



BCG-specific T cell proliferation and cytotoxic capacity in infants at risk of tuberculosis disease, following newborn BCG vaccination

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Declaration

I, *Alana Keyser*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Signed by candidate

Date: 10 May 2014

Acknowledgements

I would like to express my sincerest gratitude to:

My supervisor and mentor, Prof. Willem Hanekom. Thank you for placing your trust in me and granting me multiple opportunities which allowed me to grow both professionally and personally. Your teachings extend way beyond the pages of this thesis. I proudly regard myself as a product of the “School of Hanekom” and will be forever grateful for the privilege to have trained with one of the leading minds in TB vaccine immunology.

My co-supervisor, Prof. Thomas Scriba. Thank you for your availability and willingness to see me through to the end of this thesis; words cannot express my gratitude. I am equally honored to have been guided through this process by one of the most brilliant, and yet, modest scientists currently associated with IDM, UCT.

Ms. Jane Hughes who have not only been a guiding hand in my career but also laid the groundwork for these studies. Your leadership has been inspirational and without you, this degree would not have been a possibility. Thank you very much, Lady Jane.

Dr. Benjamin Kagina and Dr. Brian Abel. We have not had face-to-face contact in years, but I will be forever grateful for not only sharing your mounts of knowledge but for also enstilling confidence and humality in me. I feel blessed that fate crossed our livepaths.

My husband, Marquin Crotz. Thank you for your patience and constant support. Your love and positivity have been motivational.

My mom, Esther Keyser. Life’s difficulties has knocked us quite a few times, yet you have always remained a pillar of strength. Thank you for always keeping the faith and remaining positive when all seemed dark and dismal at times. Thank you for being my mother and friend.

My sons, Connor and Skye. With this chapter of my life at an end, I can now divert my attention to watch you grow and shape into the confident, yet humble men, I hope for you to become. And don’t ever forget my hug andkiss!

All my colleagues who have contributed to the studies in this thesis, as well as the following funders without whom these studies would not have been possible: Bill and Melinda Gates Foundation, EDCTP, Aeras and the National Institutes of Health.

I would like to dedicate this thesis to my father,

Alfred David Keyser

(20 September 1950 – 12 December 2011)

Your departure from our lives has been unexpectedly premature. Your absence is still felt with the greatest sadness every day as my lifepath follows in the footprints of yours. Yet, your words of encouragement carried me through to the end of this thesis. Miss you dearly daddy!

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Abbreviations

⁵¹ Cr	Chromium
APC	Antigen presenting cells
B cell	B lymphocytes
BCG	Bacille Calmette-Guerin
Bcl-6	B-cell lymphoma 6
BrdU	5-bromo-2-deoxyuridine
CCR	Chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CO ₂	Carbon dioxide
CR	Complement receptor
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTO	Cell Tracker Orange
CXCR	C-X-C chemokine receptor
Cy	Cyanine
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	Deoxyribonucleic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colon-stimulating factor
H ₂ SO ₄	Sulfuric acid
HBV	Hepatitis B virus
HiB	<i>Haemophilus influenzae</i> type B
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

ICOS	Inducible T-cell COStimulator
ICS	Intracellular cytokine stain
IFN- γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iTreg	Induced regulatory T cell
kDa	kiloDalton
LAM	Lipoarabinomannan
LCMV	Lymphocytic choriomeningitis virus
LTBI	Latent tuberculosis infection
<i>M.bovis</i>	<i>Mycobacterium bovis</i>
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MACPF	Membrane attack complex protein family
MAD	Median absolute deviations
ManLAM	Mannosylated lipoarabinomannan
MES	2-[N-Morpholino] ethanesulfonic acid hydrate
MFI	Mean fluorescence intensity
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
M ϕ	Macrophage
ng	Nanogram
NK T cell	Natural killer T cell
nm	Nanometer
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
NOS	Nitric oxide synthase
nTreg	Natural regulatory T cell
OG	Oregon Green
OPV	Oral polio vaccine

PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
pg	Picogram
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative of <i>M.tb</i>
PRR	Pattern recognition receptors
Qdot	Quantum dot
RD	Region of difference
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROR γ t	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute medium
SATVI	South African Tuberculosis Vaccine Initiative
SEB	Staphylococcus enterotoxin B
Sulfo-NHS	N-hydroxysulfosuccinimide
TB	Tuberculosis
TCR	T cell receptor
Tfh	T follicular helper cell
TGF- β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
US	United States
V	Volt
Vivid	LIVE/DEAD [®] Fixable Violet Dead Cell Stain
WBA	Whole blood assay
WHO	World health organisation
$\gamma\delta$ T cell	Gamma/delta T cell
μ g	Microgram
μ L	Microlitre

