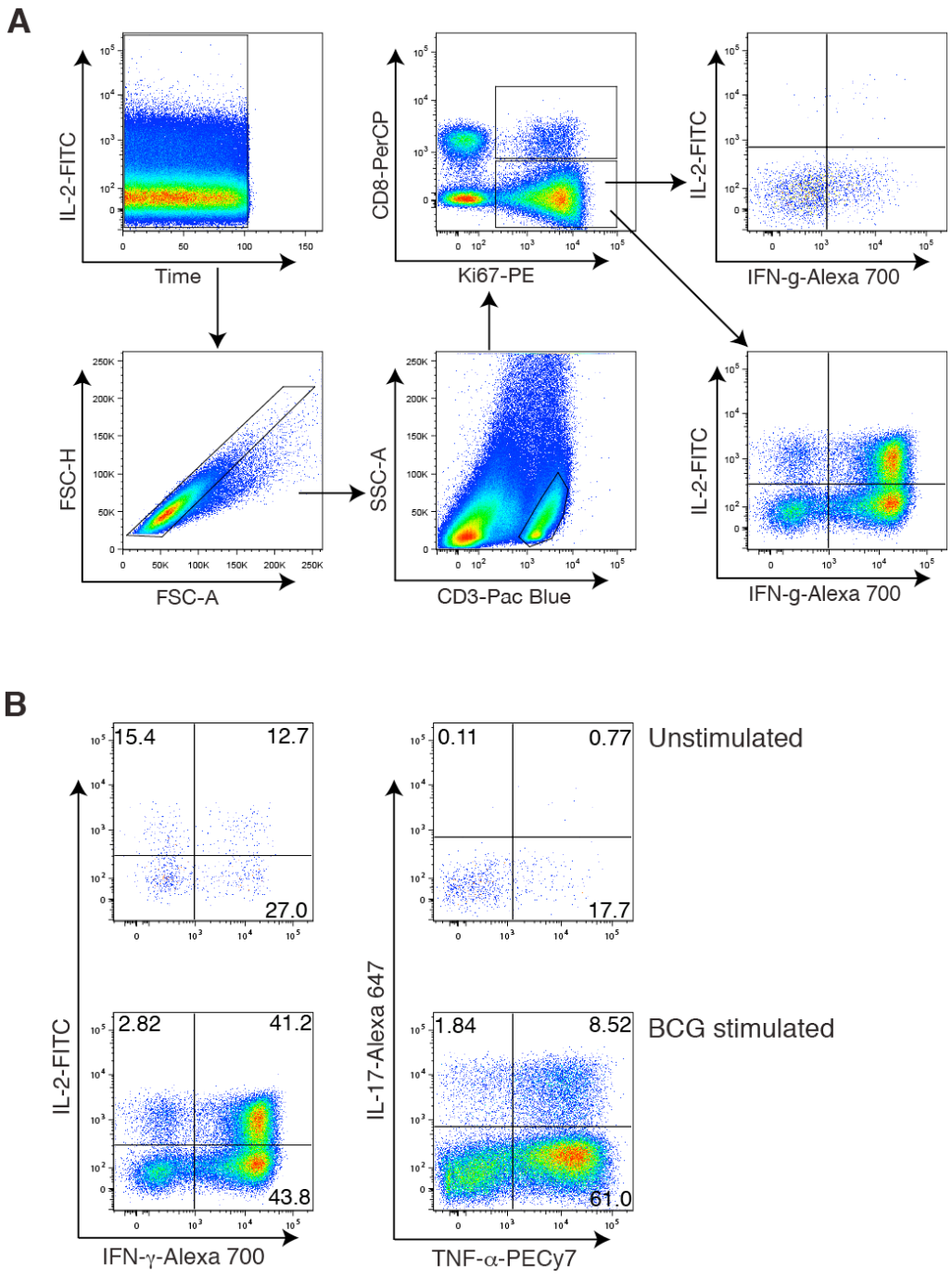

Figure 40. Gating strategy. Flow cytometry gating strategy of proliferating BCG-specific $CD4^+$ ($CD8^-$) and $CD8^+$ T cell and the associated cytokine responses evaluated in the 6-day whole blood ICS assay. We first used a time gate to select cells acquired only at the same fluorescence intensity over time. Then, doublet cells were excluded by gating on forward scatter-area (FSC-A) against forward scatter-height (FSC-H). T cells were selected by gating on CD3 expressing cells, which were further divided to $CD4^+$ and $CD8^+$ T cells (**A**). Representative flow cytometry data of $Ki67^+$ CD4 T cells expressing cytokines in a negative control (unstimulated) sample (top row) or BCG stimulated sample (bottom row) from a 9 month-old infant vaccinated with BCG at birth (**B)**

7.3.3. No difference in proliferative capacity or cytokine expression of specific $CD4^+$ T cells in infants vaccinated with BCG at birth or 6 weeks of age

The ability of cells to proliferate and respond to secondary antigen encounter is an important feature of memory and vaccine-induced T cell responses (Combadiere et al., 2004). Our aim was to compare the capacity of CD4 T cells to proliferate and produce cytokines (IL-2, IL-17, IFN-γ and TNF-α) in infants vaccinated at birth or at 6 weeks of age. We observed a detectable proliferative response of specific $CD4^+$ T cells in majority of infants in both groups. However, there was no difference in proliferative capacity of $CD4^+$ T cells between the two groups of infants (**Figure 41A**).

We also assessed the cytokine producing capacity of specific proliferating $CD4^+$ T cells, after re-stimulating the cells on day 6 with PMA/ionomycin. The capacity of the BCG-specific cells expressing either IL-2, IL-17, IFN-γ or TNF-α was not different between the two groups of infants. (**Figure. 41B**).

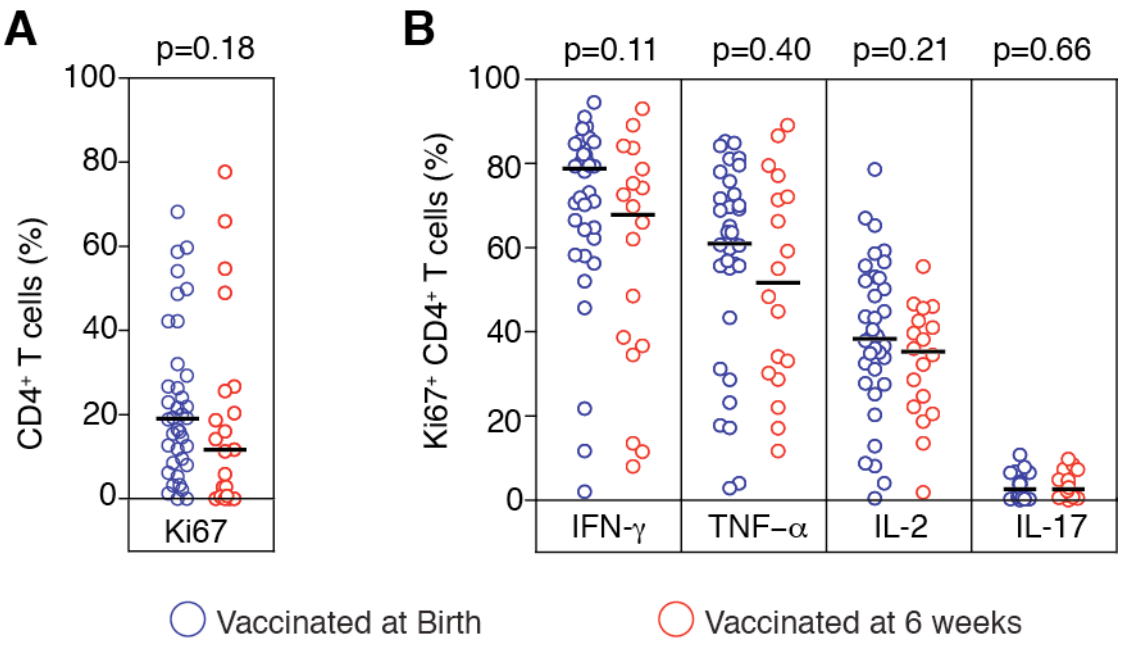

Figure 41. Proliferative capacity: BCG-specific $CD4^+$ T cell proliferative capacity and cytokine production by the proliferating T cells. Scatter plots depict the proportions of proliferating ($Ki67^+$) $CD4^+$ T cells (**A**) and the capacity of the proliferating $CD4^+$ T cells to produce IL-2, IL-17, IFN-γ or TNF-α cytokines (**B**). Horizontal lines represent medians. The blue and red dots represent the infants vaccinated at birth or 6 weeks of age respectively. The *Mann-Whitney* U test was used to compare the difference in frequencies between the 2 groups.

7.3.4. Greater capacity of specific proliferating $CD4^+$ T cells to co-express IL-2, IFN-γ and TNF-α in infants vaccinated at birth, compared with infants vaccinated at 6 weeks of age

Our next aim was to compare the capacity of proliferating $CD4^+$ T cells to co-express IL-2, IL-17, IFN-γ and TNF-α in infants vaccinated at birth or at 6 weeks of age. Our results showed that cells from infants vaccinated at birth had greater capacity to express IL-2 and IFN-γ and TNF-α together, compared with those vaccinated at 6 weeks of age (**Figure. 42**). However, the proportion of these cells able to express only TNF-α was lower in infants vaccinated at birth, compared with infants vaccinated at 6 weeks of age (**Figure 42**).

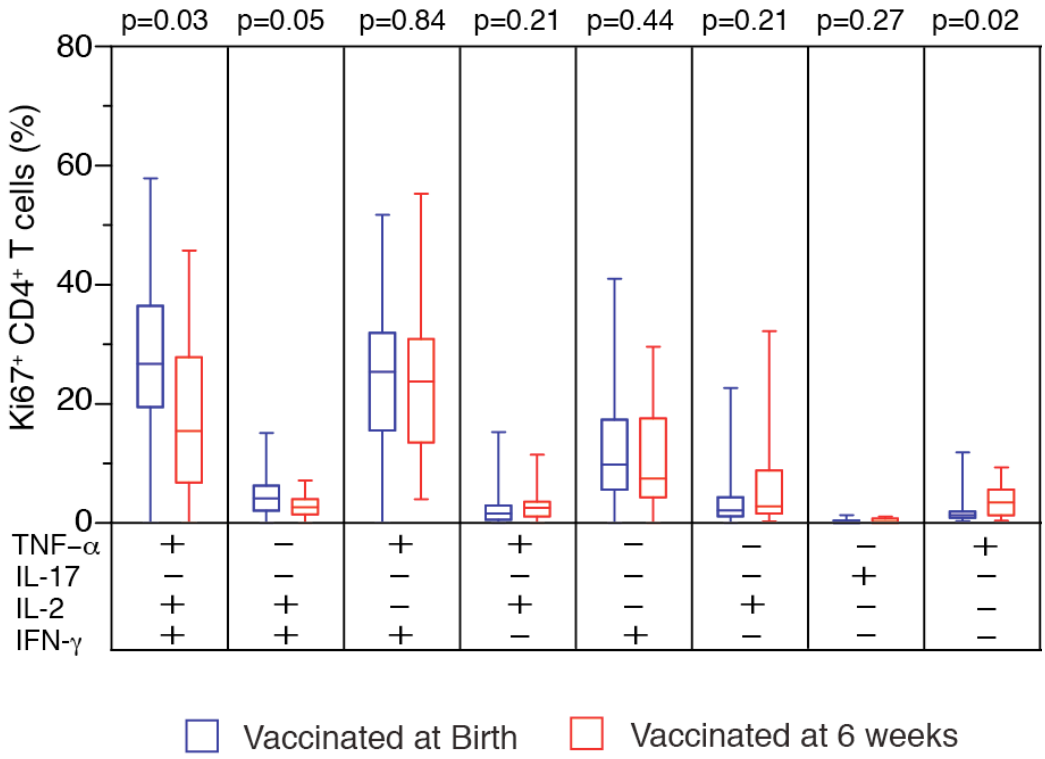

Figure 42. Capacity of proliferating $CD4^+$ T cells to co-express cytokines. The blue and red bars represent the birth and 6 weeks BCG vaccinated infants respectively. Whiskers represent the maximum and minimum value; the box represents the interquartile range, while the line in the box represents the median. The *Mann–Whitney* U test was used to compare the difference in the two groups.

7.3.5. No difference in capacity of specific proliferating $CD8^+$ T cells to produce IL-2, IFN-γ or TNF-α in infants vaccinated at birth or at 6 weeks of age

We then assessed the capacity of BCG-specific $CD8^+$ T cell to proliferate and produce cytokines in the two groups of infants. As for $CD4^+$ T cells, we observed no difference in the capacity of specific $CD8^+$ T cells to proliferate (**Figure 43A**) nor in their production of IL-2, IL-17, IFN-γ or TNF-α (**Figure 43B**) in the two groups of infants.

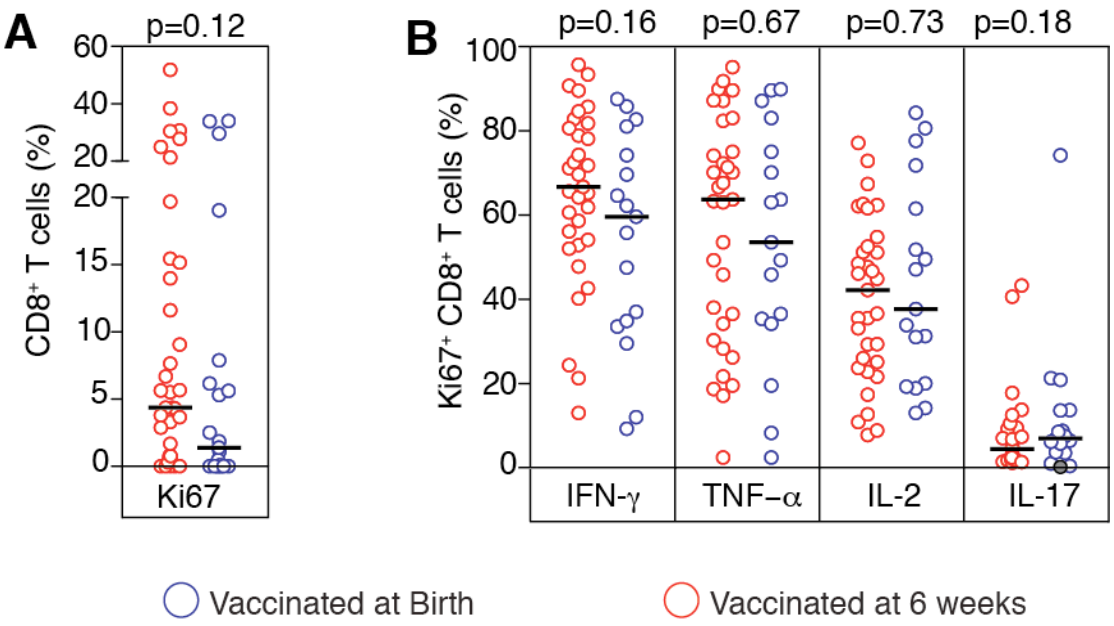

Figure 43. BCG-specific $CD8^+$ T cell proliferative capacity and cytokine production by the proliferating T cells. Scatter plots depict the proportions of proliferating ($Ki67^+$) $CD8^+$ T cells (**A**) and the capacity of these proliferating $CD8^+$ T cells to produce IL-2, IL-17, IFN-γ or TNF-α cytokines (**B**). Horizontal lines represent the median frequencies. The blue and red dots represent the birth and 6 weeks BCG vaccinated infants respectively. The *Mann-Whitney* U test was used to compare the difference in frequencies of Ki67 expressing $CD8^+$ T cells between the 2 groups.

7.3.6. No difference in the capacity of specific proliferating $CD8^+$ T cells to co-express IL-2, IFN-γ and TNF-α in the two groups of infants

We then evaluated the capacity of BCG-specific proliferating $CD8^+$ T cells to co-expressing IL-2, IL-17, IFN-γ, and TNF-α. We observed no difference in the capacity of proliferating $CD8^+$ T cells to express either IL-2, IL-17, IFN-γ or TNF-α alone or in different combinations between infants vaccinated at birth or 6 weeks of age (**Figure 44**).

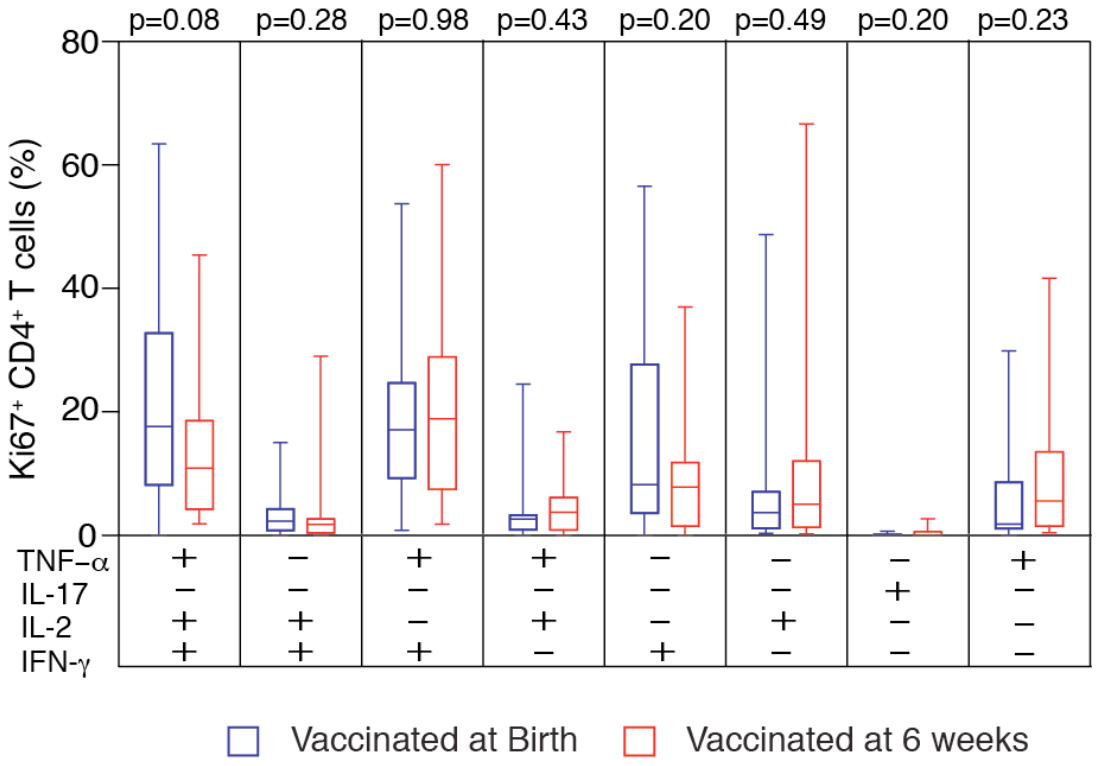

Figure 44. Capacity of proliferating CD8$^+$ T cells to co-express cytokines as measured in the 6-day proliferation assay. The blue and red bars represent infants vaccinated with BCG at birth or 6 weeks of age respectively. Whiskers represent the maximum and minimum value; the box represents the interquartile range, while the line in the box represents the median. The *Mann–Whitney* U test was used to compare the proportions of Ki67 expressing CD8$^+$ T cells between the two groups.

7.4. Discussion

In this chapter, we showed that, at 9 months of age, the two groups of infants had similar proportions of BCG-specific proliferating $CD4^+$ and $CD8^+$ T cells. However, greater capacity of the proliferating $CD4^+$ T cells to co-express IL-2^+, IFN-γ^+, and TNF-α^+ was observed in infants vaccinated at birth than at 6 weeks of age. While infants vaccinated at 6 weeks of age had greater proportions of single TNF-α production compared to those vaccinated at birth.