μM

Micromolar

μm

Micron

Summary

BCG is the only vaccine against tuberculosis and has been used for over 90 years. BCG efficacy is variable, especially in countries with high TB prevalence, where over a million deaths due to tuberculosis, are still reported annually. New TB vaccines are under development to either replace or boost the BCG vaccine. However, our understanding of the immune response required for protection against TB disease, remains inadequate. Identification of a protective immune response is only possible in a clinical trial of an efficacious vaccine, allowing comparison of vaccine-induced immune responses in protected and unprotected individuals. In the absence of such a vaccine, as is the case with TB, we can only explore biomarkers of risk of disease.

The most commonly measured outcomes of anti-mycobacterial immunity in clinical trials, specific Th1 cells, are typically thought to be protective in TB. However, to date, human mycobacteria-specific Th1 responses have not correlated with risk of TB disease. New approaches are urgently required to identify other factors at play in conferring protection against TB.

In this thesis, we explored BCG-specific cytotoxic T cells as candidate correlates of risk of TB disease in BCG-vaccinated infants. We hypothesized that reduced production of cytotoxic molecules by T cells in response to BCG are associated with risk of developing TB disease. We designed a case/control study nested within a large trial of newborn BCG-vaccination. Blood was collected at 10 weeks and infants, were followed up for two years. We compared outcomes in infants ultimately diagnosed with TB (at risk of TB disease) and two groups of healthy infants (not at risk of TB disease), the first group had household contact with TB cases, the second group were randomly selected from the community, which is endemic for TB. Amongst these groups, we designated a training and a test cohort to allow validation of candidate correlates of risk of TB.

We observed increased frequencies of BCG-specific T cells expressing cytotoxic molecules at 10 weeks of age in infants at risk of TB disease compared with infants not at risk in the training cohort. However, these

findings could not be validated in the test cohort. We also measured proliferative potential of BCG-specific T cells and Th1 cytokine production by proliferating cells in these groups. We found no differences in BCG-induced capacity of T cells to proliferate, and to express Th1 cytokines between the infant groups.

We investigated cytotoxic T cell secretion of cytotoxic molecules and the capacity of these cells to kill BCG-infected monocytes. We optimised a flow cytometry-based cellular assay that measured monocyte killing by BCG-specific cytotoxic T cells. Lastly, we optimised a flow cytometric bead array assay that quantifies soluble molecules in culture supernatants.

In summary, our data suggest that increased frequencies of BCG-specific cytotoxic T cells, with capability to kill mycobacteria-infected cells, may be associated with risk of TB disease. However, this was not validated in a second, test cohort. Further studies may explain the paradoxical nature of this finding, and elude to the underlying mechanisms involved.

Chapter 1: General Introduction

1.1. Epidemiology of TB disease

Mycobacterium tuberculosis (M.tb), the causative pathogen of tuberculosis, still remains responsible for 1.3 million deaths per year [1]. A third of the world's population is estimated to be infected with *M.tb* of which approximately 10% will develop active tuberculosis (TB) disease [2]. South Africa ranks among the three countries with the highest burden of tuberculosis in the world. TB incidence rate in South Africa was estimated at 925 per 100 000 in 2012 with 2% of reported new cases in children 15 years and younger [1]. This is coupled to a TB mortality rate of 51 per 100 000. Clearly, combating TB disease remains unsuccessful despite a global vaccination coverage of 89% of newborns in TB endemic countries with Bacille Calmette-Guerin (BCG), the only licensed TB vaccine [3].

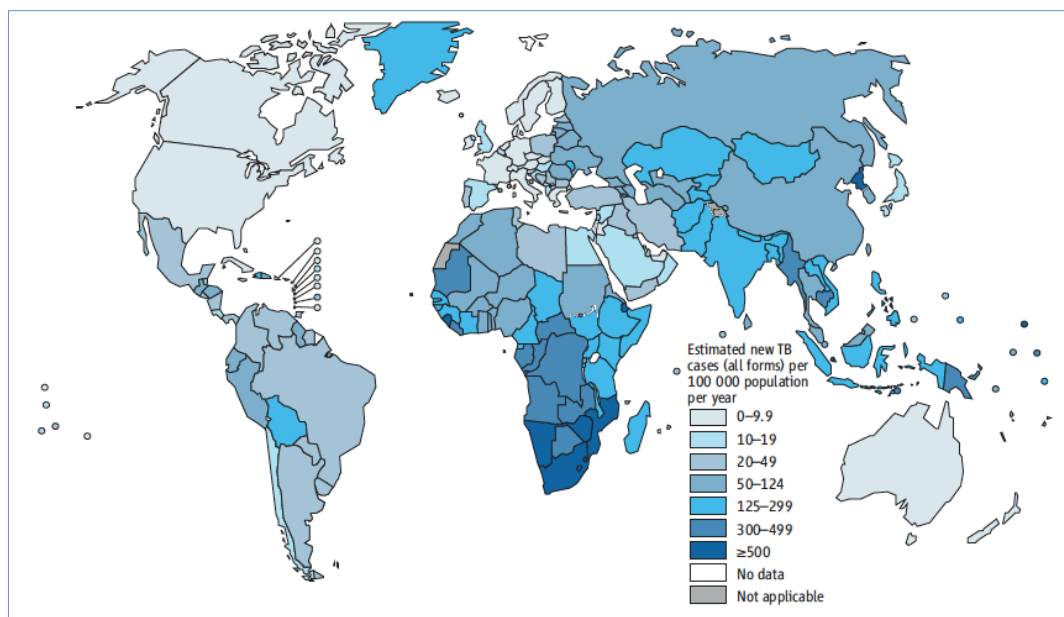


Figure 1.1. Global presentation of TB incidence rates as estimated in 2012 . (From WHO Global Tuberculosis Report 2013 [1]).

1.2. TB Pathogenesis

TB is primarily a chronic disease of the lung. Infection is established through inhalation of *M.tb*-containing aerosols expelled by TB diseased individuals through coughing. There are three possible outcomes after inhalation of *M.tb* (Figure 1.2). An indeterminate group of individuals appear to clear the infection as these individuals remain unsensitized to *M.tb*-specific antigens, even after known exposure to TB patients. About 90% of individuals do

establish infection but do not develop TB disease, a phenomenon known as latent TB infection (LTBI). In these individuals, the pathogen is maintained within granulomas, highly organized structures comprising an array of immune cells concentrated around a central region of infected macrophages [4]. In addition, about 10% of infected individuals progress to disease due to loss of control of *M.tb* in the lesion so that the pathogen can replicate and escape the granuloma. The pathogen may spread to other sites of the lung and the individual becomes diseased, displaying symptoms such as coughing, weight-loss and night sweats [5]. Such individuals become infectious and spread the pathogen. In more severe cases, the infection can spread beyond the lungs leading to disseminated disease such as TB meningitis.