T_{CM} have the ability to proliferate and differentiate into effector memory T cells with cytokine producing potential (Wherry et al., 2003). Our findings of similar T cell proliferative potential were in agreement with the phenotypic characteristics we had observed by use of the short-term assay in Chapter 5. Phenotypic characterization of the memory T cells showed no difference between the two groups of infants in the proportions of T_{CM}. However, we observed differences in the capacity of the proliferating cells to co-express IL-2^+, IFN-γ^+, and TNF-α^+.

We speculate that greater IL-10 levels observed in infants vaccinated at 6 weeks may have attenuated ability to expand into polyfunctional $CD4^+$ T cells. IL-10 inhibits IL-12 production (Liu et al., 2011), a cytokine known to indirectly drive proliferation of T cells through positive regulation of IL-2 (Clerici et al., 1993). Therefore, it is possible that there were greater levels of IL-12 cytokine at 9 months in birth-vaccinated infants resulting in increased IL-2 and subsequent proliferation of polyfunctional $CD4^+$ T cells with enhanced capacity to produce multiple cytokines simultaneously. Our speculation of greater IL-12 levels in birth-vaccinated compared with 6 week old vaccinated infants is supported by our findings of increased IFN-γ production in the former group.

Polyfunctional $CD4^+$ T cells are thought to potentially play an important role in TB immunity (Forbes et al., 2008) and are therefore routinely measured in clinical trials assessing the immunogenicity of novel TB vaccines (Abel et al., 2010,

Sander et al., 2009, Scriba et al., 2012). However, a clinical study from our laboratory reported no association between greater proportions of BCG-specific polyfunctional T cell and risk of developing TB (Kagina et al., 2010). In our study, greater capacity of proliferating BCG-specific $CD4^+$ T cells to co-express IL-2, IFN-γ, and TNF-α in infants vaccinated at birth, compared with infants vaccinated at 6 weeks, could be again a reflection of more effective vaccine-take in the former group. However, in the absence of known immune correlates of risk of TB, we can only speculate about clinical relevance of this observation in humans.

In our study, we observed frequencies of BCG-specific T cell proliferation in the same range as those reported by Soares, et al., in a study that reported a longitudinal assessment of BCG-specific immune responses of infants in the first year of life (Soares et al., 2013). However, our results of greater capacity of proliferating $CD4^+$ T cells to co-express IL-2, IFN-γ, and TNF-α in infants vaccinated at birth compared to those vaccinated at 6 weeks of age differ from those in a previous study of delayed BCG vaccination (Kagina et al., 2009). The differences between our study finding and that previously reported by Kagina, et al. may be explained by the difference in the study design. We used an observational study design whereas Kagina, et al. performed a randomized controlled trial.

In conclusion, we show greater capacity of proliferating $CD4^+$ T cells to co-express IL-2, IFN-γ, and TNF-α, when BCG is given at birth versus 6 weeks of age. Further follow up studies are needed to give more insight into clinical relevance of the results, in terms of risk of developing TB disease.

7.5. Contributions

Dr. F. Lutwama designed the experiments, conducted the laboratory assays and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B.M.N. Kagina, Dr. C.L. Day and Dr T.J Scriba.

Chapter 8: General conclusions

We report evaluation of specific T cell responses in Ugandan infants who received BCG either at birth or at 6 weeks of age. We showed that age of vaccination impacted BCG-specific immune response measured at 9 months of age. Specifically, infants vaccinated at birth had higher frequencies of BCG-specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ alone, or co-expressing IFN-γ and perforin. Further, birth vaccination induced proliferating cells that had greater capacity to produce IFN-γ, TNF-α and IL-2 together, compared with delayed vaccination at 9 months of age. Finally, we also reported that these infants had lower levels of non-specific IL-10, compared with those vaccinated at 6 weeks. We propose that this diversity in T cell responses induced when BCG was given at birth and at 6 weeks of age might impact protective immunity against TB.

Importantly, contributions from this study differ from previous reports on effects of delayed BCG vaccination. The lack of agreement observed between our study and those previously reported may be explained by the following: first, the differences in the study design (Burl et al., 2010, Hussey et al., 2002, Kagina et al., 2009). We studied a “real-life” African situation, where BCG was either given at birth or not because of hospital or home delivery of the infant. This is different from the randomized controlled trial designs in previous published studies (Burl et al., 2010, Hussey et al., 2002, Kagina et al., 2009); Second, different assays were used to measure outcomes in previous studies. For example, we used BCG as antigen, as did Burl *et al*. (Burl et al., 2010), while others used PPD (Hussey et al., 2002); Third, choice of T cell outcomes differed (Burl et al., 2010, Hussey et al., 2002, Kagina et al., 2009). Finally, differences between our results and those of delayed BCG vaccination studies in South Africa and the Gambia suggest that environmental and genetic background of the populations may impact the mycobacterial immune response, as demonstrated by diverse patterns of antigen recognition and cytokine production in *M.tb*-infected persons from the Gambia, Uganda and South Africa (Black et al., 2001). Environmental

factors may include exposure to helminthes (Elias et al., 2001), other infections and diverse nutritional practices (Rodriguez et al., 2005). Genetic variation across Africa that is associated with characteristics of BCG-specific immune response is well documented in descriptions of single nucleotide polymorphisms (Randhawa et al., 2011). Currently, ongoing studies in our laboratory are investigating whether there is an association between differential gene expression profiles and the responsivesness to BCG.

We report that a higher proportion of infants vaccinated at 6 weeks of age were from families of lower social economic status than infants vaccinated at birth. Lower social economic status may negatively influence health seeking behavior, helminth exposure, nutrition possibly leading to altered immune responses (Rodriguez et al., 2005). Furthermore, we observed a significant association between the frequency of BCG-specific IFN-γ-expressing $CD4^+$ T cells and household income. This is in agreement with previous studies that suggest that co-administration of BCG with nutritional supplements (Vitamin A) may impact vaccine-specific immune responses (Benn et al., 2005, Humphrey et al., 1996). For example, greater in PPD-specific IFN-γ expression was observed at 6 weeks post vaccination in infants who received BCG with Vitamin A compared to BCG administered with a vitamin placebo (Diness et al., 2007). We therefore propose that future studies evaluating delayed BCG-induced immunity should take into consideration nutritional parameters as potential covariates.

Our study also highlights a potential interaction that may occur when EPI vaccines are co-administered. In this regard, we speculate that the lower Th1 responses observed in infants vaccinated at 6 weeks of age may be due to co-administration of BCG with Alum, present in other vaccines such as DPT. In a randomized controlled trial, lower vaccine induced Th1 immune responses were observed when a novel TB vaccine, MVA85A was co-administered with DPT compared to when the MVA85A was given alone (Ota et al., 2011). Alum, the adjuvant for DPT, induces predominantly Th2 immune responses (Bungener et

al., 2008), which may have attenuated the BCG-induced Th1 responses (Abbas et al., 1996). Also, observations made by *Sartono et al.*, indicate that co-administration of OPV with BCG may attenuate PPD-specific responses (Sartono et al., 2010). However, in a subsequent study, no differences were observed between infants who received BCG with OPV at birth compared to those who received BCG without OPV (Lund et al., 2012). In our study, BCG was co-administered with OPV in both study groups. Therefore, it is unlikely that the differences we observed in our study could be explained by BCG co-administration with OPV. Nevertheless, any modification in vaccination programs when incorporating novel TB vaccines, or revising the timing or vaccines such as BCG, into the EPI schedule should take into account the potential interactions that may occur when different vaccines are co-administered.

We assessed immune responses at 9 months of age. We do not know if other differences in BCG-induced immunity may exist at earlier or later post-vaccination time points. Our study setting limited us from accessing the participants at the peak of the BCG-induced T cell immune response, which occurs 6-10 weeks after vaccination (Soares et al., 2013). However, in a clinical study of delayed BCG vaccination, Kagina *et al*. showed the greatest difference in BCG-induced T cell immunity was at one year of age and not at the peak time point post-vaccination (Kagina et al., 2009). Another potential limitation in our study stems from the fact that the priming of the BCG-specific cells occurs in lymphoid tissues, such as lymph nodes that drain the vaccination site, at the time of vaccine administration. Again we can only speculate that the soluble cytokine levels we measured in plasma may be reflective of the cytokine milieu at the time of T cell priming.

In summary, our findings appear to support WHO recommendations that infants in high endemic areas for TB, such as Uganda, be vaccinated as soon as possible after birth. In this setting, delayed vaccination resulting from home births does not appear to hold vaccine induced immunological advantages. We believe

these studies provide new insights into how timing of BCG vaccination may affect the vaccine induced immune responses. The novel clinical data presented here are relevant to the general medical community, particularly paediatricians and infectious disease physicians, but also to epidemiologists, immunologists and policy makers.

8.1. Contributions

Dr. F. Lutwama wrote this chapter under supervision of Dr. B.M.N. Kagina, Dr. T.J. Scriba and Prof. W.A. Hanekom.

References

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

Abbas, A. K., Murphy, K. M. & Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature,* 383**,** 787-93.

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

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

Abubakar, I., Zignol, M., Falzon, D., Raviglione, M., Ditiu, L., Masham, S., Adetifa, I., Ford, N., Cox, H., Lawn, S. D., Marais, B. J., Mchugh, T. D., Mwaba, P., Bates, M., Lipman, M., Zijenah, L., Logan, S., Mcnerney, R., Zumla, A., Sarda, K., Nahid, P., Hoelscher, M., Pletschette, M., Memish, Z. A., Kim, P., Hafner, R., Cole, S., Migliori, G. B., Maeurer, M., Schito, M. & Zumla, A. 2013. Drug-resistant tuberculosis: time for visionary political leadership. *Lancet Infect Dis,* 13**,** 529-39.

Adekambi, T., Ibegbu, C. C., Kalokhe, A. S., Yu, T., Ray, S. M. & Rengarajan, J. 2012. Distinct effector memory CD4+ T cell signatures in latent Mycobacterium tuberculosis infection, BCG vaccination and clinically resolved tuberculosis. *PLoS One,* 7**,** e36046.

Algood, H. M., Lin, P. L. & Flynn, J. L. 2005. Tumor necrosis factor and chemokine interactions in the formation and maintenance of granulomas in tuberculosis. *Clin Infect Dis,* 41 Suppl 3**,** S189-93.

Anderson, E. J., Webb, E. L., Mawa, P. A., Kizza, M., Lyadda, N., Nampijja, M. & Elliott, A. M. 2012. The influence of BCG vaccine strain on mycobacteria-specific and non-specific immune responses in a prospective cohort of infants in Uganda. *Vaccine,* 30**,** 2083-9.

Andersson, J., Samarina, A., Fink, J., Rahman, S. & Grundstrom, S. 2007. Impaired expression of perforin and granulysin in CD8+ T cells at the site of infection in human chronic pulmonary tuberculosis. *Infect Immun,* 75**,** 5210-22.

Aronson, J. D. 1948. Protective vaccination against tuberculosis with special reference to BCG vaccination. *Am Rev Tuberc,* 58**,** 255-81.

Aronson, N. E., Santosham, M., Comstock, G. W., Howard, R. S., Moulton, L. H., Rhoades, E. R. & Harrison, L. H. 2004. Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: A 60-year follow-up study. *JAMA,* 291**,** 2086-91.

Avgustin, B., Kotnik, V., Skoberne, M., Malovrh, T., Skralovnik-Stern, A. & Tercelj, M. 2005. CD69 expression on CD4+ T lymphocytes after in vitro stimulation with tuberculin is an indicator of immune sensitization against Mycobacterium tuberculosis antigens. *Clin Diagn Lab Immunol,* 12**,** 101-6.

Bachmann, M. F., Lutz, M. B., Layton, G. T., Harris, S. J., Fehr, T., Rescigno, M. & Ricciardi-Castagnoli, P. 1996. Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8+ cytotoxic T lymphocytes. *Eur J Immunol,* 26**,** 2595-600.

Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A. & Sher, A. 2005. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med,* 202**,** 1715-24.

Baily, G. V. 1980. Tuberculosis prevention Trial, Madras. *Indian J Med Res,* 72 Suppl**,** 1-74.

Balcells, M. E., Thomas, S. L., Godfrey-Faussett, P. & Grant, A. D. 2006. Isoniazid preventive therapy and risk for resistant tuberculosis. *Emerg Infect Dis,* 12**,** 744-51.

Bastian, M., Braun, T., Bruns, H., Rollinghoff, M. & Stenger, S. 2008. Mycobacterial lipopeptides elicit CD4+ CTLs in Mycobacterium tuberculosis-infected humans. *J Immunol,* 180**,** 3436-46.

Baumgarth, N. & Roederer, M. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods,* 243**,** 77-97.

Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D. & Britton, W. J. 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *J Immunol,* 162**,** 3504-11.

Behr, M. A., Wilson, M. A., Gill, W. P., Salamon, H., Schoolnik, G. K., Rane, S. & Small, P. M. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science,* 284**,** 1520-3.

Bellamy, R., Ruwende, C., Corrah, T., Mcadam, K. P., Whittle, H. C. & Hill, A. V. 1998. Assessment of the interleukin 1 gene cluster and other candidate gene polymorphisms in host susceptibility to tuberculosis. *Tuber Lung Dis,* 79**,** 83-9.

Belyhun, Y., Medhin, G., Amberbir, A., Erko, B., Hanlon, C., Alem, A., Venn, A., Britton, J. & Davey, G. 2010. Prevalence and risk factors for soil-transmitted helminth infection in mothers and their infants in Butajira, Ethiopia: a population based study. *BMC Public Health,* 10**,** 21.

Benn, C. S., Martins, C., Rodrigues, A., Jensen, H., Lisse, I. M. & Aaby, P. 2005. Randomised study of effect of different doses of vitamin A on childhood morbidity and mortality. *BMJ,* 331**,** 1428-32.

Berrington, W. R. & Hawn, T. R. 2007. Mycobacterium tuberculosis, macrophages, and the innate immune response: does common variation matter? *Immunol Rev,* 219**,** 167-86.

Black, G. F., Dockrell, H. M., Crampin, A. C., Floyd, S., Weir, R. E., Bliss, L., Sichali, L., Mwaungulu, L., Kanyongoloka, H., Ngwira, B., Warndorff, D. K. & Fine, P. E. 2001. Patterns and implications of naturally acquired immune responses to environmental and tuberculous mycobacterial antigens in northern Malawi. *J Infect Dis,* 184**,** 322-9.

Bogunovic, D., Byun, M., Durfee, L. A., Abhyankar, A., Sanal, O., Mansouri, D., Salem, S., Radovanovic, I., Grant, A. V., Adimi, P., Mansouri, N., Okada, S., Bryant, V. L., Kong, X. F., Kreins, A., Velez, M. M., Boisson, B., Khalilzadeh, S., Ozcelik, U., Darazam, I. A., Schoggins, J. W., Rice, C. M., Al-Muhsen, S., Behr, M., Vogt, G., Puel, A., Bustamante, J., Gros, P., Huibregtse, J. M., Abel, L., Boisson-Dupuis, S. & Casanova, J. L. 2012.

Mycobacterial disease and impaired IFN-gamma immunity in humans with inherited ISG15 deficiency. *Science,* 337**,** 1684-8.

Bonah, C. 2005. The 'experimental stable' of the BCG vaccine: safety, efficacy, proof, and standards, 1921-1933. *Stud Hist Philos Biol Biomed Sci,* 36**,** 696-721.

Bonecini-Almeida, M. G., Ho, J. L., Boechat, N., Huard, R. C., Chitale, S., Doo, H., Geng, J., Rego, L., Lazzarini, L. C., Kritski, A. L., Johnson, W. D., Jr., Mccaffrey, T. A. & Silva, J. R. 2004. Down-modulation of lung immune responses by interleukin-10 and transforming growth factor beta (TGF-beta) and analysis of TGF-beta receptors I and II in active tuberculosis. *Infect Immun,* 72**,** 2628-34.

Bonifachich, E., Chort, M., Astigarraga, A., Diaz, N., Brunet, B., Pezzotto, S. M. & Bottasso, O. 2006. Protective effect of Bacillus Calmette-Guerin (BCG) vaccination in children with extra-pulmonary tuberculosis, but not the pulmonary disease. A case-control study in Rosario, Argentina. *Vaccine,* 24**,** 2894-9.

Brahmajothi, V., Pitchappan, R. M., Kakkanaiah, V. N., Sashidhar, M., Rajaram, K., Ramu, S., Palanimurugan, K., Paramasivan, C. N. & Prabhakar, R. 1991. Association of pulmonary tuberculosis and HLA in south India. *Tubercle,* 72**,** 123-32.

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

Brandt, L., Skeiky, Y. A., Alderson, M. R., Lobet, Y., Dalemans, W., Turner, O. C., Basaraba, R. J., Izzo, A. A., Lasco, T. M., Chapman, P. L., Reed, S. G. & Orme, I. M. 2004. The protective effect of the Mycobacterium bovis BCG vaccine is increased by coadministration with the Mycobacterium tuberculosis 72-kilodalton fusion polyprotein Mtb72F in M. tuberculosis-infected guinea pigs. *Infect Immun,* 72**,** 6622-32.

Brewer, T. F. 2000. Preventing tuberculosis with bacillus Calmette-Guerin vaccine: a meta-analysis of the literature. *Clin Infect Dis,* 31 Suppl 3**,** S64-7.

Brewer, T. F. & Colditz, G. A. 1995. Relationship between bacille Calmette-Guerin (BCG) strains and the efficacy of BCG vaccine in the prevention of tuberculosis. *Clin Infect Dis,* 20**,** 126-35.

Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J. & Modlin, R. L. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science,* 285**,** 732-6.

Brittle, W., Marais, B. J., Hesseling, A. C., Schaaf, H. S., Kidd, M., Wasserman, E. & Botha, T. 2009. Improvement in mycobacterial yield and reduced time to detection in pediatric samples by use of a nutrient broth growth supplement. *J Clin Microbiol,* 47**,** 1287-9.

Brown, R. M., Cruz, O., Brennan, M., Gennaro, M. L., Schlesinger, L., Skeiky, Y. A. & Hoft, D. F. 2003. Lipoarabinomannan-reactive human secretory immunoglobulin A responses induced by mucosal bacille Calmette-Guerin vaccination. *J Infect Dis,* 187**,** 513-7.

Bruns, H., Meinken, C., Schauenberg, P., Harter, G., Kern, P., Modlin, R. L., Antoni, C. & Stenger, S. 2009. Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J Clin Invest,* 119**,** 1167-77.

Bundy, D., Sher, A. & Michael, E. 2000. Good worms or bad worms: do worm infections affect the epidemiological patterns of other diseases? *Parasitol Today,* 16**,** 273-4.

Bungener, L., Geeraedts, F., Ter Veer, W., Medema, J., Wilschut, J. & Huckriede, A. 2008. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. *Vaccine,* 26**,** 2350-9.

Burl, S., Adetifa, U. J., Cox, M., Touray, E., Ota, M. O., Marchant, A., Whittle, H., McShane, H., Rowland-Jones, S. L. & Flanagan, K. L. 2010. Delaying bacillus Calmette-Guerin vaccination from birth to 4 1/2 months of age reduces postvaccination Th1 and IL-17 responses but leads to comparable mycobacterial responses at 9 months of age. *J Immunol,* 185**,** 2620-8.

Caccamo, N., Meraviglia, S., La Mendola, C., Guggino, G., Dieli, F. & Salerno, A. 2006. Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens. *J Immunol,* 177**,** 1780-5.

Campbell, J. J. & Butcher, E. C. 2000. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr Opin Immunol,* 12**,** 336-41.