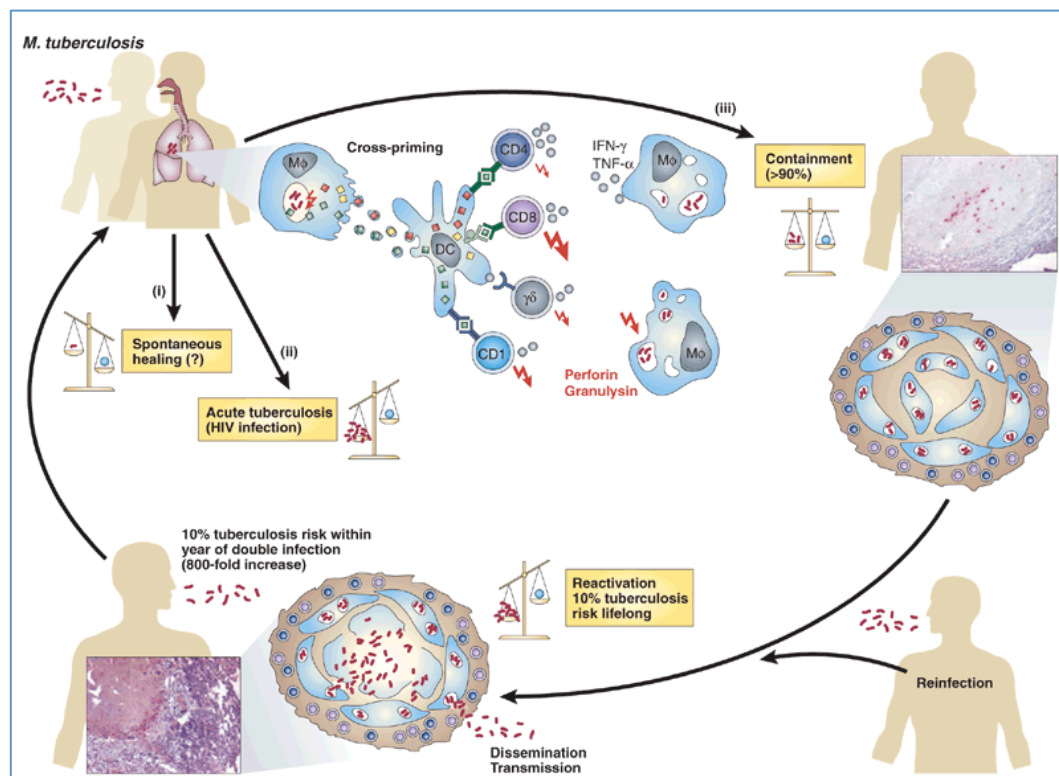


Figure 1.2. Three infection outcomes of *Mycobacterium tuberculosis*. (i) A small proportion of individuals are able to clear *M.tb* before infection is established. (ii) Some individuals, such as HIV+ patients, can develop acute TB disease upon infection. (iii) The majority of individuals are able to contain *M.tb* infection, however 10% of these may develop disease due to a weakened immune system, becoming infectious if untreated. (From Kaufmann *et al*, Nature Medicine Reviews, 2005 [6]).

1.3. The host immune response to *M.tb*

1.3.1. Recognition and Uptake of M.tb by APCs

The *M.tb* cell wall consists of an array of components including polysaccharides, lipids and proteins. This provides a selection of pathogen associated molecular patterns (PAMPs) for recognition and uptake of *M.tb* by various pattern recognition receptors (PRR). PRRs, such as toll-like receptors (TLRs), are expressed either on the surface of phagocytic cells, granulocytes, macrophages (M ϕ) and dendritic cells (DCs) or intracellularly. TLRs are a group of thirteen mammalian PRRs able to recognize microbial molecules. Of these, only TLR-1 to TLR-10 have been identified in humans, with TLR-11, -12 and -13 being restricted to mice [7]. TLR-2 recognises and binds the glycolipid lipoarabinomannan (LAM), which is a major component of the cell wall of *M.tb* [8]. TLR-2 can also form functional heterodimeric pairs with TLR-1 or TLR-6 inducing macrophage activation upon recognition of mycobacterial lipopeptide [9] [10]. Mycobacterial lipopolysaccharides and β -glucans are recognised by TLR-4 and the c-type lectin receptor, Dectin-1, respectively [11], [12]. Dendritic cells are also equipped with an additional c-type lectin receptor, DC-SIGN, for detection of β -glucan, ManLAM and lipomannans. Through the recognition of ManLAM, DC-SIGN also mediates internalisation of *M.tb* [13]. In addition, intracellular recognition is mediated by the endocytic TLR-9 and the cytoplasmic NOD-like receptor, NOD-2, which detects bacterial CpG DNA and peptidoglycans, respectively [14].

Phagocytosis of *M.tb* is mainly mediated by three receptors expressed on the surface of the phagocytic cell. Complement receptors (CR1 and CR3) mediate engulfment of *M.tb* opsonised with complement [15]. Uptake of non-opsonised *M.tb* is mediated through another c-type lectin receptor, mannose receptor [16], and scavenger receptors which binds mannose and lipopolysaccharides, respectively. Interestingly, the fate of *M.tb* appears to be dependent on the PRR-PAMP interactions and binding of these PRRs appears to elicit different immune responses to *M.tb* [17]. For example, engagement of the mannose receptor, induces an anti-inflammatory response and blocks phagolysosome fusion promoting intracellular survival of *M.tb* [17] [18] [20].

1.3.2. Innate mechanisms of killing *M.tb*

In the lung, *M.tb* is taken up by resident alveolar M ϕ through phagocytosis where it resides within phagosomes. Typically, phagosomes undergo maturation through acidification in order to fuse with lysosomes. Within the phagolysosome *M.tb* comes under the attack of degradative enzymes that are active at low pH. In addition to lysosomal/proteolytic enzymes, infected M ϕ also produce free radicals that are detrimental to cell survival. Infected M ϕ increase oxygen consumption, undergoing oxidative stress leading to the subsequent production of microbicidal reactive oxygen intermediates (ROI). Activation of murine M ϕ by IFN- γ and TNF- α also trigger the production of reactive nitrogen intermediates (RNI) which have been shown to kill mycobacteria [21].

Much of the success of *M.tb* to survive intracellularly is ascribed to its ability to arrest phagolysosome formation and phagosome escape [22]. However, M ϕ are able to circumvent *M.tb*-induced phagosome arrest through autophagy, usually a homeostatic process through which cells recycle essential nutrients during starvation. Autophagy involves the formation of double-membrane vesicles around a specific organelle in the cell, which is then targeted for degradation through lysosomal activity. Induction of autophagy through IFN- γ has been shown to increase acidification and promote maturation of mycobacteria-containing phagosomes [23]. A vitamin D-dependant antimicrobial pathway has also been identified in M ϕ and neutrophils. Through this pathway, vitamin D increases the production of the microbicidal peptide, cathelicidin, which in turn induces autophagy [24]. Cathelicidin has been shown to have a direct killing effect on *BCG* and *M.tb*, possibly through the disruption of the mycobacterial cell wall [25], [26].

1.3.3. Engagement of the host adaptive immune system

In addition to the innate mechanisms described above, M ϕ and DCs also engage cells of the adaptive immune system, comprising B lymphocytes (B cells) and T lymphocytes (T cells) [27]. B cells primarily generate antibodies while T cells produce cytokines and cytotoxins to combat infection. Initial engagement of these cells during infection, ultimately leads to immunological memory, the hallmark of the adaptive immune system.

Establishing pathogen-specific immunological memory is the aim of vaccination and essential to effectiveness of vaccines, including BCG, to prevent disease development. Initiation of an adaptive immune response, however, requires antigen recognition and presentation by antigen presenting cells (APC).

1.3.4. Antigen processing and presentation

B cells can function as both an APC and as an effector cell to establish immunological memory. Cell-surface expression of B-cell receptors or immunoglobulins allow B cells to recognize and engulf specific antigens. These include proteins, lipids, polysaccharides, nucleic acids and some chemicals termed haptens associated with extracellular pathogens. B cells are able to process these antigens, which are loaded onto MHC class II molecules for presentation to CD4 T cells. A specific group of Th cells, T follicular helper cells (Tfh), induce maturation of B cells to become plasma cells which produce antigen-specific antibody.

Unlike B cells, T cells require APCs for antigen presentation of intracellular pathogen. APCs utilize two pathways of antigen processing and ultimate presentation to T cells. An exogenous pathway is used to present antigens from phagocytosed pathogens, which are retained within a phagolysosome for degradation. MHC class II containing vesicles, derived from the Golgi apparatus, fuse with the phagolysosome to allow binding of pathogenic peptides by MHC class II molecules. The peptide-bound MHC molecules are transported to the cell surface for presentation of antigen to CD4 T cells. The MHC class II exogenous pathway is predominantly used to present antigens of mycobacteria retained within the phagolysosome. An endogenous pathway entails degradation of intracellular proteins, including antigens from pathogens in the cytoplasm. Typically, viral proteins are degraded into peptides via this pathway through a cytoplasmic protein complex known as the proteasome. The peptide is transported to the endoplasmic reticulum where it is loaded onto major histocompatibility complex (MHC) class I molecules and the complex is then moved to the cell surface through the Golgi apparatus. At this point, peptides bound to MHC

class I molecules (MHCI) are presented to a T lymphocyte subset called CD8 T cells.

Mycobacteria can induce the endogenous MHCI antigen presentation pathway through cross-priming [28][29]. This involves induction of programmed cell death leading to the formation of apoptotic vesicles which are taken up by bystander DC. The phagocytosed pathogen product is translocated from the phagosome, possibly through the action of saposin, to the cytosol for loading onto MHCI. However, *M.tb* has been shown to block apoptosis of host M ϕ through induction of anti-apoptotic proteins [30]. These evasion tactics are examples of a large number of mechanisms by which *M.tb* has managed to manipulate the host immune system to promote its survival [31]. CD4 and CD8 T cells are regarded as conventional T cells characterized by surface-expression of the $\alpha\beta$ T cell receptors. In addition, APCs are also able to present microbial lipids to a group of unconventional T cells through engagement of the MHCI-related molecule, CD1. Lipids are loaded onto CD1 molecules for presentation to CD1-restricted T cells, including natural killer (NK T cells) and gammadelta T cells ($\gamma\delta$ T cells). In essence, antigen processing and presentation enables APCs to activate T cells of the adaptive immune system if they fail to resolve the infection.