Canaday, D. H., Wilkinson, R. J., Li, Q., Harding, C. V., Silver, R. F. & Boom, W. H. 2001. CD4(+) and CD8(+) T cells kill intracellular Mycobacterium tuberculosis by a perforin and Fas/Fas ligand-independent mechanism. *J Immunol,* 167**,** 2734-42.

Caruso, A. M., Serbina, N., Klein, E., Triebold, K., Bloom, B. R. & Flynn, J. L. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol,* 162**,** 5407-16.

Cattamanchi, A., Dowdy, D. W., Davis, J. L., Worodria, W., Yoo, S., Joloba, M., Matovu, J., Hopewell, P. C. & Huang, L. 2009. Sensitivity of direct versus concentrated sputum smear microscopy in HIV-infected patients suspected of having pulmonary tuberculosis. *BMC Infect Dis,* 9**,** 53.

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

Chan, J., Fan, X. D., Hunter, S. W., Brennan, P. J. & Bloom, B. R. 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. *Infect Immun,* 59**,** 1755-61.

Chattopadhyay, P. K., Price, D. A., Harper, T. F., Betts, M. R., Yu, J., Gostick, E., Perfetto, S. P., Goepfert, P., Koup, R. A., De Rosa, S. C., Bruchez, M. P. & Roederer, M. 2006. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat Med,* 12**,** 972-7.

Chowdhury, D. & Lieberman, J. 2008. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol,* 26**,** 389-420.

Clayberger, C. & Krensky, A. M. 2003. Granulysin. *Curr Opin Immunol,* 15**,** 560-5.

Clemens, D. L. & Horwitz, M. A. 1995. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med,* 181**,** 257-70.

Clerici, M., Lucey, D. R., Berzofsky, J. A., Pinto, L. A., Wynn, T. A., Blatt, S. P., Dolan, M. J., Hendrix, C. W., Wolf, S. F. & Shearer, G. M. 1993. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. *Science,* 262**,** 1721-4.

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

Comstock, G. W., Livesay, V. T. & Woolpert, S. F. 1974. Evaluation of BCG vaccination among Puerto Rican children. *Am J Public Health,* 64**,** 283-91.

Comstock, G. W. & Palmer, C. E. 1966. Long-term results of BCG vaccination in the southern United States. *Am Rev Respir Dis,* 93**,** 171-83.

Cooper, A. M., Magram, J., Ferrante, J. & Orme, I. M. 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med,* 186**,** 39-45.

Corbett, N. P., Blimkie, D., Ho, K. C., Cai, B., Sutherland, D. P., Kallos, A., Crabtree, J., Rein-Weston, A., Lavoie, P. M., Turvey, S. E., Hawkins, N. R., Self, S. G., Wilson, C. B., Hajjar, A. M., Fortuno, E. S., 3rd & Kollmann, T. R. 2010. Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells. *PLoS One,* 5**,** e15041.

Croft, M. 2009. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol,* 9**,** 271-85.

Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K. & Mescher, M. F. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol,* 162**,** 3256-62.

D'andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M. & Trinchieri, G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med,* 178**,** 1041-8.

Dahl, K. E., Shiratsuchi, H., Hamilton, B. D., Ellner, J. J. & Toossi, Z. 1996. Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of Mycobacterium tuberculosis. *Infect Immun,* 64**,** 399-405.

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

Davids, V., Hanekom, W. A., Mansoor, N., Gamieldien, H., Gelderbloem, S. J., Hawkridge, A., Hussey, G. D., Hughes, E. J., Soler, J., Murray, R. A., Ress, S. R. & Kaplan, G. 2006. The effect of bacille Calmette-Guerin vaccine strain and route of administration on induced immune responses in vaccinated infants. *J Infect Dis,* 193**,** 531-6.

Davis, J. M. & Ramakrishnan, L. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell,* 136**,** 37-49.

De Jong, R., Altare, F., Haagen, I. A., Elferink, D. G., Boer, T., Van Breda Vriesman, P. J., Kabel, P. J., Draaisma, J. M., Van Dissel, J. T., Kroon, F. P., Casanova, J. L. & Ottenhoff, T. H. 1998. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science,* 280**,** 1435-8.

De Jong, R., Brouwer, M., Hooibrink, B., Van Der Pouw-Kraan, T., Miedema, F. & Van Lier, R. A. 1992. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. *Eur J Immunol,* 22**,** 993-9.

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

Deng, A., Chen, S., Li, Q., Lyu, S. C., Clayberger, C. & Krensky, A. M. 2005. Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator. *J Immunol,* 174**,** 5243-8.

Desjardins, M., Huber, L. A., Parton, R. G. & Griffiths, G. 1994. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J Cell Biol,* 124**,** 677-88.

Di Liberto, D., Buccheri, S., Caccamo, N., Meraviglia, S., Romano, A., Di Carlo, P., Titone, L., Dieli, F., Krensky, A. M. & Salerno, A. 2007. Decreased serum granulysin levels in childhood tuberculosis which reverse after therapy. *Tuberculosis (Edinb),* 87**,** 322-8.

Diedrich, C. R., Mattila, J. T., Klein, E., Janssen, C., Phuah, J., Sturgeon, T. J., Montelaro, R. C., Lin, P. L. & Flynn, J. L. 2010. Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. *PLoS One,* 5**,** e9611.

Dieli, F., Friscia, G., Di Sano, C., Ivanyi, J., Singh, M., Spallek, R., Sireci, G., Titone, L. & Salerno, A. 1999. Sequestration of T lymphocytes to body fluids in tuberculosis: reversal of anergy following chemotherapy. *J Infect Dis,* 180**,** 225-8.

Diness, B. R., Fisker, A. B., Roth, A., Yazdanbakhsh, M., Sartono, E., Whittle, H., Nante, J. E., Lisse, I. M., Ravn, H., Rodrigues, A., Aaby, P. & Benn, C. S. 2007. Effect of high-dose vitamin A supplementation on the immune response to Bacille Calmette-Guerin vaccine. *Am J Clin Nutr,* 86**,** 1152-9.

Ding, A., Nathan, C. F., Graycar, J., Derynck, R., Stuehr, D. J. & Srimal, S. 1990. Macrophage deactivating factor and transforming growth factors-beta 1 -beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. *J Immunol,* 145**,** 940-4.

Dintwe, O. B., Day, C. L., Smit, E., Nemes, E., Gray, C., Tameris, M., Mcshane, H., Mahomed, H., Hanekom, W. A. & Scriba, T. J. 2013. Heterologous vaccination against human tuberculosis modulates antigen-specific CD4+ T-cell function. *Eur J Immunol,* 43**,** 2409-20.

Dudani, R., Chapdelaine, Y., Faassen Hv, H., Smith, D. K., Shen, H., Krishnan, L. & Sad, S. 2002. Multiple mechanisms compensate to enhance tumor-protective CD8(+) T cell response in the long-term despite poor CD8(+) T cell priming initially: comparison between an acute versus a chronic intracellular bacterium expressing a model antigen. *J Immunol,* 168**,** 5737-45.

Dye, C., Glaziou, P., Floyd, K. & Raviglione, M. 2013. Prospects for tuberculosis elimination. *Annu Rev Public Health,* 34**,** 271-86.

Edwards, L. B., Acquaviva, F. A. & Livesay, V. T. 1973. Identification of tuberculous infected. Dual tests and density of reaction. *Am Rev Respir Dis,* 108**,** 1334-9.

Eisenhut, M., Paranjothy, S., Abubakar, I., Bracebridge, S., Lilley, M., Mulla, R., Lack, K., Chalkley, D. & Mcevoy, M. 2009. BCG vaccination reduces risk of infection with Mycobacterium tuberculosis as detected by gamma interferon release assay. *Vaccine,* 27**,** 6116-20.

Elias, D., Britton, S., Aseffa, A., Engers, H. & Akuffo, H. 2008. Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine,* 26**,** 3897-902.

Elias, D., Wolday, D., Akuffo, H., Petros, B., Bronner, U. & Britton, S. 2001. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. *Clin Exp Immunol,* 123**,** 219-25.

Elliott, A. M., Mawa, P. A., Webb, E. L., Nampijja, M., Lyadda, N., Bukusuba, J., Kizza, M., Namujju, P. B., Nabulime, J., Ndibazza, J., Muwanga, M. & Whitworth, J. A. 2010. Effects of maternal and infant co-infections, and of maternal immunisation, on the infant response to BCG and tetanus immunisation. *Vaccine,* 29**,** 247-55.

Endsley, J. J., Furrer, J. L., Endsley, M. A., Mcintosh, M. A., Maue, A. C., Waters, W. R., Lee, D. R. & Estes, D. M. 2004. Characterization of bovine homologues of granulysin and NK-lysin. *J Immunol,* 173**,** 2607-14.

Eriksen, J., Chow, J. Y., Mellis, V., Whipp, B., Walters, S., Abrahamson, E. & Abubakar, I. 2010. Protective effect of BCG vaccination in a nursery outbreak in 2009: time to reconsider the vaccination threshold? *Thorax,* 65**,** 1067-71.

Ewer, K., Deeks, J., Alvarez, L., Bryant, G., Waller, S., Andersen, P., Monk, P. & Lalvani, A. 2003. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak. *Lancet,* 361**,** 1168-73.

Fadnes, L. T., Nankabirwa, V., Sommerfelt, H., Tylleskar, T., Tumwine, J. K. & Engebretsen, I. M. 2011. Is vaccination coverage a good indicator of age-appropriate vaccination? A prospective study from Uganda. *Vaccine,* 29**,** 3564-70.

Ferebee, S. H. 1970. Controlled chemoprophylaxis trials in tuberculosis. A general review. *Bibl Tuberc,* 26**,** 28-106.

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

Fine Pem, C. I., Milstien Jb, Clements Cj. 1999. Issues relating to the use of BCG immunisation programmes: A discussion document. *World Health Organisation Document.* Geneva.

Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W. & O'garra, A. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol,* 146**,** 3444-51.

Flaherty, D. K., Vesosky, B., Beamer, G. L., Stromberg, P. & Turner, J. 2006. Exposure to Mycobacterium avium can modulate established immunity against Mycobacterium tuberculosis infection generated by Mycobacterium bovis BCG vaccination. *J Leukoc Biol,* 80**,** 1262-71.

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

Fremond, C. M., Yeremeev, V., Nicolle, D. M., Jacobs, M., Quesniaux, V. F. & Ryffel, B. 2004. Fatal Mycobacterium tuberculosis infection despite adaptive immune response in the absence of MyD88. *J Clin Invest,* 114**,** 1790-9.

Fritsch, R. D., Shen, X., Sims, G. P., Hathcock, K. S., Hodes, R. J. & Lipsky, P. E. 2005. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J Immunol,* 175**,** 6489-97.

Gallegos, A. M., Van Heijst, J. W., Samstein, M., Su, X., Pamer, E. G. & Glickman, M. S. 2011. A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo. *PLoS Pathog,* 7**,** e1002052.

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

Goldfeld, A. E., Delgado, J. C., Thim, S., Bozon, M. V., Uglialoro, A. M., Turbay, D., Cohen, C. & Yunis, E. J. 1998. Association of an HLA-DQ allele with clinical tuberculosis. *JAMA,* 279**,** 226-8.

Gong, J. H., Zhang, M., Modlin, R. L., Linsley, P. S., Iyer, D., Lin, Y. & Barnes, P. F. 1996. Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. *Infect Immun,* 64**,** 913-8.

Goonetilleke, N. P., Mcshane, H., Hannan, C. M., Anderson, R. J., Brookes, R. H. & Hill, A. V. 2003. Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine

using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J Immunol,* 171**,** 1602-9.

Gorak-Stolinska, P., Weir, R. E., Floyd, S., Lalor, M. K., Stenson, S., Branson, K., Blitz, R., Luke, S., Nazareth, B., Ben-Smith, A., Fine, P. E. & Dockrell, H. M. 2006. Immunogenicity of Danish-SSI 1331 BCG vaccine in the UK: comparison with Glaxo-Evans 1077 BCG vaccine. *Vaccine,* 24**,** 5726-33.

Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol,* 3**,** 23-35.

Goriely, S., Van Lint, C., Dadkhah, R., Libin, M., De Wit, D., Demonte, D., Willems, F. & Goldman, M. 2004. A defect in nucleosome remodeling prevents IL-12(p35) gene transcription in neonatal dendritic cells. *J Exp Med,* 199**,** 1011-6.

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I. & Deretic, V. 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell,* 119**,** 753-66.

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

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

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

Harada, N. 2006. [Characteristics of a diagnostic method for tuberculosis infection based on whole blood interferon-gamma assay]. *Kekkaku,* 81**,** 681-6.

Harari, A., Vallelian, F., Meylan, P. R. & Pantaleo, G. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J Immunol,* 174**,** 1037-45.

Harari, A., Vallelian, F. & Pantaleo, G. 2004. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur J Immunol,* 34**,** 3525-33.

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

Harris, J., De Haro, S. A., Master, S. S., Keane, J., Roberts, E. A., Delgado, M. & Deretic, V. 2007. T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis. *Immunity,* 27**,** 505-17.

Harris, J., Hope, J. C. & Lavelle, E. C. 2009. Autophagy and the immune response to TB. *Transbound Emerg Dis,* 56**,** 248-54.

Hart, P. D., Armstrong, J. A., Brown, C. A. & Draper, P. 1972. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. *Infect Immun,* 5**,** 803-7.

Hart, P. D. & Sutherland, I. 1977. BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. *Br Med J,* 2**,** 293-5.

Harth, G. & Horwitz, M. A. 1999. An inhibitor of exported Mycobacterium tuberculosis glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. *J Exp Med,* 189**,** 1425-36.

Hawkridge, A., Hatherill, M., Little, F., Goetz, M. A., Barker, L., Mahomed, H., Sadoff, J., Hanekom, W., Geiter, L. & Hussey, G. 2008. Efficacy of percutaneous versus intradermal BCG in the prevention of tuberculosis in South African infants: randomised trial. *BMJ,* 337**,** a2052.

Hayday, A. C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol,* 18**,** 975-1026.

Henao-Tamayo, M. I., Ordway, D. J., Irwin, S. M., Shang, S., Shanley, C. & Orme, I. M. 2010. Phenotypic definition of effector and memory T-lymphocyte subsets in mice chronically infected with Mycobacterium tuberculosis. *Clin Vaccine Immunol,* 17**,** 618-25.

Henderson, R. A., Watkins, S. C. & Flynn, J. L. 1997. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J Immunol,* 159**,** 635-43.

Herbst, S., Schaible, U. E. & Schneider, B. E. 2011. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *PLoS One,* 6**,** e19105.

Hernandez-Pando, R., Orozcoe, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J. M. & Madrid, M. V. 1996. Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. *Immunology,* 89**,** 26-33.

Hersperger, A. R., Makedonas, G. & Betts, M. R. 2008. Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry A,* 73**,** 1050-7.

Hesseling, A. C., Caldwell, J., Cotton, M. F., Eley, B. S., Jaspan, H. B., Jennings, K., Marais, B. J., Nuttall, J., Rabie, H., Roux, P. & Schaaf, H. S. 2009. BCG vaccination in South African HIV-exposed infants--risks and benefits. *S Afr Med J,* 99**,** 88-91.

Hesseling, A. C., Cotton, M. F., Fordham Von Reyn, C., Graham, S. M., Gie, R. P. & Hussey, G. D. 2008. Consensus statement on the revised World Health Organization recommendations for BCG vaccination in HIV-infected infants. *Int J Tuberc Lung Dis,* 12**,** 1376-9.

Hintzen, R. Q., De Jong, R., Lens, S. M. & Van Lier, R. A. 1994. CD27: marker and mediator of T-cell activation? *Immunol Today,* 15**,** 307-11.

Hiromatsu, K., Dascher, C. C., Leclair, K. P., Sugita, M., Furlong, S. T., Brenner, M. B. & Porcelli, S. A. 2002. Induction of CD1-restricted immune responses in guinea pigs by immunization with mycobacterial lipid antigens. *J Immunol,* 169**,** 330-9.

Hoft, D. F., Blazevic, A., Abate, G., Hanekom, W. A., Kaplan, G., Soler, J. H., Weichold, F., Geiter, L., Sadoff, J. C. & Horwitz, M. A. 2008. A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J Infect Dis,* 198**,** 1491-501.

Hoft, D. F., Blazevic, A., Stanley, J., Landry, B., Sizemore, D., Kpamegan, E., Gearhart, J., Scott, A., Kik, S., Pau, M. G., Goudsmit, J., Mcclain, J. B. & Sadoff, J. 2012. A recombinant adenovirus expressing immunodominant

TB antigens can significantly enhance BCG-induced human immunity. *Vaccine,* 30**,** 2098-108.

Hoft, D. F., Brown, R. M. & Roodman, S. T. 1998. Bacille Calmette-Guerin vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. *J Immunol,* 161**,** 1045-54.

Holland, D., Booy, R., De Looze, F., Eizenberg, P., Mcdonald, J., Karrasch, J., Mckeirnan, M., Salem, H., Mills, G., Reid, J., Weber, F. & Saville, M. 2008. Intradermal influenza vaccine administered using a new microinjection system produces superior immunogenicity in elderly adults: a randomized controlled trial. *J Infect Dis,* 198**,** 650-8.

Holtmeier, W. & Kabelitz, D. 2005. gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy,* 86**,** 151-83.

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

Howie, S. R. 2011. Blood sample volumes in child health research: review of safe limits. *Bull World Health Organ,* 89**,** 46-53.

Hughes, A. J., Hutchinson, P., Gooding, T., Freezer, N. J., Holdsworth, S. R. & Johnson, P. D. 2005. Diagnosis of Mycobacterium tuberculosis infection using ESAT-6 and intracellular cytokine cytometry. *Clin Exp Immunol,* 142**,** 132-9.

Humphrey, J. H., Agoestina, T., Wu, L., Usman, A., Nurachim, M., Subardja, D., Hidayat, S., Tielsch, J., West, K. P., Jr. & Sommer, A. 1996. Impact of neonatal vitamin A supplementation on infant morbidity and mortality. *J Pediatr,* 128**,** 489-96.

Humphreys, I. R., Stewart, G. R., Turner, D. J., Patel, J., Karamanou, D., Snelgrove, R. J. & Young, D. B. 2006. A role for dendritic cells in the dissemination of mycobacterial infection. *Microbes Infect,* 8**,** 1339-46.

Hussey, G. D., Watkins, M. L., Goddard, E. A., Gottschalk, S., Hughes, E. J., Iloni, K., Kibel, M. A. & Ress, S. R. 2002. Neonatal mycobacterial specific cytotoxic T-lymphocyte and cytokine profiles in response to distinct BCG vaccination strategies. *Immunology,* 105**,** 314-24.

Ildirim, I., Sapan, N. & Cavusoglu, B. 1992. Comparison of BCG vaccination at birth and at third month of life. *Arch Dis Child,* 67**,** 80-2.

Inaba, K., Turley, S., Iyoda, T., Yamaide, F., Shimoyama, S., Reis E Sousa, C., Germain, R. N., Mellman, I. & Steinman, R. M. 2000. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J Exp Med,* 191**,** 927-36.

Jagannath, C., Lindsey, D. R., Dhandayuthapani, S., Xu, Y., Hunter, R. L., Jr. & Eissa, N. T. 2009. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med,* 15**,** 267-76.

Juffermans, N. P., Florquin, S., Camoglio, L., Verbon, A., Kolk, A. H., Speelman, P., Van Deventer, S. J. & Van Der Poll, T. 2000. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J Infect Dis,* 182**,** 902-8.

Jung, Y. J., Ryan, L., Lacourse, R. & North, R. J. 2003. Increased interleukin-10 expression is not responsible for failure of T helper 1 immunity to resolve airborne Mycobacterium tuberculosis infection in mice. *Immunology,* 109**,** 295-9.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K. J., Podack, E. R., Zinkernagel, R. M. & Hengartner, H. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature,* 369**,** 31-7.

Kagina, B. M., Abel, B., Bowmaker, M., Scriba, T. J., Gelderbloem, S., Smit, E., Erasmus, M., Nene, N., Walzl, G., Black, G., Hussey, G. D., Hesseling, A. C. & Hanekom, W. A. 2009. Delaying BCG vaccination from birth to 10 weeks of age may result in an enhanced memory CD4 T cell response. *Vaccine,* 27**,** 5488-95.

Kagina, B. M., Abel, B., Scriba, T. J., Hughes, E. J., Keyser, A., Soares, A., Gamieldien, H., Sidibana, M., Hatherill, M., Gelderbloem, S., Mahomed, H., Hawkridge, A., Hussey, G., Kaplan, G. & Hanekom, W. A. 2010. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am J Respir Crit Care Med,* 182**,** 1073-9.

Kaspar, A. A., Okada, S., Kumar, J., Poulain, F. R., Drouvalakis, K. A., Kelekar, A., Hanson, D. A., Kluck, R. M., Hitoshi, Y., Johnson, D. E., Froelich, C. J.,

Thompson, C. B., Newmeyer, D. D., Anel, A., Clayberger, C. & Krensky, A. M. 2001. A distinct pathway of cell-mediated apoptosis initiated by granulysin. *J Immunol,* 167**,** 350-6.

Kaufmann, S. H. 2001. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol,* 1**,** 20-30.

Kaufmann, S. H. 2012. Tuberculosis vaccine development: strength lies in tenacity. *Trends Immunol,* 33**,** 373-9.

Kaveh, D. A., Bachy, V. S., Hewinson, R. G. & Hogarth, P. J. 2011. Systemic BCG immunization induces persistent lung mucosal multifunctional CD4 T(EM) cells which expand following virulent mycobacterial challenge. *PLoS One,* 6**,** e21566.

Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W. D., Siegel, J. N. & Braun, M. M. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med,* 345**,** 1098-104.

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

Kleinnijenhuis, J., Oosting, M., Joosten, L. A., Netea, M. G. & Van Crevel, R. 2011. Innate immune recognition of Mycobacterium tuberculosis. *Clin Dev Immunol,* 2011**,** 405310.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A., Ifrim, D. C., Saeed, S., Jacobs, C., Van Loenhout, J., De Jong, D., Stunnenberg, H. G., Xavier, R. J., Van Der Meer, J. W., Van Crevel, R. & Netea, M. G. 2012. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A,* 109**,** 17537-42.

Klucar, P., Barnes, P. F., Kong, Y., Samten, B., Tvinnereim, A., Spallek, R., Nepom, G. T., Singh, M. & Shams, H. 2008. Characterization of effector functions of human peptide-specific CD4+ T-cell clones for an intracellular pathogen. *Hum Immunol,* 69**,** 475-83.

Knight, S. C. & Stagg, A. J. 1993. Antigen-presenting cell types. *Curr Opin Immunol,* 5**,** 374-82.

Kornfeld, S. 1987. Trafficking of lysosomal enzymes. *FASEB J,* 1**,** 462-8.

Kurtz, J. 2005. Specific memory within innate immune systems. *Trends Immunol,* 26**,** 186-92.

Ladel, C. H., Szalay, G., Riedel, D. & Kaufmann, S. H. 1997. Interleukin-12 secretion by Mycobacterium tuberculosis-infected macrophages. *Infect Immun,* 65**,** 1936-8.

Lalloo, U. G. 2010. Drug-resistant tuberculosis: reality and potential threat. *Int J Tuberc Lung Dis,* 14**,** 255-8.

Lalor, M. K., Floyd, S., Gorak-Stolinska, P., Ben-Smith, A., Weir, R. E., Smith, S. G., Newport, M. J., Blitz, R., Mvula, H., Branson, K., Mcgrath, N., Crampin, A. C., Fine, P. E. & Dockrell, H. M. 2011. BCG vaccination induces different cytokine profiles following infant BCG vaccination in the UK and Malawi. *J Infect Dis,* 204**,** 1075-85.

Lalor, M. K., Smith, S. G., Floyd, S., Gorak-Stolinska, P., Weir, R. E., Blitz, R., Branson, K., Fine, P. E. & Dockrell, H. M. 2010. Complex cytokine profiles induced by BCG vaccination in UK infants. *Vaccine,* 28**,** 1635-41.

Larsen, M. H., Biermann, K., Chen, B., Hsu, T., Sambandamurthy, V. K., Lackner, A. A., Aye, P. P., Didier, P., Huang, D., Shao, L., Wei, H., Letvin, N. L., Frothingham, R., Haynes, B. F., Chen, Z. W. & Jacobs, W. R., Jr. 2009. Efficacy and safety of live attenuated persistent and rapidly cleared Mycobacterium tuberculosis vaccine candidates in non-human primates. *Vaccine,* 27**,** 4709-17.

Law, K., Weiden, M., Harkin, T., Tchou-Wong, K., Chi, C. & Rom, W. N. 1996. Increased release of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis. *Am J Respir Crit Care Med,* 153**,** 799-804.

Lawn, S. D., Kranzer, K. & Wood, R. 2009. Antiretroviral therapy for control of the HIV-associated tuberculosis epidemic in resource-limited settings. *Clin Chest Med,* 30**,** 685-99, viii.

Leroux-Roels, I., Forgus, S., De Boever, F., Clement, F., Demoitie, M. A., Mettens, P., Moris, P., Ledent, E., Leroux-Roels, G. & Ofori-Anyinam, O.

2013. Improved CD4(+) T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: a randomized trial. *Vaccine,* 31**,** 2196-206.

Levidiotou, S., Papamichael, D., Gessouli, E., Golegou, S., Anagnostou, S., Galanakis, E., Papadopoulou, C. & Antoniadis, G. 1999. Detection of mycobacteria in clinical specimen using the mycobacteria growth indicator tube (MGIT) and the Lowenstein Jensen medium. *Microbiol Res,* 154**,** 151-5.

Lienhardt, C., Sillah, J., Fielding, K., Donkor, S., Manneh, K., Warndorff, D., Bennett, S. & Mcadam, K. 2003. Risk factors for tuberculosis infection in children in contact with infectious tuberculosis cases in the Gambia, West Africa. *Pediatrics,* 111**,** e608-14.

Liu, B., Tonkonogy, S. L. & Sartor, R. B. 2011. Antigen-presenting cell production of IL-10 inhibits T-helper 1 and 17 cell responses and suppresses colitis in mice. *Gastroenterology,* 141**,** 653-62, 662 e1-4.

Lund, N., Andersen, A., Monteiro, I., Aaby, P. & Benn, C. S. 2012. No effect of oral polio vaccine administered at birth on mortality and immune response to BCG. A natural experiment. *Vaccine,* 30**,** 6694-9.

Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. S., Culpepper, J. A., Wysocka, M., Trinchieri, G., Murphy, K. M. & O'garra, A. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol,* 154**,** 5071-9.

Macmicking, J. D., North, R. J., Lacourse, R., Mudgett, J. S., Shah, S. K. & Nathan, C. F. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A,* 94**,** 5243-8.

Madhan Kumar, M. & Raja, A. 2010. Cytotoxicity responses to selected ESAT-6 and CFP-10 peptides in tuberculosis. *Cell Immunol,* 265**,** 146-55.

Maecker, H. T., Frey, T., Nomura, L. E. & Trotter, J. 2004. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A,* 62**,** 169-73.

Maecker, H. T. & Trotter, J. 2006. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A,* 69**,** 1037-42.

Magalhaes, I., Sizemore, D. R., Ahmed, R. K., Mueller, S., Wehlin, L., Scanga, C., Weichold, F., Schirru, G., Pau, M. G., Goudsmit, J., Kuhlmann-Berenzon, S., Spangberg, M., Andersson, J., Gaines, H., Thorstensson, R., Skeiky, Y. A., Sadoff, J. & Maeurer, M. 2008. rBCG induces strong antigen-specific T cell responses in rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector. *PLoS One,* 3**,** e3790.

Maglione, P. J., Xu, J., Casadevall, A. & Chan, J. 2008. Fc gamma receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. *J Immunol,* 180**,** 3329-38.

Mahnke, Y. D. & Roederer, M. 2007. Optimizing a multicolor immunophenotyping assay. *Clin Lab Med,* 27**,** 469-85, v.

Makedonas, G., Banerjee, P. P., Pandey, R., Hersperger, A. R., Sanborn, K. B., Hardy, G. A., Orange, J. S. & Betts, M. R. 2009. Rapid up-regulation and granule-independent transport of perforin to the immunological synapse define a novel mechanism of antigen-specific CD8+ T cell cytotoxic activity. *J Immunol,* 182**,** 5560-9.

Makedonas, G., Hutnick, N., Haney, D., Amick, A. C., Gardner, J., Cosma, G., Hersperger, A. R., Dolfi, D., Wherry, E. J., Ferrari, G. & Betts, M. R. 2010. Perforin and IL-2 upregulation define qualitative differences among highly functional virus-specific human CD8 T cells. *PLoS Pathog,* 6**,** e1000798.

Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H., Kazura, J. W. & King, C. L. 1999. Helminth- and Bacillus Calmette-Guerin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. *J Immunol,* 162**,** 6843-8.

Manetti, R., Gerosa, F., Giudizi, M. G., Biagiotti, R., Parronchi, P., Piccinni, M. P., Sampognaro, S., Maggi, E., Romagnani, S., Trinchieri, G. & Et Al. 1994. Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones. *J Exp Med,* 179**,** 1273-83.

Mangtani, P., Abubakar, I., Ariti, C., Beynon, R., Pimpin, L., Fine, P. E., Rodrigues, L. C., Smith, P. G., Lipman, M., Whiting, P. F. & Sterne, J. A. 2014. Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. *Clin Infect Dis,* 58**,** 470-80.

Mansoor, N., Scriba, T. J., De Kock, M., Tameris, M., Abel, B., Keyser, A., Little, F., Soares, A., Gelderbloem, S., Mlenjeni, S., Denation, L., Hawkridge, A., Boom, W. H., Kaplan, G., Hussey, G. D. & Hanekom, W. A. 2009. HIV-1 infection in infants severely impairs the immune response induced by Bacille Calmette-Guerin vaccine. *J Infect Dis,* 199**,** 982-90.

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

Marrack, P., Mckee, A. S. & Munks, M. W. 2009. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol,* 9**,** 287-93.

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

Martinez, F. O., Helming, L. & Gordon, S. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol,* 27**,** 451-83.

Mckinstry, K. K., Golech, S., Lee, W. H., Huston, G., Weng, N. P. & Swain, S. L. 2007. Rapid default transition of CD4 T cell effectors to functional memory cells. *J Exp Med,* 204**,** 2199-211.

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

Meallet-Renault, R., Herault, A., Vachon, J. J., Pansu, R. B., Amigoni-Gerbier, S. & Larpent, C. 2006. Fluorescent nanoparticles as selective Cu(II) sensors. *Photochem Photobiol Sci,* 5**,** 300-10.

Means, T. K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D. T. & Fenton, M. J. 1999. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. *J Immunol,* 163**,** 3920-7.

Mempel, T. R., Henrickson, S. E. & Von Andrian, U. H. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature,* 427**,** 154-9.

Michie, C. A., Mclean, A., Alcock, C. & Beverley, P. C. 1992. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature,* 360**,** 264-5.

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

Milstien, J. B. & Gibson, J. J. 1990. Quality control of BCG vaccine by WHO: a review of factors that may influence vaccine effectiveness and safety. *Bull World Health Organ,* 68**,** 93-108.

Mittrucker, H. W., Steinhoff, U., Kohler, A., Krause, M., Lazar, D., Mex, P., Miekley, D. & Kaufmann, S. H. 2007. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc Natl Acad Sci U S A,* 104**,** 12434-9.

Miyazaki, T., Liu, Z. J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Permutter, R. M. & Taniguchi, T. 1995. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. *Cell,* 81**,** 223-31.

Molloy, A., Laochumroonvorapong, P. & Kaplan, G. 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. *J Exp Med,* 180**,** 1499-509.

Moore, K. W., De Waal Malefyt, R., Coffman, R. L. & O'garra, A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol,* 19**,** 683-765.

Mori, A., Oleszycka, E., Sharp, F. A., Coleman, M., Ozasa, Y., Singh, M., O'hagan, D. T., Tajber, L., Corrigan, O. I., Mcneela, E. A. & Lavelle, E. C. 2012. The vaccine adjuvant alum inhibits IL-12 by promoting PI3 kinase signaling while chitosan does not inhibit IL-12 and enhances Th1 and Th17 responses. *Eur J Immunol,* 42**,** 2709-19.

Murray, R. A., Mansoor, N., Harbacheuski, R., Soler, J., Davids, V., Soares, A., Hawkridge, A., Hussey, G. D., Maecker, H., Kaplan, G. & Hanekom, W. A.

2006. Bacillus Calmette Guerin vaccination of human newborns induces a specific, functional CD8+ T cell response. *J Immunol,* 177**,** 5647-51.

Nacci, F. & Matucci-Cerinic, M. 2011. Tuberculosis and other infections in the anti-tumour necrosis factor-alpha (anti-TNF-alpha) era. *Best Pract Res Clin Rheumatol,* 25**,** 375-88.

Nankabirwa, V., Tumwine, J. K., Tylleskar, T., Nankunda, J. & Sommerfelt, H. 2011. Perinatal mortality in eastern Uganda: a community based prospective cohort study. *PLoS One,* 6**,** e19674.

Newport, M. J., Huxley, C. M., Huston, S., Hawrylowicz, C. M., Oostra, B. A., Williamson, R. & Levin, M. 1996. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med,* 335**,** 1941-9.

Nolte, M. A., Van Olffen, R. W., Van Gisbergen, K. P. & Van Lier, R. A. 2009. Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology. *Immunol Rev,* 229**,** 216-31.

Nomura, L. E., Walker, J. M. & Maecker, H. T. 2000. Optimization of whole blood antigen-specific cytokine assays for CD4(+) T cells. *Cytometry,* 40**,** 60-8.

O'garra, A. & Vieira, P. 2004. Regulatory T cells and mechanisms of immune system control. *Nat Med,* 10**,** 801-5.

O'garra, A. & Vieira, P. 2007. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol,* 7**,** 425-8.

Oddo, M., Renno, T., Attinger, A., Bakker, T., Macdonald, H. R. & Meylan, P. R. 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. *J Immunol,* 160**,** 5448-54.

Okada, S., Li, Q., Whitin, J. C., Clayberger, C. & Krensky, A. M. 2003. Intracellular mediators of granulysin-induced cell death. *J Immunol,* 171**,** 2556-62.

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., De Waal-Malefyt, R., Hannum, C., Bazan, J. F. &

Kastelein, R. A. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity,* 13**,** 715-25.

Orme, I. M. 2010. The Achilles heel of BCG. *Tuberculosis (Edinb),* 90**,** 329-32.

Ota, M. O., Odutola, A. A., Owiafe, P. K., Donkor, S., Owolabi, O. A., Brittain, N. J., Williams, N., Rowland-Jones, S., Hill, A. V., Adegbola, R. A. & Mcshane, H. 2011. Immunogenicity of the tuberculosis vaccine MVA85A is reduced by coadministration with EPI vaccines in a randomized controlled trial in Gambian infants. *Sci Transl Med,* 3**,** 88ra56.

Ota, M. O., Vekemans, J., Schlegel-Haueter, S. E., Fielding, K., Sanneh, M., Kidd, M., Newport, M. J., Aaby, P., Whittle, H., Lambert, P. H., Mcadam, K. P., Siegrist, C. A. & Marchant, A. 2002. Influence of Mycobacterium bovis bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. *J Immunol,* 168**,** 919-25.

Ottenhoff, T. H., De Boer, T., Verhagen, C. E., Verreck, F. A. & Van Dissel, J. T. 2000. Human deficiencies in type 1 cytokine receptors reveal the essential role of type 1 cytokines in immunity to intracellular bacteria. *Microbes Infect,* 2**,** 1559-66.

Pai, M., Riley, L. W. & Colford, J. M., Jr. 2004. Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect Dis,* 4**,** 761-76.

Palmer, C. E. & Long, M. W. 1966. Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *Am Rev Respir Dis,* 94**,** 553-68.

Parida, S. K. & Kaufmann, S. H. 2010. Novel tuberculosis vaccines on the horizon. *Curr Opin Immunol,* 22**,** 374-84.

Patel, N. & Trapathi, S. B. 2003. Improved cure rates in pulmonary tuberculosis category II (retreatment) with mycobacterium w. *J Indian Med Assoc,* 101**,** 680, 682.

Pena, S. V. & Krensky, A. M. 1997. Granulysin, a new human cytolytic granule-associated protein with possible involvement in cell-mediated cytotoxicity. *Semin Immunol,* 9**,** 117-25.

Perfetto, S. P., Chattopadhyay, P. K. & Roederer, M. 2004. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol,* 4**,** 648-55.

Pitt, J. M., Stavropoulos, E., Redford, P. S., Beebe, A. M., Bancroft, G. J., Young, D. B. & O'garra, A. 2012. Blockade of IL-10 signaling during bacillus Calmette-Guerin vaccination enhances and sustains Th1, Th17, and innate lymphoid IFN-gamma and IL-17 responses and increases protection to Mycobacterium tuberculosis infection. *J Immunol,* 189**,** 4079-87.

Qin, L., Gilbert, P. B., Corey, L., Mcelrath, M. J. & Self, S. G. 2007. A framework for assessing immunological correlates of protection in vaccine trials. *J Infect Dis,* 196**,** 1304-12.

Raby, E., Moyo, M., Devendra, A., Banda, J., De Haas, P., Ayles, H. & Godfrey-Faussett, P. 2008. The effects of HIV on the sensitivity of a whole blood IFN-gamma release assay in Zambian adults with active tuberculosis. *PLoS One,* 3**,** e2489.

Radosevic, K., Wieland, C. W., Rodriguez, A., Weverling, G. J., Mintardjo, R., Gillissen, G., Vogels, R., Skeiky, Y. A., Hone, D. M., Sadoff, J. C., Van Der Poll, T., Havenga, M. & Goudsmit, J. 2007. Protective immune responses to a recombinant adenovirus type 35 tuberculosis vaccine in two mouse strains: CD4 and CD8 T-cell epitope mapping and role of gamma interferon. *Infect Immun,* 75**,** 4105-15.

Rahman, S., Magalhaes, I., Rahman, J., Ahmed, R. K., Sizemore, D. R., Scanga, C. A., Weichold, F., Verreck, F., Kondova, I., Sadoff, J., Thorstensson, R., Spangberg, M., Svensson, M., Andersson, J., Maeurer, M. & Brighenti, S. 2012. Prime-boost vaccination with rBCG/rAd35 enhances CD8(+) cytolytic T-cell responses in lesions from Mycobacterium tuberculosis-infected primates. *Mol Med,* 18**,** 647-58.

Randhawa, A. K., Shey, M. S., Keyser, A., Peixoto, B., Wells, R. D., De Kock, M., Lerumo, L., Hughes, J., Hussey, G., Hawkridge, A., Kaplan, G., Hanekom, W. A. & Hawn, T. R. 2011. Association of human TLR1 and TLR6 deficiency with altered immune responses to BCG vaccination in South African infants. *PLoS Pathog,* 7**,** e1002174.

Raviglione, M. C. 2007. The new Stop TB Strategy and the Global Plan to Stop TB, 2006-2015. *Bull World Health Organ,* 85**,** 327.

Remus, N., El Baghdadi, J., Fieschi, C., Feinberg, J., Quintin, T., Chentoufi, M., Schurr, E., Benslimane, A., Casanova, J. L. & Abel, L. 2004. Association of IL12RB1 polymorphisms with pulmonary tuberculosis in adults in Morocco. *J Infect Dis,* 190**,** 580-7.

Ritz, N. & Curtis, N. 2009. Mapping the global use of different BCG vaccine strains. *Tuberculosis (Edinb),* 89**,** 248-51.

Ritz, N., Strach, M., Yau, C., Dutta, B., Tebruegge, M., Connell, T. G., Hanekom, W. A., Britton, W. J., Robins-Browne, R. & Curtis, N. 2012. A comparative analysis of polyfunctional T cells and secreted cytokines induced by Bacille Calmette-Guerin immunisation in children and adults. *PLoS One,* 7**,** e37535.

Rodriguez, L., Gonzalez, C., Flores, L., Jimenez-Zamudio, L., Graniel, J. & Ortiz, R. 2005. Assessment by flow cytometry of cytokine production in malnourished children. *Clin Diagn Lab Immunol,* 12**,** 502-7.

Roederer, M. 2001. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry,* 45**,** 194-205.

Roederer, M., Darzynkiewicz, Z. & Parks, D. R. 2004. Guidelines for the presentation of flow cytometric data. *Methods Cell Biol,* 75**,** 241-56.

Roederer, M., Nozzi, J. L. & Nason, M. C. 2011. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry A,* 79**,** 167-74.

Rojas, R. E., Balaji, K. N., Subramanian, A. & Boom, W. H. 1999. Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor beta. *Infect Immun,* 67**,** 6461-72.

Romaszko, J., Bucinski, A., Wasinski, R., Roslan, A. & Bednarski, K. 2008. Incidence and risk factors for pulmonary tuberculosis among the poor in the northern region of Poland. *Int J Tuberc Lung Dis,* 12**,** 430-5.

Rook, G. A. 2007. Th2 cytokines in susceptibility to tuberculosis. *Curr Mol Med,* 7**,** 327-37.

Roth, D. E., Soto, G., Arenas, F., Bautista, C. T., Ortiz, J., Rodriguez, R., Cabrera, L. & Gilman, R. H. 2004. Association between vitamin D receptor

gene polymorphisms and response to treatment of pulmonary tuberculosis. *J Infect Dis,* 190**,** 920-7.

Russell, D. G., Cardona, P. J., Kim, M. J., Allain, S. & Altare, F. 2009. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat Immunol,* 10**,** 943-8.

Sadeghi, K., Berger, A., Langgartner, M., Prusa, A. R., Hayde, M., Herkner, K., Pollak, A., Spittler, A. & Forster-Waldl, E. 2007. Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. *J Infect Dis,* 195**,** 296-302.

Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol,* 6**,** 345-52.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature,* 401**,** 708-12.

Samant, Y., Lanjewar, H., Parker, D., Block, L., Tomar, G. S. & Stein, B. 2007. Evaluation of the cold-chain for oral polio vaccine in a rural district of India. *Public Health Rep,* 122**,** 112-21.

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

Sartono, E., Lisse, I. M., Terveer, E. M., Van De Sande, P. J., Whittle, H., Fisker, A. B., Roth, A., Aaby, P., Yazdanbakhsh, M. & Benn, C. S. 2010. Oral polio vaccine influences the immune response to BCG vaccination. A natural experiment. *PLoS One,* 5**,** e10328.

Saunders, B. M., Frank, A. A., Orme, I. M. & Cooper, A. M. 2000. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection. *Infect Immun,* 68**,** 3322-6.

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S. C. & Dinarello, C. A. 1990. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood,* 75**,** 40-7.

Schreiber, T., Ehlers, S., Heitmann, L., Rausch, A., Mages, J., Murray, P. J., Lang, R. & Holscher, C. 2009. Autocrine IL-10 induces hallmarks of alternative activation in macrophages and suppresses antituberculosis effector mechanisms without compromising T cell immunity. *J Immunol,* 183**,** 1301-12.

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

Scriba, T. J., Tameris, M., Smit, E., Van Der Merwe, L., Hughes, E. J., Kadira, B., Mauff, K., Moyo, S., Brittain, N., Lawrie, A., Mulenga, H., De Kock, M., Makhethe, L., Janse Van Rensburg, E., Gelderbloem, S., Veldsman, A., Hatherill, M., Geldenhuys, H., Hill, A. V., Hawkridge, A., Hussey, G. D., Hanekom, W. A., Mcshane, H. & Mahomed, H. 2012. A phase IIa trial of the new tuberculosis vaccine, MVA85A, in HIV- and/or Mycobacterium tuberculosis-infected adults. *Am J Respir Crit Care Med,* 185**,** 769-78.

Sebina, I., Cliff, J. M., Smith, S. G., Nogaro, S., Webb, E. L., Riley, E. M., Dockrell, H. M., Elliott, A. M., Hafalla, J. C. & Cose, S. 2012. Long-lived memory B-cell responses following BCG vaccination. *PLoS One,* 7**,** e51381.

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

Seder, R. A. & Paul, W. E. 1994. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol,* 12**,** 635-73.

Semple, P. L., Watkins, M., Davids, V., Krensky, A. M., Hanekom, W. A., Kaplan, G. & Ress, S. 2011. Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth. *Clin Dev Immunol,* 2011**,** 438463.

Siegrist, C. A. 2001. Neonatal and early life vaccinology. *Vaccine,* 19**,** 3331-46.

Skjot, R. L., Oettinger, T., Rosenkrands, I., Ravn, P., Brock, I., Jacobsen, S. & Andersen, P. 2000. Comparative evaluation of low-molecular-mass proteins from Mycobacterium tuberculosis identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infect Immun,* 68**,** 214-20.

Skolimowska, K. H., Rangaka, M. X., Meintjes, G., Pepper, D. J., Seldon, R., Matthews, K., Wilkinson, R. J. & Wilkinson, K. A. 2012. Altered ratio of IFN-gamma/IL-10 in patients with drug resistant Mycobacterium tuberculosis and HIV- Tuberculosis Immune Reconstitution Inflammatory Syndrome. *PLoS One,* 7**,** e46481.

Slifka, M. K., Rodriguez, F. & Whitton, J. L. 1999. Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. *Nature,* 401**,** 76-9.

Smith, S. M., Malin, A. S., Pauline, T., Lukey, Atkinson, S. E., Content, J., Huygen, K. & Dockrell, H. M. 1999. Characterization of human Mycobacterium bovis bacille Calmette-Guerin-reactive CD8+ T cells. *Infect Immun,* 67**,** 5223-30.

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

Soares, A. P., Kwong Chung, C. K., Choice, T., Hughes, E. J., Jacobs, G., Van Rensburg, E. J., Khomba, G., De Kock, M., Lerumo, L., Makhethe, L., Maneli, M. H., Pienaar, B., Smit, E., Tena-Coki, N. G., Van Wyk, L., Boom, W. H., Kaplan, G., Scriba, T. J. & Hanekom, W. A. 2013. Longitudinal Changes in CD4+ T-Cell Memory Responses Induced by BCG Vaccination of Newborns. *J Infect Dis,* 207**,** 1084-94.

Soares, A. P., Scriba, T. J., Joseph, S., Harbacheuski, R., Murray, R. A., Gelderbloem, S. J., Hawkridge, A., Hussey, G. D., Maecker, H., Kaplan, G. & Hanekom, W. A. 2008. Bacillus Calmette-Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J Immunol,* 180**,** 3569-77.

Soysal, A., Millington, K. A., Bakir, M., Dosanjh, D., Aslan, Y., Deeks, J. J., Efe, S., Staveley, I., Ewer, K. & Lalvani, A. 2005. Effect of BCG vaccination on risk of Mycobacterium tuberculosis infection in children with household tuberculosis contact: a prospective community-based study. *Lancet,* 366**,** 1443-51.

Spencer, C. T., Abate, G., Blazevic, A. & Hoft, D. F. 2008. Only a subset of phosphoantigen-responsive gamma9delta2 T cells mediate protective tuberculosis immunity. *J Immunol,* 181**,** 4471-84.

Spencer, C. T., Abate, G., Sakala, I. G., Xia, M., Truscott, S. M., Eickhoff, C. S., Linn, R., Blazevic, A., Metkar, S. S., Peng, G., Froelich, C. J. & Hoft, D. F. 2013. Granzyme A produced by gamma(9)delta(2) T cells induces human macrophages to inhibit growth of an intracellular pathogen. *PLoS Pathog,* 9**,** e1003119.

Stefanova, T., Chouchkova, M., Hinds, J., Butcher, P. D., Inwald, J., Dale, J., Palmer, S., Hewinson, R. G. & Gordon, S. V. 2003. Genetic composition of Mycobacterium bovis BCG substrain Sofia. *J Clin Microbiol,* 41**,** 5349.

Steinman, R. M. & Hemmi, H. 2006. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol,* 311**,** 17-58.

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

Stenger, S. & Modlin, R. L. 1998. Cytotoxic T cell responses to intracellular pathogens. *Curr Opin Immunol,* 10**,** 471-7.

Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. & Russell, D. G. 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. *Science,* 263**,** 678-81.

Tadokera, R., Meintjes, G., Skolimowska, K. H., Wilkinson, K. A., Matthews, K., Seldon, R., Chegou, N. N., Maartens, G., Rangaka, M. X., Rebe, K., Walzl, G. & Wilkinson, R. J. 2011. Hypercytokinaemia accompanies HIV-tuberculosis immune reconstitution inflammatory syndrome. *Eur Respir J,* 37**,** 1248-59.

Tameris, M. D., Hatherill, M., Landry, B. S., Scriba, T. J., Snowden, M. A., Lockhart, S., Shea, J. E., Mcclain, J. B., Hussey, G. D., Hanekom, W. A., Mahomed, H. & Mcshane, H. 2013. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet,* 381**,** 1021-8.

Tena-Coki, N. G., Scriba, T. J., Peteni, N., Eley, B., Wilkinson, R. J., Andersen, P., Hanekom, W. A. & Kampmann, B. 2010. CD4 and CD8 T-cell responses to mycobacterial antigens in African children. *Am J Respir Crit Care Med,* 182**,** 120-9.

Testi, R., D'ambrosio, D., De Maria, R. & Santoni, A. 1994. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol Today,* 15**,** 479-83.

Thiery, J., Keefe, D., Boulant, S., Boucrot, E., Walch, M., Martinvalet, D., Goping, I. S., Bleackley, R. C., Kirchhausen, T. & Lieberman, J. 2011. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol,* 12**,** 770-7.

Thomas, D. A., Du, C., Xu, M., Wang, X. & Ley, T. J. 2000. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity,* 12**,** 621-32.

Toossi, Z., Gogate, P., Shiratsuchi, H., Young, T. & Ellner, J. J. 1995. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. *J Immunol,* 154**,** 465-73.

Tortoli, E. 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev,* 16**,** 319-54.

Trombetta, E. S. & Mellman, I. 2005. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol,* 23**,** 975-1028.

Trunz, B. B., Fine, P. & Dye, C. 2006. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet,* 367**,** 1173-80.

Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M. & Mellman, I. 2000. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science,* 288**,** 522-7.

Turner, J. & Dockrell, H. M. 1996. Stimulation of human peripheral blood mononuclear cells with live Mycobacterium bovis BCG activates cytolytic CD8+ T cells in vitro. *Immunology,* 87**,** 339-42.

Ulrichs, T., Moody, D. B., Grant, E., Kaufmann, S. H. & Porcelli, S. A. 2003. T-cell responses to CD1-presented lipid antigens in humans with Mycobacterium tuberculosis infection. *Infect Immun,* 71**,** 3076-87.

Umemura, M., Yahagi, A., Hamada, S., Begum, M. D., Watanabe, H., Kawakami, K., Suda, T., Sudo, K., Nakae, S., Iwakura, Y. & Matsuzaki, G. 2007. IL-17-mediated regulation of innate and acquired immune response against

pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. *J Immunol,* 178**,** 3786-96.

Unepi 2003. Uganda national expanded programe on immunisation (UNEPI) standards.

Van Crevel, R., Ottenhoff, T. H. & Van Der Meer, J. W. 2002. Innate immunity to Mycobacterium tuberculosis. *Clin Microbiol Rev,* 15**,** 294-309.

Van Grol, J., Subauste, C., Andrade, R. M., Fujinaga, K., Nelson, J. & Subauste, C. S. 2010. HIV-1 inhibits autophagy in bystander macrophage/monocytic cells through Src-Akt and STAT3. *PLoS One,* 5**,** e11733.

Van Pinxteren, L. A., Cassidy, J. P., Smedegaard, B. H., Agger, E. M. & Andersen, P. 2000. Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. *Eur J Immunol,* 30**,** 3689-98.

Vermijlen, D., Brouwer, M., Donner, C., Liesnard, C., Tackoen, M., Van Rysselberge, M., Twite, N., Goldman, M., Marchant, A. & Willems, F. 2010. Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *J Exp Med,* 207**,** 807-21.

Vesosky, B., Rottinghaus, E. K., Davis, C. & Turner, J. 2009. CD8 T Cells in old mice contribute to the innate immune response to Mycobacterium tuberculosis via interleukin-12p70-dependent and antigen-independent production of gamma interferon. *Infect Immun,* 77**,** 3355-63.

Vidard, L., Rock, K. L. & Benacerraf, B. 1992. Heterogeneity in antigen processing by different types of antigen-presenting cells. Effect of cell culture on antigen processing ability. *J Immunol,* 149**,** 1905-11.

Von Reyn, C. F., Mtei, L., Arbeit, R. D., Waddell, R., Cole, B., Mackenzie, T., Matee, M., Bakari, M., Tvaroha, S., Adams, L. V., Horsburgh, C. R. & Pallangyo, K. 2010. Prevention of tuberculosis in Bacille Calmette-Guerin-primed, HIV-infected adults boosted with an inactivated whole-cell mycobacterial vaccine. *AIDS,* 24**,** 675-85.

Waldrop, S. L., Pitcher, C. J., Peterson, D. M., Maino, V. C. & Picker, L. J. 1997. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J Clin Invest,* 99**,** 1739-50.

Walker, L. & Lowrie, D. B. 1981. Killing of Mycobacterium microti by immunologically activated macrophages. *Nature,* 293**,** 69-71.

Walker, V., Selby, G. & Wacogne, I. 2006. Does neonatal BCG vaccination protect against tuberculous meningitis? *Arch Dis Child,* 91**,** 789-91.

Wallis, R. S. 2007. Reactivation of latent tuberculosis by TNF blockade: the role of interferon gamma. *J Investig Dermatol Symp Proc,* 12**,** 16-21.

Walzl, G., Ronacher, K., Hanekom, W., Scriba, T. J. & Zumla, A. 2011. Immunological biomarkers of tuberculosis. *Nat Rev Immunol,* 11**,** 343-54.

Wang, C. H., Liu, C. Y., Lin, H. C., Yu, C. T., Chung, K. F. & Kuo, H. P. 1998. Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. *Eur Respir J,* 11**,** 809-15.

Wang, J., Wakeham, J., Harkness, R. & Xing, Z. 1999. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest,* 103**,** 1023-9.

Warner, J. O. 2004. The early life origins of asthma and related allergic disorders. *Arch Dis Child,* 89**,** 97-102.

Watanabe, Y., Watari, E., Matsunaga, I., Hiromatsu, K., Dascher, C. C., Kawashima, T., Norose, Y., Shimizu, K., Takahashi, H., Yano, I. & Sugita, M. 2006. BCG vaccine elicits both T-cell mediated and humoral immune responses directed against mycobacterial lipid components. *Vaccine,* 24**,** 5700-7.

Webb, E. L., Mawa, P. A., Ndibazza, J., Kizito, D., Namatovu, A., Kyosiimire-Lugemwa, J., Nanteza, B., Nampijja, M., Muhangi, L., Woodburn, P. W., Akurut, H., Mpairwe, H., Akello, M., Lyadda, N., Bukusuba, J., Kihembo, M., Kizza, M., Kizindo, R., Nabulime, J., Ameke, C., Namujju, P. B., Tweyongyere, R., Muwanga, M., Whitworth, J. A. & Elliott, A. M. 2011. Effect of single-dose anthelmintic treatment during pregnancy on an infant's response to immunisation and on susceptibility to infectious diseases in infancy: a randomised, double-blind, placebo-controlled trial. *Lancet,* 377**,** 52-62.

Weir, R. E., Gorak-Stolinska, P., Floyd, S., Lalor, M. K., Stenson, S., Branson, K., Blitz, R., Ben-Smith, A., Fine, P. E. & Dockrell, H. M. 2008. Persistence of the immune response induced by BCG vaccination. *BMC Infect Dis,* 8**,** 9.

Wenner, C. A., Guler, M. L., Macatonia, S. E., O'garra, A. & Murphy, K. M. 1996. Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. *J Immunol,* 156**,** 1442-7.

Wherry, E. J., Teichgraber, V., Becker, T. C., Masopust, D., Kaech, S. M., Antia, R., Von Andrian, U. H. & Ahmed, R. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol,* 4**,** 225-34.

WHO 1972. BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. *Bull World Health Organ,* 46**,** 371-85.

WHO 1982. Efficacy of various durations of isoniazid preventive therapy for tuberculosis: five years of follow-up in the IUAT trial. International Union Against Tuberculosis Committee on Prophylaxis. *Bull World Health Organ,* 60**,** 555-64.

WHO 2004. BCG vaccine: WHO position paper. 79**,** 27-38.

WHO 2013a. Baccille Calmette Guérin vaccine: Reported estimates of BCG coverage.

WHO 2013b. Global Tuberculosis Report 2013.

Winau, F., Weber, S., Sad, S., De Diego, J., Hoops, S. L., Breiden, B., Sandhoff, K., Brinkmann, V., Kaufmann, S. H. & Schaible, U. E. 2006. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity,* 24**,** 105-17.

Woodworth, J. S., Wu, Y. & Behar, S. M. 2008. Mycobacterium tuberculosis-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. *J Immunol,* 181**,** 8595-603.

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

Yamada, H., Mizumo, S., Horai, R., Iwakura, Y. & Sugawara, I. 2000. Protective role of interleukin-1 in mycobacterial infection in IL-1 alpha/beta double-knockout mice. *Lab Invest,* 80**,** 759-67.

Yan, S. R., Qing, G., Byers, D. M., Stadnyk, A. W., Al-Hertani, W. & Bortolussi, R. 2004. Role of MyD88 in diminished tumor necrosis factor alpha production by newborn mononuclear cells in response to lipopolysaccharide. *Infect Immun,* 72**,** 1223-9.

Yazdanbakhsh, M., Kremsner, P. G. & Van Ree, R. 2002. Allergy, parasites, and the hygiene hypothesis. *Science,* 296**,** 490-4.

Young, D. & Dye, C. 2006. The development and impact of tuberculosis vaccines. *Cell,* 124**,** 683-7.

Young, J. & O'connor, M. E. 2005. Risk factors associated with latent tuberculosis infection in Mexican American children. *Pediatrics,* 115**,** e647-53.

Young, S. L., Slobbe, L., Wilson, R., Buddle, B. M., De Lisle, G. W. & Buchan, G. S. 2007. Environmental strains of Mycobacterium avium interfere with immune responses associated with Mycobacterium bovis BCG vaccination. *Infect Immun,* 75**,** 2833-40.

Zarkowsky, D., Lamoreaux, L., Chattopadhyay, P., Koup, R. A., Perfetto, S. P. & Roederer, M. 2011. Heavy metal contaminants can eliminate quantum dot fluorescence. *Cytometry A,* 79**,** 84-9.

Zeng, Z., Castano, A. R., Segelke, B. W., Stura, E. A., Peterson, P. A. & Wilson, I. A. 1997. Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. *Science,* 277**,** 339-45.

Zhou, F. 2009. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. *Int Rev Immunol,* 28**,** 239-60.

Ziegler, S. F., Ramsdell, F. & Alderson, M. R. 1994. The activation antigen CD69. *Stem Cells,* 12**,** 456-65.

Appendix

Journal of Infectious Diseases Advance Access published November 27, 2013

MAJOR ARTICLE

Distinct T-Cell Responses When BCG Vaccination Is Delayed From Birth to 6 Weeks of Age in Ugandan Infants

F. Lutwama,[1,2,3] B. M. Kagina,[1,2] A. Wajja,[3] F. Waiswa,[3] N. Mansoor,[1,2] S. Kirimunda,[3] E. J. Hughes,[1,2] N. Kiwanuka,[3] M. L. Joloba,[3] P. Musoke,[3] T. J. Scriba,[1,2] H. Mayanja-Kizza,[3] C. L. Day,[1,2,4,5] and W. A. Hanekom[1,2]

[1]South African Tuberculosis Vaccine Initiative, Institute of Infectious Diseases and Molecular Medicine, and [2]School of Child and Adolescent Health, University of Cape Town, Cape Town, South Africa; [3]Infectious Diseases Institute, Makerere University College of Health Sciences, Kampala, Uganda; and [4]Department of Global Health, Rollins School of Public Health, and [5]Emory Vaccine Center, Emory University, Atlanta, Georgia

Background. In Uganda, the tuberculosis vaccine BCG is administered on the first day of life. Infants delivered at home receive BCG vaccine at their first healthcare facility visit at 6 weeks of age. Our aim was to determine the effect of this delay in BCG vaccination on the induced immune response.

Methods. We assessed $CD4^+$ and $CD8^+$ T-cell responses with a 12-hour whole-blood intracellular cytokine/cytotoxic marker assay, and with a 6-day proliferation assay.

Results. We enrolled 92 infants: 50 had received BCG vaccine at birth and 42 at 6 weeks of age. Birth vaccination was associated with (1) greater induction of $CD4^+$ and $CD8^+$ T cells expressing either interferon γ (IFN-γ) alone or IFN-γ together with perforin and (2) induction of proliferating cells that had greater capacity to produce IFN-γ, tumor necrosis factor α (TNF-α), and interleukin 2 together, compared with delayed vaccination.

Conclusions. Distinct patterns of T-cell induction occurred when BCG vaccine was given at birth and at 6 weeks of age. We propose that this diversity might impact protection against tuberculosis. Our results differ from those of studies of delayed BCG vaccination in South Africa and the Gambia, suggesting that geographical and population heterogeneity may affect the BCG vaccine–induced T-cell response.

Keywords. Uganda; BCG; vaccination; birth; delayed; $CD4^+$ and $CD8^+$ T-cell responses.

BCG vaccine is the only vaccine licensed for prevention of childhood tuberculosis [1]. BCG protects infants against severe forms of tuberculosis (meningitis and miliary tuberculosis) [2] and has a positive influence on overall infant morbidity and mortality [3]. Therefore, in settings where the *Mycobacterium tuberculosis* exposure risk is high, the World Health Organization (WHO) recommends BCG vaccination soon after birth [4].

In Uganda, tuberculosis is endemic [5], and BCG vaccine is routinely administered within 24 hours after birth among infants born in a healthcare facility. However, up to 50% of babies are born at home [6, 7]. These infants commonly receive BCG vaccine at the first contact with a healthcare facility, usually at 6 weeks of age, when other WHO Expanded Programme on Immunization–recommended vaccines are administered [8]. We aimed to assess the effect of this delay on the immune response induced by BCG vaccine.

We proposed to investigate $CD4^+$ and $CD8^+$ T-cell immunity, which is thought to be critical in the control of *M. tuberculosis*. BCG vaccine induces a T-helper type 1 (Th1) response, characterized by production of interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and interleukin 2 (IL-2) [9–12]. BCG vaccine also induces cytotoxic T cells [13], as well as interleukin 17 (IL-17; Th17)–producing T cells [14].

Compared with adults, neonates' innate immune cells produce less Th1-promoting interleukin 12 (IL-12) [15]

Received 20 May 2013; accepted 6 September 2013.

Presented in part: 6th EDCTP forum, Addis Ababa, Ethiopia, 9–12 October 2011; Federations of African Immunologists Societies Conference, Durban, South Africa, 3–5 December 2012; TB Vaccine Global Forum, Cape Town, South Africa, 24–27 March 2013.

Correspondence: Willem A. Hanekom, FCP (SA), University of Cape Town, Faculty of Health Sciences, Anzio Rd, Observatory 7925, Cape Town, South Africa (willem.hanekom@uct.ac.za).

The Journal of Infectious Diseases

DOI: 10.1093/infdis/jit570

and display diminished Toll-like receptor 4 (TLR) expression and signaling [16]. This potentially results in a suboptimal response to BCG vaccine. At 6 weeks of age, the infant immune system would start adapting to the ex utero environment [17], and enhanced priming of BCG-specific immune responses might occur. Multiple studies have investigated the effects of delaying BCG vaccination. Comparable purified protein derivative (PPD)–induced IFN-γ production was shown between infants vaccinated at birth or later [18–20]. In contrast, a South African study showed that delaying BCG vaccination from birth to 10 weeks of age induced a greater frequency of BCG-specific polyfunctional $CD4^+$ T cells (ie, cells that express IFN-γ, TNF-α, and IL-2 together) [21]. Importantly, in all previous studies, infants were randomized to receive BCG vaccine at birth or later. In contrast, we addressed the effects in a setting where delayed vaccination occurred due to home births. We hypothesized that BCG vaccination at 6 weeks of age would result in an enhanced specific T-cell response, compared with administration at birth. We report a comprehensive immunological assessment of delayed BCG vaccination.

METHODS

Study Population and Sample Collection

Healthy 9-month-old infants were enrolled at the Child Health and Development Center in Mulago National Referral Hospital, Kampala, Uganda. A child health growth card was used to identify infants who received BCG vaccine at birth or at 6 weeks of age.

Infants were excluded if the mother had documented evidence of a positive HIV test result or had not participated in a program to prevent mother-to-child HIV transmission and if the infant lacked a BCG vaccination scar, was born before 37 weeks of gestation, had significant perinatal complications, had any acute or chronic disease symptoms at the time of enrollment or clinically apparent anemia, had a household contact with tuberculosis, or had an unexplained persistent cough or confirmed active tuberculosis.

The study was approved by the institutional review board of the School of Public Health, Makerere University College of Health Sciences, and the Uganda National Council for Science and Technology. Good clinical practice procedures were adhered to.

Blood Collection and Processing

From each study participant, a 4-mL whole-blood specimen was collected in a sodium-heparin tube and was transported to the laboratory for processing within 1 hour. One milliliter of heparinized whole blood was incubated with either BCG (Danish strain 1331; Statens Serum Institut; 1.2×10^6 colony-forming units [CFU]/mL) or phytohemagglutinin (PHA; Sigma-Aldrich; 5 μg/mL) or was left unstimulated, as previously described [22]. The costimulatory antibodies anti-CD28 and anti-CD49d (at 1 μg/mL each; BD Biosciences, San Jose, CA) were added to all assay conditions to enhance the responses [23]. Blood was incubated at 37°C for 7 hours, after which plasma was removed and stored at −80°C for later measurement of soluble cytokines. Thereafter, brefeldin A (Sigma-Aldrich; 10 μg/m) was added, and the blood was incubated for a further 5 hours. Cells were harvested, fixed in BD FACS Lysing Solution (BD Biosciences), and cryopreserved for later measurement of T-cell–associated cytokine expression.

A further 1 mL of whole blood was diluted in 9 mL of Roswell Park Memorial Institute medium and mixed in a sterile polypropylene tube. One milliliter of diluted blood was incubated with either BCG (Danish strain 1331; Statens Serum Institut; 1×10^5 CFU/mL) or PHA (Sigma-Aldrich; 1 μg/mL, added on the third day of incubation) or was left unstimulated. Incubation continued for 6 days at 37°C in 5% CO_2 as previously described [24]. Four hours before the end of the cell culture, phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; 20 ng/mL), ionomycin (Sigma-Aldrich; 2 μg/mL), and brefeldin A (Sigma-Aldrich; 10 μg/mL) were added to induce cytokine expression. Later, red cells were lysed with BD FACS Lysing Solution (BD Biosciences), and white blood cells were fixed and cryopreserved for measurement of the proliferation and cytokine-producing potential of proliferating cells.

Antibodies

The following monoclonal antibodies were used: anti-CD3 Pacific Blue (UCHT1), anti-CD8 PerCP-Cy5.5 (SK-1), anti-CD8 Horizon V500 (RPA-T8), anti-IFN-γ Alexa Fluor 700 (B27), anti-IL-2 FITC (5344.111), anti-Ki67 PE (B56), anti-CD45RA FITC (HI100), anti-CD27 APC (L128), and anti-CCR7 PE (150 503), from BD Biosciences (San Jose, CA); anti-CD4 QDot605 (S3.5) and anti-CD8 QDot565 (3B5), from Invitrogen (Eugene, OR); anti-TNF-αPE-Cy7 (Mab11) and anti-IL-17 Alexa Fluor 647 (eBio64CAP17), from eBiosciences (San Diego, CA); anti-CD69 PerCP-Cy5.5 (FN50), from Biolegend (San Diego, CA); and anti-Perforin PE (B-D48), from Diaclone (Besancon, France).

Intracellular Cytokine Staining (ICS) Assay

Fixed, cryopreserved white cells from the stimulated whole blood were thawed, washed, and permeabilized before staining, as previously described [22]. Stained cells were acquired on a LSRII flow cytometer (BD Biosciences). After acquisition, data were analyzed using FlowJo software (v9.4.11; Tree Star). Compensation was done with positive and negative anti-mouse immunoglobulin kappa beads (BD Biosciences) labeled with the respective fluorochrome-conjugated antibodies. Cytometer setting and tracking beads (BD Biosciences) were used for daily settings.

Flow cytometry data were exported to Pestle v1.7 (Mario Roederer, Vaccine Research Center, National Institute of

Allergy and Infectious Diseases, National Institutes of Health) and Spice (v5.1) for analysis [25].

Multiplex Soluble Cytokine Assay

Plasma collected after 7 hours of incubation during the short-term whole-blood assay was used to measure levels of IFN-γ, IL-2, interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), and interleukin 13 (IL-13) with the Milliplex MAP assay (Millipore, Billerica, MA), in accordance with the manufacturer's instructions. Fluorescence was detected using a Luminex 100 IS machine (xMAP technology; Luminex). Data were acquired using the Bio-Plex Manager Software. The standards for each assay ranged from 3.2 to 10 000 pg/mL.

Data Analysis

The response detected in the negative controls (unstimulated) was subtracted from the response detected in the BCG-stimulated samples. For short-term ICS, proliferation, and multiplex analyses, samples were excluded from the final analysis if the response detected in the positive control (PHA) was lower than the median plus 3 times the median absolute deviations of the negative control samples for all infants.

For memory phenotype analysis, in addition to the above criteria, data were excluded if (1) the frequency of BCG-specific cells was <0.01%, (2) the ratio of BCG to unstimulated frequencies was <2, and (3) there were <20 positive events in the BCG-stimulated sample minus the number of events in the unstimulated sample.

For multiplex analysis, data were excluded if (1) the positive control response was less than that of the negative control and (2) the positive control response was <3.2 pg/mL.

The Mann-Whitney *U* test was used to compare immunological outcomes between the 2 groups. A *P* value of <.05 was considered statistically significant. Spearman rank correlation was used to test for associations between frequencies of the specific T cells and the levels of soluble cytokines. Prism v5.0 (GraphPad Software) was used for statistical analyses. The influence of vaccination group, sex, household income, and weight on the frequency of cytokine-expressing T cells was determined by linear regression analysis.

RESULTS

Participants

We enrolled 92 infants at 9 months of age between October 2008 and February 2009 in Uganda. Fifty of these infants received BCG vaccine at birth and 42 received the vaccine at 6 weeks of age. Six infants who had received BCG vaccine at birth and 2 infants who had received BCG vaccine at 6 weeks of age were excluded because of inadequate blood volumes. The body weight and sex distribution between the 2 groups were not different at recruitment (Table 1). The birth weight for homeborn infants was not available. Infants who received BCG vaccine at birth were more likely to be from a household with higher income than infants vaccinated at 6 weeks of age (Table 1).

Greater Frequencies of BCG-Specific CD4$^+$ and CD8$^+$ T Cells Expressing IFN-γ, With or Without Perforin, in Infants Vaccinated at Birth, Compared With Infants Vaccinated at 6 Weeks of Age

We compared the frequency of BCG-specific IL-2–, IL-17–, IFN-γ–, TNF-α–, and perforin-expressing CD4$^+$ T cells in infants who received BCG vaccine at birth or at 6 weeks of age, using a short-term WB-ICS assay (Figure 1*A* and Supplementary Figure 1). The great majority of infants vaccinated at either time point had a detectable specific IL-2, IL-17, IFN-γ, TNF-α, and perforin CD4$^+$ T-cell response (Figure 1*B* and 1*C*). The frequencies of BCG-specific CD4$^+$ T cells expressing IL-2, IL-17, TNF-α, or perforin were comparable in the 2 vaccination groups (Figure 1*B* and 1*C*). However, infants who received BCG vaccine at birth had greater frequencies of IFN-γ–expressing CD4$^+$ T cells, compared with infants vaccinated at 6 weeks of age (Figure 1*B*).

Next, we compared the profile of BCG-specific CD4$^+$ T cells expressing IL-2, IL-17, IFN-γ, or TNF-α alone or in different combinations between the 2 groups of infants. We did not observe coexpression of IL-17 with any of the Th1 cytokines (Figure 1*D* and data not shown), whereas perforin was coexpressed with IFN-γ only (Figure 1*E* and data not shown). Frequencies of BCG-specific polyfunctional (IL-2$^+$IFN-γ$^+$TNF-

Table 1. Demographic Characteristics of the Study Participants at 9 Months of Age

Variable	Vaccinated at Birth (n = 44)	Vaccinated at 6 Weeks of Age (n = 40)	*P*
Sex			
Female	18 (41)	20 (53)	.29[a]
Male	26 (59)	18 (47)	
Income			
Less than $125	12 (27)	25 (64)	.001[a]
Greater than $125	32 (73)	14 (36)	
Season of BCG vaccination			
Dry	28 (64)	24 (60)	.73[a]
Rainy	16 (36)	16 (40)	
Weight at 9 mo of age, kg	9.0 (8–9.55)	8.5 (8–9.45)	.26[b]
Weight-for-age *z* score at 9 mo of age	0.27 (−0.70 to 1.08)	0.04 (−0.42 to 0.89)	.53[b]

Data are no. (%) of infants or median (interquartile range).

[a] By χ^2 analysis.

[b] By the Mann-Whitney *U* test.

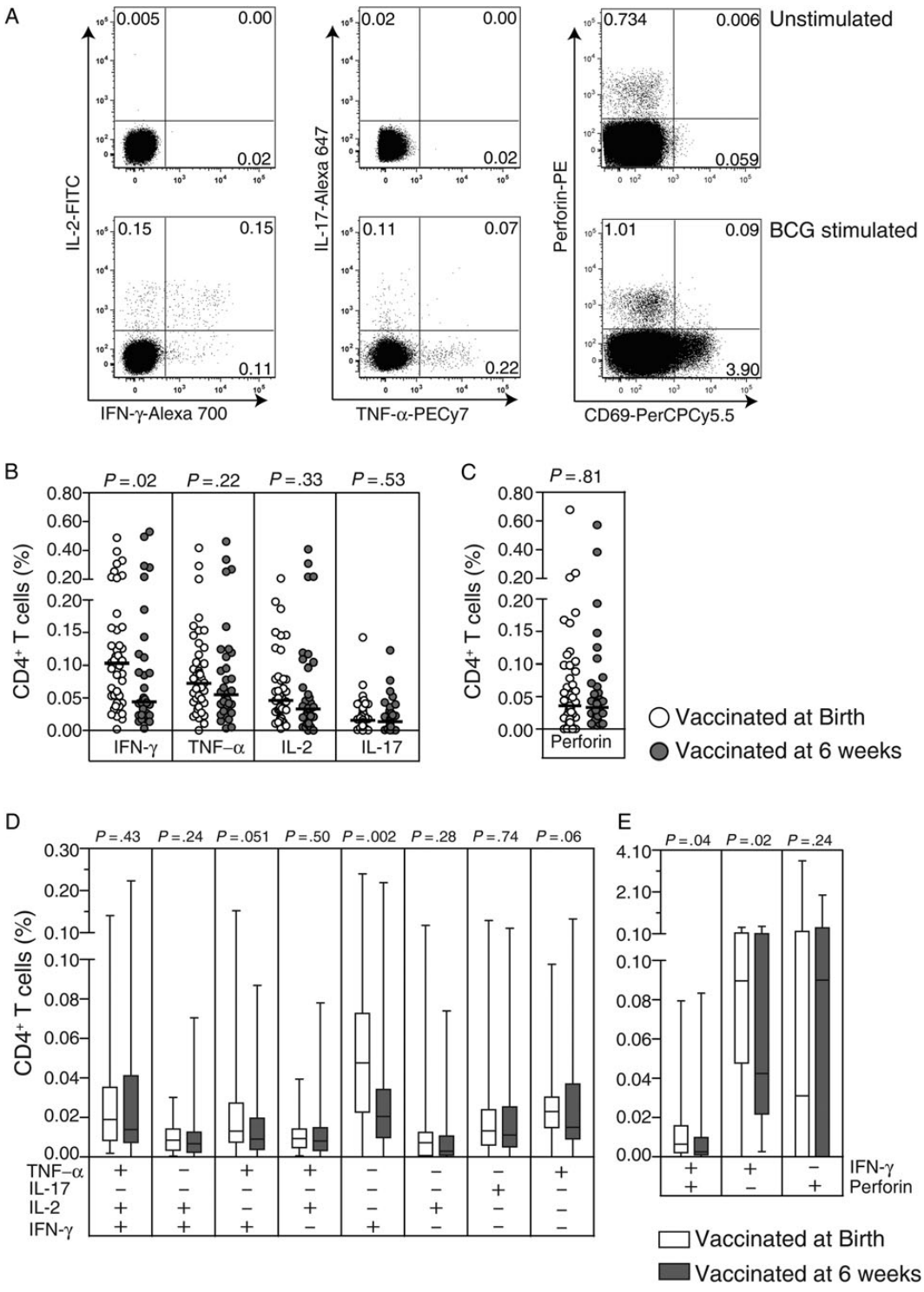

Figure 1. Specific CD4$^+$ T-cell cytokine and perforin responses measured in the short-term whole-blood assay. Representative flow cytometry data of cytokine and perforin expression in a negative control (unstimulated) sample (top row) or BCG-stimulated sample (bottom row) from a 9-month-old infant who received BCG vaccine at birth (*A*). Scatterplots depict frequencies of total: interferon γ (IFN-γ)–, tumor necrosis factor α (TNF-α)–, interleukin 2 (IL-2)–, and interleukin 17 (IL-17)–expressing CD4$^+$ T cells (*B*) and perforin-expressing CD69$^+$CD4$^+$ T cells (*C*). In the scatterplots, the horizontal lines represent the median frequencies. Box and whisker plots show the frequencies of distinct subsets of specific CD4$^+$ T cells based on combinations of expression of IFN-γ, TNF-α, IL-2, and IL-17 (*D*) and the frequencies of CD69$^+$CD4$^+$ T cells expressing IFN-γ and perforin singly or in combination (*E*). For box and whiskers plots, the horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles. Values shown are corrected for background responses in the negative control condition. The Mann-Whitney *U* test was used to assess differences in frequencies of cytokine- or perforin-expressing CD4$^+$ T cells between infants vaccinated at birth (open dots/bars) and 6 weeks of age (closed dots/bars).

α+), double positive (IL-2+IFN-γ+, IL-2+TNF-α+, or IFN-γ+TNF-α+), and single-positive (IL-2+, IL-17+, TNF-α+, or perforin+) CD4+ T-cell subsets were not different between the 2 groups (Figure 1*D* and 1*E*). However, the group vaccinated at birth had greater frequencies of BCG-specific IFN-γ single-positive and IFN-γ+perforin+ double-positive CD4+ T cells, compared with those vaccinated at 6 weeks (Figure 1*D* and 1*E*).

Next, we analyzed specific CD8+ T cells. In both groups, BCG-specific CD8+ T-cell responses were dominated by IFN-γ and perforin expression (Figure 2*B* and 2*C*). As observed for CD4+ T cells, frequencies of BCG-specific IL-2–, IL-17–, TNF-α–, and perforin-expressing CD8+ T cells in both groups were not different, whereas infants who received BCG vaccine at birth had greater frequencies of IFN-γ–expressing CD8+ T cells, compared with infants vaccinated at 6 weeks of age (Figure 2*B*). Also, similar to CD4+ T cells, the group vaccinated at birth had greater frequencies of specific CD8+ T cells expressing IFN-γ alone (Figure 2*D*) or in combination with perforin (Figure 2*E*).

No Difference in Proportions of BCG-Specific CD4+ and CD8+ T-Cell Memory Phenotypes Between the 2 Groups

Next, we evaluated whether the observed differences in frequencies of BCG-specific IFN-γ–expressing CD4+ and CD8+ T cells could be associated with differential T-cell memory phenotypes, as defined by CCR7 and CD45RA expression (Figure 3*A*) [26]. We hypothesized that birth vaccinated infants would show a greater proportion of BCG-specific effector memory (T_{EM}) cells, compared with those vaccinated at 6 weeks of age. Phenotypic markers were measured following stimulation of whole blood for 12 hours.

The majority of BCG-specific IFN-γ–expressing CD4+ T cells showed a central memory (T_{CM}) phenotype, in both groups of infants (Figure 3*B*). Proportions of BCG-specific IFN-γ–expressing CD4+ T cells showing a naive-like (T_{Naive}), T_{CM}, T_{EM}, and effector memory RA (T_{EMRA}) phenotype were not different in the infant groups (Figure 3*B*). We also measured memory phenotypes of specific CD8+ T cells. BCG-specific IFN-γ–expressing CD8+ T cells mainly showed T_{EM} and T_{EMRA} phenotypes in both groups of infants, and no differences were observed between the 2 vaccination groups (Figure 3*C*).

Higher IL-10 Levels in Infants Vaccinated at 6 Weeks of Age, Compared With Those Vaccinated at Birth

We also evaluated levels of Th1 and Th2 cytokines and IL-10 in plasma. We hypothesized that the cytokine milieu would impact the BCG-primed T-cell response (eg, the presence of high levels of Th2 cytokines [IL-4, IL-5, and IL-13], as well as IL-10, might attenuate Th1 immunity) [27, 28]. Levels of BCG-induced IL-10 were higher in the infants vaccinated at 6 weeks of age, compared with the group vaccinated at birth (Figure 4*A*–*C* and 4*E*). However, infants vaccinated at birth had higher levels of IL-10 in the unstimulated controls, compared with infants vaccinated at 6 weeks of age (Figure 4*D*). We therefore calculated a stimulation index and could confirm higher specific induction of IL-10 when vaccinated later (Figure 4*E*); IL-10 was not measured with the WB-ICS, because preliminary experiments showed that T-cell–specific expression was too low for valid analysis. Levels of all other cytokines were not different between the 2 groups of infants (Figure 4*A*–*C* and 4*E*).

Greater Capacity of Specific Proliferating CD4+ T Cells to Coexpress IL-2, IFN-γ, and TNF-α in Infants Vaccinated at Birth Than at 6 Weeks of Age

The ability of T cells to proliferate in response to secondary antigen encounter is an important feature of memory responses [29]. We measured this ability of the T cells in a 6-day assay by measuring upregulation of Ki67, a nuclear protein expressed during the active phases of cell division [30], as a marker for proliferation (Figure 5*A* and 5*B*) [24, 31]. The proliferative capacity of CD4+ and CD8+ T cells were similar between the 2 groups (Figure 5*C* and 5*D*).

We also assessed the cytokine-producing capacity of specific proliferating CD4+ T cells after stimulating the cells on day 6 with PMA/ionomycin. Although the capacity of the specific cells (ie, Ki67+ cells) to produce any of the cytokines did not differ (data not shown), we observed that cells from infants vaccinated at birth had a greater capacity to coexpress IL-2, IFN-γ, and TNF-α, compared with those vaccinated at 6 weeks of age. However, the proportion of these cells expressing TNF-α only was lower in the group vaccinated at birth, compared with those vaccinated at 6 weeks of age (Figure 5*E*). Cytokine production capacity among specific CD8+ T cells did not differ between the groups (data not shown).

DISCUSSION

We compared BCG vaccine–induced immunity in Ugandan infants either vaccinated at birth or at 6 weeks of age. We showed that age of vaccination impacted specific immune response measured at 9 months of age. Infants vaccinated at birth had higher frequencies of BCG-specific CD4+ and CD8+ T cells producing IFN-γ alone or coexpressing IFN-γ and perforin. Furthermore, although the T-cell proliferative potential was similar in the 2 groups, a higher proportion of proliferating BCG-specific CD4+ T cells coexpressed IL-2, IFN-γ, and TNF-α in birth vaccinated infants. These infants also had lower levels of specific IL-10, compared with those vaccinated at 6 weeks of age.

We measured specific Th1 cytokine– and perforin-producing cells based on the proposed roles for these molecules in control of mycobacteria. For example, humans with mutations in the IL-12/IFN-γ pathway show an increased risk of mycobacterial disease [32, 33]. The role of perforin in mediating immunity against

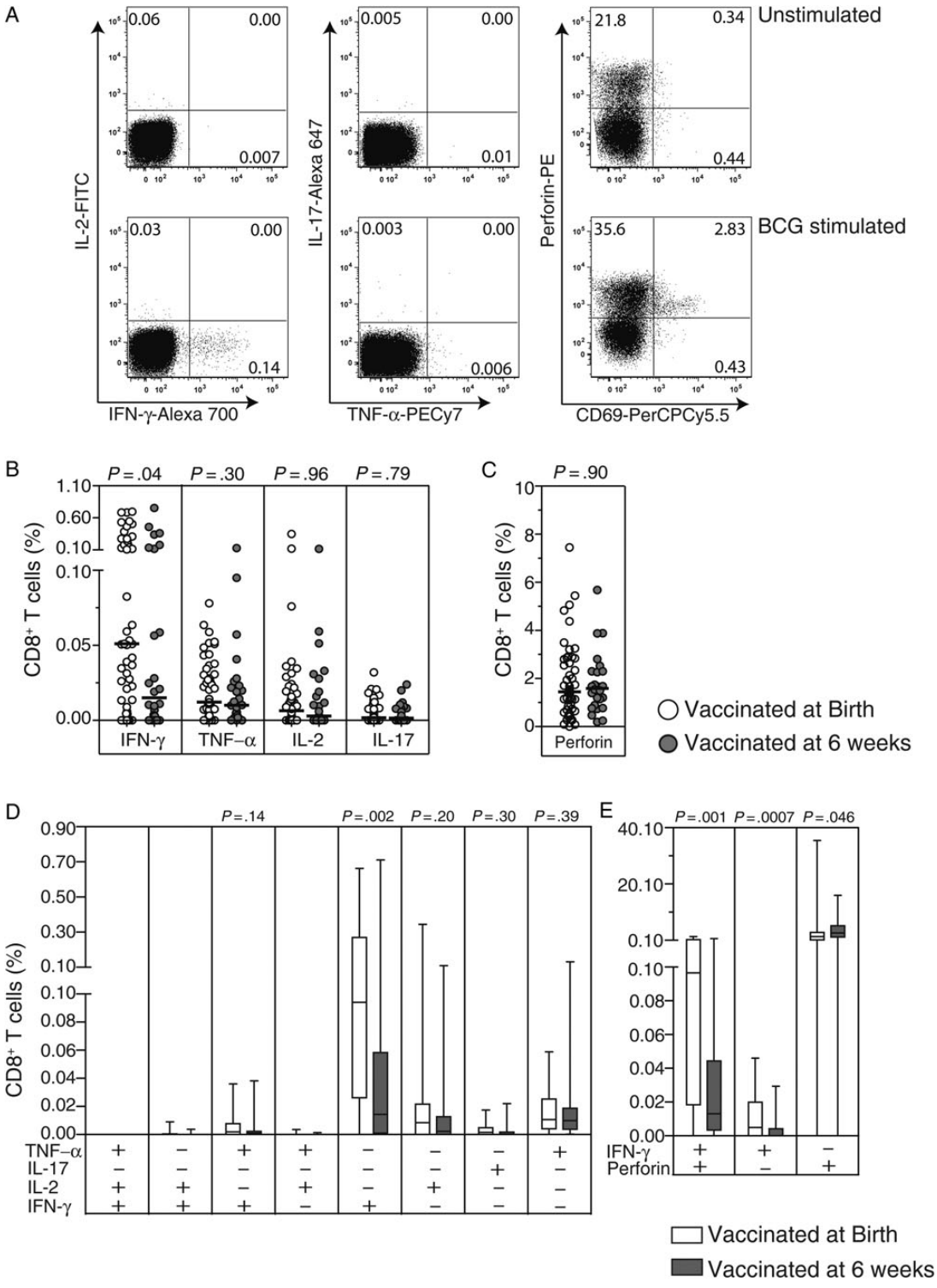

Figure 2. Specific $CD8^+$ T-cell cytokine and perforin responses measured in the short-term whole-blood assay. Representative flow cytometry data of cytokine and perforin expression in a control (unstimulated) sample (top row) or a BCG-stimulated sample (bottom row) from a 9-month-old infant who received BCG vaccine at birth (*A*). Scatterplots depict frequencies of total: interferon γ (IFN-γ)–, tumor necrosis factor α (TNF-α)–, interleukin 2 (IL-2)–, and interleukin 17 (IL-17)–expressing $CD8^+$ T cells (*B*) and perforin-expressing $CD69^+CD8^+$ T cells (*C*). On the scatterplots, the horizontal lines represent the median frequencies. Box and whisker plots show the frequencies of distinct subsets of specific $CD8^+$ T cells based on combinations of cytokine expression of IFN-γ TNF-α IL-2, and IL-17 (*D*) and the frequencies of $CD69^+CD8^+$ T cells expressing IFN-γ and perforin singly or in combination (*E*). For box and whisker plots, the horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles. Values shown are corrected for background responses in the negative control condition. The Mann-Whitney *U* test was used to assess the differences in frequencies of cytokine- or perforin-expressing $CD8^+$ T cells between infants vaccinated at birth (open dots/bars) and 6 weeks of age (closed dots/bars).

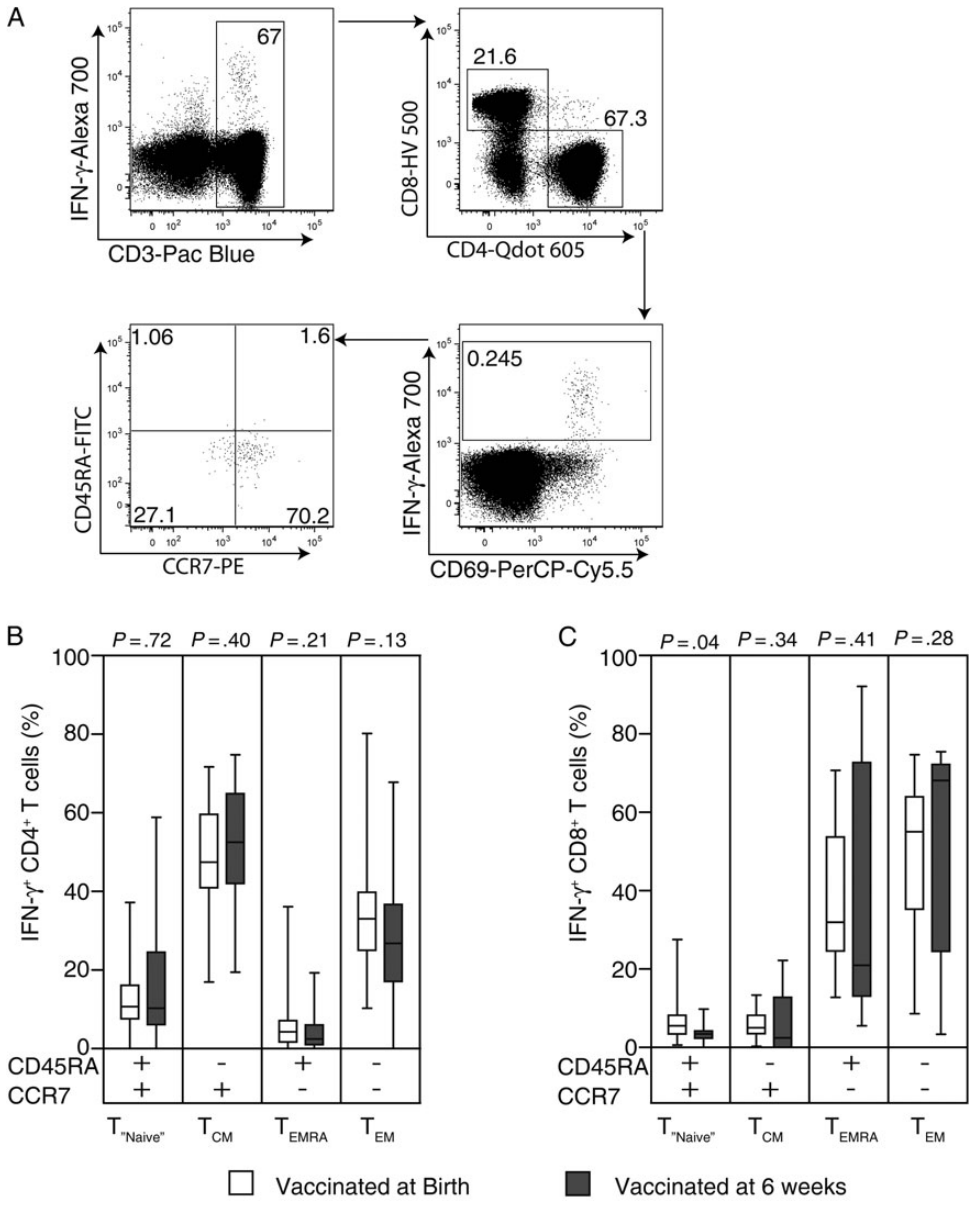

Figure 3. Memory phenotype of BCG-specific CD4$^+$ and CD8$^+$ T cells identified in the short-term whole blood intracellular cytokine assay. For the gating strategy to analyze the expression of CD45RA and CCR7 memory markers on BCG-specific CD4$^+$ T cells, CD3$^+$ cells were separated into CD4$^+$ and CD8$^+$ T cells. Then, cells expressing interferon γ (IFN-γ) or CD69, both considered specific to BCG stimulation, were selected from the CD4$^+$ T cells. Finally, CD45RA and CCR7 expression were assessed from these specific cells (*A*). A similar analysis strategy was used for CD8$^+$ T cells. Box and whiskers plots show the frequencies of BCG-specific IFN-γ$^+$ CD4$^+$ T cells (*B*) and IFN-γ$^+$ CD8$^+$ T cells (*C*) expressing the CD45RA and CCR7 memory markers singly or in combination. The open and closed bars represent the infants vaccinated at birth and 6 weeks of age, respectively. The horizontal line represents the median, the boxes represent the interquartile range, and the whiskers represent the 10th and 90th percentiles. The Mann-Whitney *U* test was used to assess the differences in proportions of memory phenotypes of specific CD4$^+$ and CD8$^+$ T cells between the 2 groups.

M. tuberculosis involves perforation of the cell membrane of an infected cell to permit entry of cytolytic granzymes that may directly kill *M. tuberculosis* or infected cells [34]. Rahman et al showed that, following vaccination of nonhuman primates with a recombinant BCG vaccine expressing a pore-forming toxin and the *M. tuberculosis* antigens Ag85A, Ag85B, and TB10.4, which was then boosted with an adenovirus 35 (rAd35) vaccine vector encoding the same *M. tuberculosis* antigens, better protection against *M. tuberculosis* challenge correlated with greater frequencies of vaccination-induced perforin-expressing T cells [35]. In our study, greater frequencies of BCG-specific CD4$^+$ and CD8$^+$ T cells expressing either IFN-γ and coexpressing IFN-γ and perforin in the group vaccinated at birth, compared with the group vaccinated at 6 weeks of age, could be a reflection of more effective

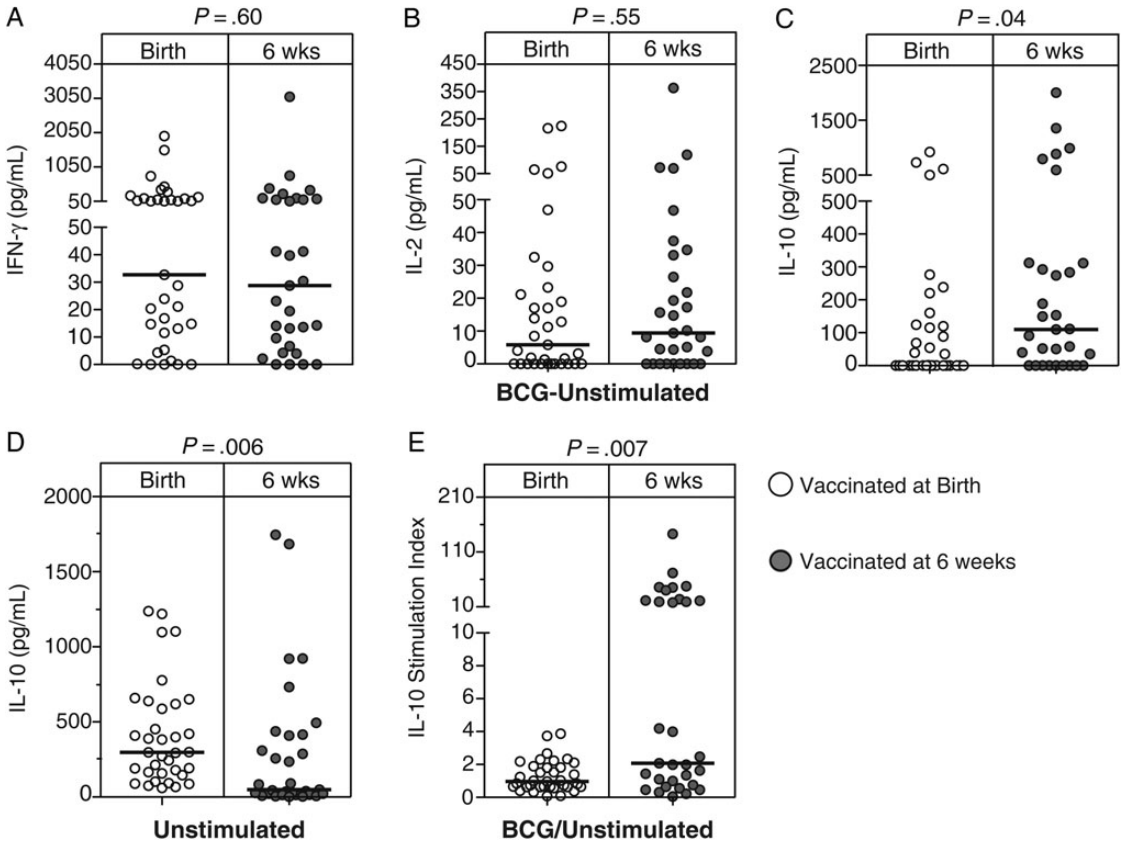

Figure 4. BCG-specific cytokine levels in plasma. The scatterplots show levels of 3 different cytokines measured by multiplex bead array in plasma collected after whole blood was incubated with BCG for 7 hours. The following cytokines are shown: interferon γ (IFN-γ; *A*), interferon 2 (IL-2; *B*), and interleukin 10 (IL-10; *C*). All values are corrected for unstimulated levels (BCG-unstimulated) implies the response detected in the unstimulated sample was subtracted from that of the stimulated sample. IL-10 levels of the unstimulated samples and the ratio of stimulated divided by unstimulated IL-10 levels are shown in panels *D* and *E*, respectively. The horizontal lines represent the median frequencies. The Mann-Whitney *U* test was used to assess for differences between the cytokine levels in the 2 groups.

vaccine-take in the former group. However, we cannot speculate about clinical relevance of this observation in human infants in terms of protection against tuberculosis [14]; BCG-induced correlates of protection are not known.

In chronic viral infections, persistence of antigen is associated with induction of effector memory cells; these cells may be functionally defined by their ability to produce IFN-γ [36]. The current study and previous studies from our laboratory have shown BCG-specific responses in infants are dominated by IFN-γ–producing $CD4^+$ and $CD8^+$ T cells [12, 21]. On the basis of this definition, it would appear as if vaccination at birth is more likely to induce an effector phenotype, compared with delayed vaccination. However, we have shown here and previously [37] that most BCG-induced IFN-γ–expressing cells have a T_{CM} surface phenotype—the cells express the surface molecules required by classical T_{CM} to home to lymph nodes [38]. The functional implication of the apparent discrepancy between functional and phenotypic definitions of T-cell populations induced by BCG is the focus of ongoing research; this may be important, given that BCG is likely to remain a prime vaccine for quite some time.

We evaluated levels of BCG-induced IL-10 on the basis of studies showing that this cytokine may attenuate Th1 responses. For example, in *M. tuberculosis*–susceptible CBA/J mice, antibody blockade of IL-10R during BCG vaccination resulted in an enhanced BCG-specific IFN-γ response and better protection against subsequent *M. tuberculosis* challenge [39]. We have shown that infants who received BCG vaccine at 6 weeks of age had greater levels of BCG-specific IL-10 production, compared with infants vaccinated at birth. However, we showed no strong negative correlation between IL-10 levels and $CD4^+$ T-cell IFN-γ (Data not shown). The greater BCG-induced IL-10 levels observed in the infants vaccinated at 6 weeks of age may potentially result in attenuation of mycobacteria-specific Th1 immune responses during infection with *M. tuberculosis* in infants vaccinated at 6 weeks of age, compared with those vaccinated at birth.

We expanded the specific memory T cells in a long-term proliferation assay and measured the capacity of the specific

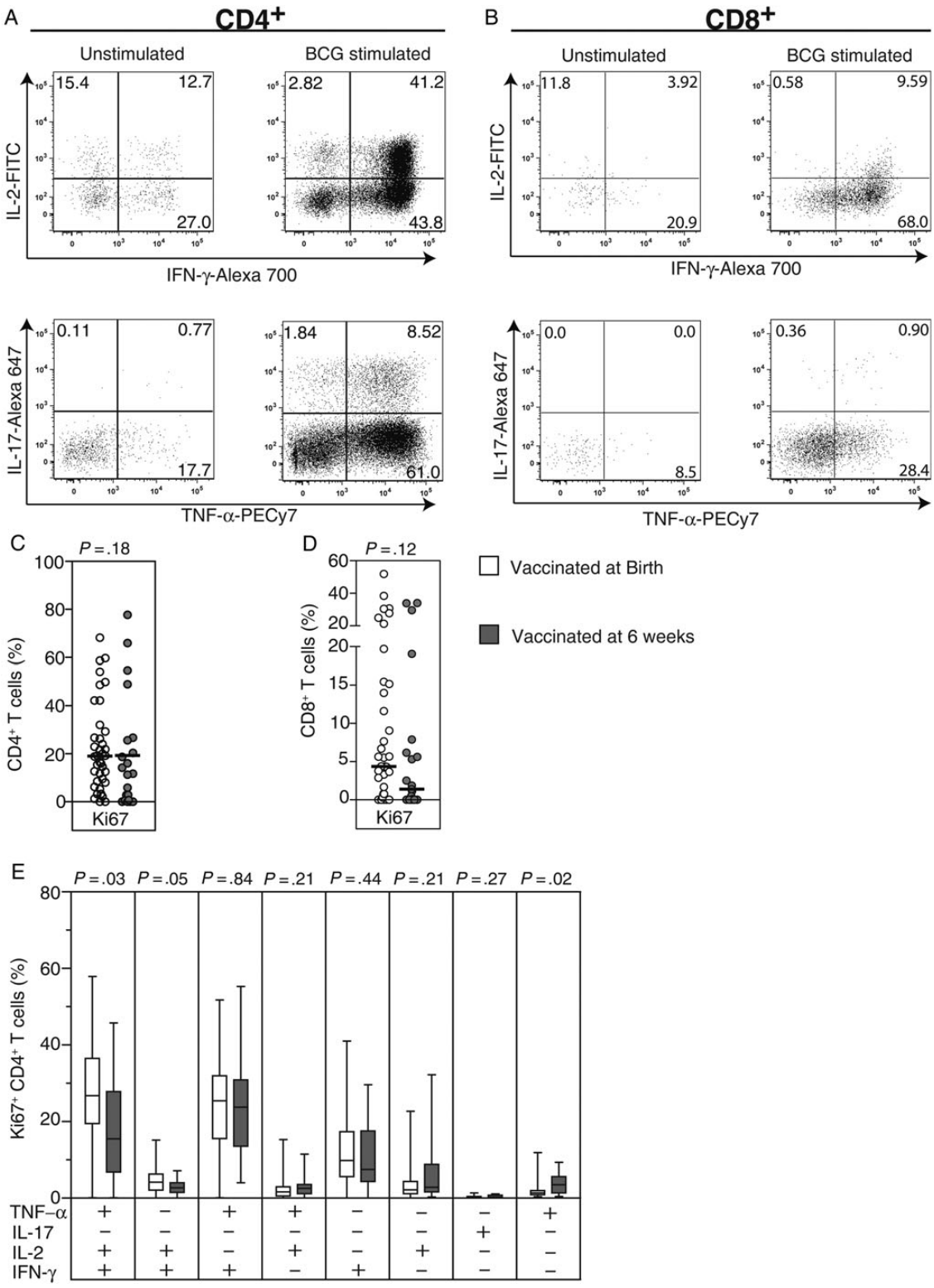

Figure 5. Capacity of BCG-specific $CD4^+$ and $CD8^+$ T cells to proliferate and produce cytokines. Representative flow cytometry data of cytokine expression by proliferating $CD8^-$ (herein referred to as $CD4^+$; *A*) and $CD8^+$ (*B*) T cells in a control (unstimulated) sample (top row) or BCG-stimulated sample (bottom row) from a 9-month-old infant who received BCG vaccine at birth. Scatterplots depict frequencies of proliferating $CD4^+$ (*C*) and $CD8^+$ (*D*) T cells. The capacity of the proliferating $CD4^+$ T cells to express interleukin 2 (IL-2), interleukin 17 (IL-17), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) alone or in combination is shown in (*E*). In the scatterplots, the horizontal lines represent the median frequencies. Finally, proportions of distinct subsets assessed by the cytokine coexpression of the proliferating $CD4^+$ T cells were analyzed (*D*). The open and closed bars or dots represent the infants vaccinated at birth and 6 weeks of age, respectively. The Mann-Whitney *U* test was used to assess for differences between the 2 groups.

proliferating cells to secrete cytokines. Proliferating $CD4^+$ T cells from the infants vaccinated at birth showed greater capacity to coexpress IL-2, IFN-γ, and TNF-α. This was surprising, given our findings from the short-term assay, which indicated that BCG vaccination at birth was more likely to induce cells able to produce IFN-γ alone. It is possible that greater IL-10 levels observed in the infants vaccinated at 6 weeks of age may have attenuated the expansion of polyfunctional $CD4^+$ T cells. Polyfunctional $CD4^+$ T cells are thought to be important in tuberculosis immunity [40] and are therefore routinely measured in clinical trials assessing the immunogenicity of novel tuberculosis vaccines [41–43]. However, a clinical study from our laboratory reported no association between greater proportions of BCG-specific polyfunctional T cells and the risk of developing tuberculosis [14].

What are the possible explanations for the differences observed in the BCG-induced T-cell immunity between the 2 groups of infants? First, we observed that a higher proportion of infants vaccinated at 6 weeks of age were from families of lower social economic status, compared with infants vaccinated at birth (Table 1). Lower social economic status may negatively impact health-seeking behavior, nutrition, helminth exposure, and other factors, possibly leading to altered immune responses [44]. We showed no difference in the frequencies of BCG-specific IFN-γ–expressing $CD4^+$ and $CD8^+$ T-cell responses in infants from families with a higher income, compared with those from families in the lower income categories (Supplementary Figure 2) A and B. Nevertheless, we observed a significant association between the frequency of IFN-γ–expressing $CD4^+$ T cells and household income (Supplementary Table 1). Second, we speculate that the lower Th1 responses observed in infants vaccinated at 6 weeks of age may be due to coadministration of BCG vaccine with alum, which is present in other vaccines [45]. Alum induces predominantly Th2 immune responses [46], which might have attenuated the BCG-induced Th1 responses [47].

We assessed the immune responses at 9 months of age. We do not know whether other differences in BCG-induced immunity may exist at earlier time points after vaccination. Our study setting limited us from accessing the participants at the peak of BCG-induced T-cell immune response [37]. However, in a clinical study of delayed BCG vaccination, Kagina et al showed that the greatest difference in BCG-induced T-cell immunity was at 1 year of age and not at the peak time point after the vaccination [21]. Differences between our study findings and those previously reported may be explained by many factors. First, we used a cross-sectional design, whereas previous studies were randomized controlled trials [18, 19, 21]. Second, diverse assays were used to measure outcomes in previous studies. For example, we used BCG as antigen, as did Burl et al [18], while others used PPD [19]. Third, the duration of incubation and choice of T-cell outcomes differed [18, 19, 21]. Finally, environmental and genetic variation may impact the mycobacterial immune response, as recently demonstrated by diverse patterns of antigen recognition and cytokine production in *M. tuberculosis*–infected persons from the Gambia, Uganda, and South Africa [48]. Environmental factors may include exposure to helminths [49], other infections, and diverse nutritional practices [44]. Genetic variation across Africa is well documented in descriptions of single-nucleotide polymorphisms [50], which may affect the immune response.

In summary, our findings appear to support WHO recommendations that infants in areas of high endemicity, such as Uganda, be vaccinated as soon as possible after birth. In this setting, delaying vaccination does not appear to hold vaccine-induced immunological advantages.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by the European and Developing Countries Clinical Trials Partnership (EDCTP CT grant 2005. 32090.003).

Potential conflict of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Fine PE, Carneiro IAM, Milstien JB. Issues relating to the use of BCG immunisation programmes. WHO/V&B/99.23. Geneva: World Health Organization, **1999**.
2. Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet **2006**; 367:1173–80.
3. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis **2011**; 204:245–52.
4. World Health Organization. BCG vaccine. WHO position paper. Wkly Epidemiol Rec **2004**; 79:27–38.
5. World Health Organization. WHO global tuberculosis control. **2011**.
6. Fadnes LT, Nankabirwa V, Sommerfelt H, Tylleskar T, Tumwine JK, Engebretsen IM. Is vaccination coverage a good indicator of age-appropriate vaccination? A prospective study from Uganda. Vaccine **2011**; 29:3564–70.
7. Nankabirwa V, Tumwine JK, Tylleskar T, Nankunda J, Sommerfelt H. Perinatal mortality in eastern Uganda: a community based prospective cohort study. PLoS One **2011**; 6:e19674.
8. Ministry of Health, Uganda. Promotion of Immunisation in Uganda: Booklet for Leaders. Vaccine Resource Library 2002. http:\\www.path.org/vaccineresources/files/Uganda_immunisation_advocacy.pdf. Accessed 11 November 2013.
9. Hoft DF, Blazevic A, Stanley J, et al. A recombinant adenovirus expressing immunodominant TB antigens can significantly enhance BCG-induced human immunity. Vaccine **2012**; 30:2098–108.

10. Murray RA, Mansoor N, Harbacheuski R, et al. Bacillus Calmette Guerin vaccination of human newborns induces a specific, functional CD8+ T cell response. J Immunol **2006**; 177:5647–51.
11. Smith SM, Malin AS, Pauline T, et al. Characterization of human Mycobacterium bovis bacille Calmette-Guerin-reactive CD8+ T cells. Infect Immun **1999**; 67:5223–30.
12. Soares AP, Scriba TJ, Joseph S, et al. Bacillus Calmette-Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. J Immunol **2008**; 180:3569–77.
13. Semple PL, Watkins M, Davids V, et al. Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth. Clin Dev Immunol **2011**; 2011:438463.
14. Kagina BM, Abel B, Scriba TJ, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. Am J Respir Crit Care Med **2010**; 182:1073–9.
15. Corbett NP, Blimkie D, Ho KC, et al. Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells. PLoS One **2010**; 5:e15041.
16. Sadeghi K, Berger A, Langgartner M, et al. Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. J Infect Dis **2007**; 195:296–302.
17. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. Science **2002**; 296:490–4.
18. Burl S, Adetifa UJ, Cox M, et al. Delaying bacillus Calmette-Guerin vaccination from birth to 4 1/2 months of age reduces postvaccination Th1 and IL-17 responses but leads to comparable mycobacterial responses at 9 months of age. J Immunol **2010**; 185:2620–8.
19. Hussey GD, Watkins ML, Goddard EA, et al. Neonatal mycobacterial specific cytotoxic T-lymphocyte and cytokine profiles in response to distinct BCG vaccination strategies. Immunology **2002**; 105:314–24.
20. Marchant A, Goetghebuer T, Ota MO, et al. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. J Immunol **1999**; 163:2249–55.
21. Kagina BM, Abel B, Bowmaker M, et al. Delaying BCG vaccination from birth to 10 weeks of age may result in an enhanced memory CD4 T cell response. Vaccine **2009**; 27:5488–95.
22. Hanekom WA, Hughes J, Mavinkurve M, et al. Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies. J Immunol Methods **2004**; 291:185–95.
23. Waldrop SL, Pitcher CJ, Peterson DM, Maino VC, Picker LJ. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J Clin Invest **1997**; 99:1739–50.
24. Soares A, Govender L, Hughes J, et al. Novel application of Ki67 to quantify antigen-specific in vitro lymphoproliferation. J Immunol Methods **2010**; 362:43–50.
25. Roederer M, Nozzi JL, Nason MX. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A **2011**; 79A:167–74.
26. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature **1999**; 401:708–12.
27. Rook GA. Th2 cytokines in susceptibility to tuberculosis. Curr Mol Med **2007**; 7:327–37.
28. Schreiber T, Ehlers S, Heitmann L, et al. Autocrine IL-10 induces hallmarks of alternative activation in macrophages and suppresses antituberculosis effector mechanisms without compromising T cell immunity. J Immunol **2009**; 183:1301–12.
29. Combadiere B, Boissonnas A, Carcelain G, et al. Distinct time effects of vaccination on long-term proliferative and IFN-gamma-producing T cell memory to smallpox in humans. J Exp Med **2004**; 199:1585–93.
30. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol **1984**; 133:1710–5.
31. Cellerai C, Harari A, Vallelian F, Boyman O, Pantaleo G. Functional and phenotypic characterization of tetanus toxoid-specific human CD4 + T cells following re-immunization. Eur J Immunol **2007**; 37:1129–38.
32. Bogunovic D, Byun M, Durfee LA, et al. Mycobacterial disease and impaired IFN-gamma immunity in humans with inherited ISG15 deficiency. Science **2012**; 337:1684–8.
33. Ottenhoff TH, de Boer T, Verhagen CE, Verreck FA, van Dissel JT. Human deficiencies in type 1 cytokine receptors reveal the essential role of type 1 cytokines in immunity to intracellular bacteria. Microbes Infect **2000**; 2:1559–66.
34. Thiery J, Keefe D, Boulant S, et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. Nat Immunol **2011**; 12:770–7.
35. Rahman S, Magalhaes I, Rahman J, et al. Prime-boost vaccination with rBCG/rAd35 enhances CD8(+) cytolytic T-cell responses in lesions from Mycobacterium tuberculosis-infected primates. Mol Med **2012**; 18:647–58.
36. Harari A, Vallelian F, Pantaleo G. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. Eur J Immunol **2004**; 34:3525–33.
37. Soares AP, Kwong Chung CK, Choice T, et al. Longitudinal changes in CD4(+) T-cell memory responses induced by BCG vaccination of newborns. J Infect Dis **2013**; 207:1084–94.
38. Campbell JJ, Butcher EC. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. Curr Opin Immunol **2000**; 12:336–41.
39. Pitt JM, Stavropoulos E, Redford PS, et al. Blockade of IL-10 signaling during bacillus Calmette-Guerin vaccination enhances and sustains Th1, Th17, and innate lymphoid IFN-gamma and IL-17 responses and increases protection to Mycobacterium tuberculosis infection. J Immunol **2012**; 189:4079–87.
40. Forbes EK, Sander C, Ronan EO, et al. Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. J Immunol **2008**; 181:4955–64.
41. Abel B, Tameris M, Mansoor N, et al. The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. Am J Respir Crit Care Med **2010**; 181:1407–17.
42. Sander CR, Pathan AA, Beveridge NE, et al. Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in Mycobacterium tuberculosis-infected individuals. Am J Respir Crit Care Med **2009**; 179:724–33.
43. Scriba TJ, Tameris M, Smit E, et al. A phase IIa trial of the new tuberculosis vaccine, MVA85A, in HIV- and/or Mycobacterium tuberculosis-infected adults. Am J Respir Crit Care Med **2012**; 185:769–78.
44. Rodriguez L, Gonzalez C, Flores L, Jimenez-Zamudio L, Graniel J, Ortiz R. Assessment by flow cytometry of cytokine production in malnourished children. Clin Diagn Lab Immunol **2005**; 12:502–7.
45. Ota MO, Odutola AA, Owiafe PK, et al. Immunogenicity of the tuberculosis vaccine MVA85A is reduced by coadministration with EPI vaccines in a randomized controlled trial in Gambian infants. Sci Transl Med **2011**; 3:88ra56.
46. Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine **2008**; 26:2350–9.
47. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature **1996**; 383:787–93.
48. Black GF, Thiel BA, Ota MO, et al. Immunogenicity of novel DosR regulon-encoded candidate antigens of Mycobacterium tuberculosis in three high-burden populations in Africa. Clin Vaccine Immunol **2009**; 16:1203–12.
49. Elias D, Wolday D, Akuffo H, Petros B, Bronner U, Britton S. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. Clin Exp Immunol **2001**; 123:219–25.
50. Shey MS, Randhawa AK, Bowmaker M, et al. Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide- and mycobacteria-induced interleukin-6 secretion. Genes Immun **2010**; 11:561–72.