1.3.5. Establishing immunological memory

T cell priming primarily takes place in the lymph node. Pathogens taken up by immature DCs that patrol mucosal sites and tissues become activated and migrate to the regional lymph nodes. Within the lymph node, DCs undergo a process of maturation characterized by a loss of phagocytic function as well as increased surface expression of MHC molecules and co-stimulatory molecules [27]. This enables DCs to prime naïve T cells to differentiate into a specific helper CD4 T (Th) cell subsets or CD8 T cells. Activation and subsequent proliferation of naïve T cells requires MHC:antigen:TCR (CD4 or CD8) engagement as well as a co-stimulatory signal. This is followed by T cell differentiation which requires a specific cytokine milieu, provided mostly by innate cell inflammation. Peptides bound to MHC molecules class I and II are presented to CD8 and CD4 T cells, respectively. These effector cells will then mount a pathogen-specific

immune response. Once the pathogen has been eradicated, a huge proportion of the effector cells die with a small surviving proportion becoming long-lived antigen-specific memory cells. Upon recognition of MHC-bound antigen, memory cells have the capacity to mount a rapid and stronger immune response. Thus, through primary infection or vaccination immunological memory is established which confers life-long protective immunity.

1.4. Cell-mediated adaptive immune mechanisms against *M.tb*

1.4.1. Role of CD4 T cell subsets in *M.tb* infection

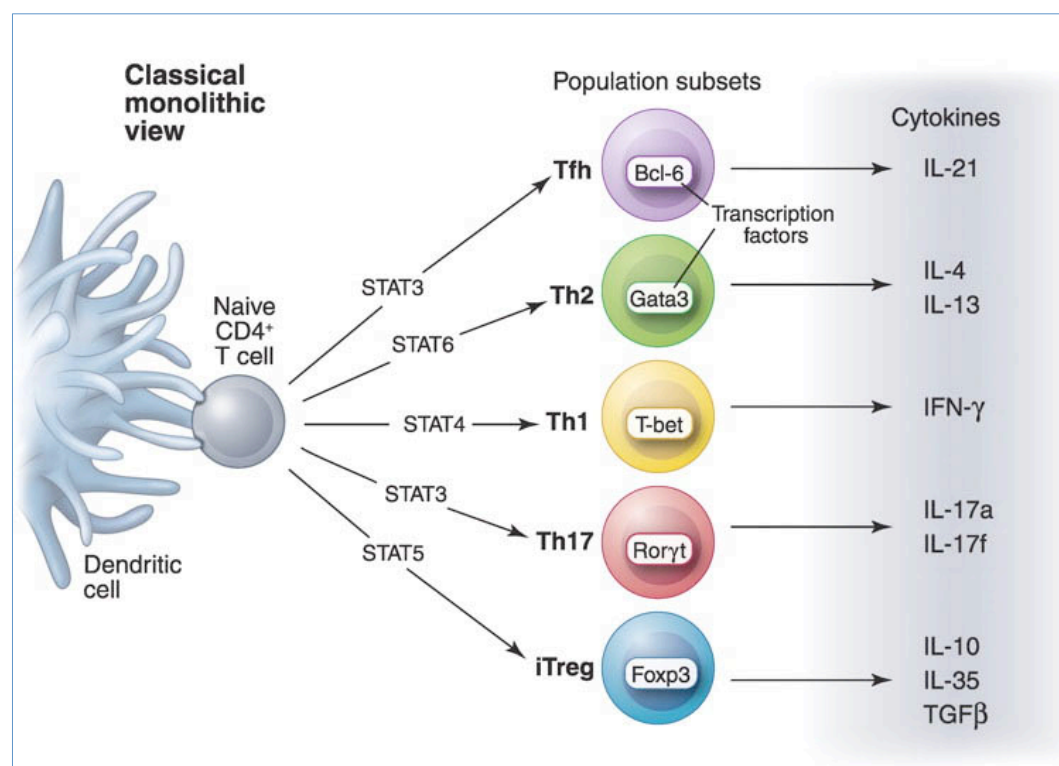


Figure 1.3. The five CD4 Th cell subsets of the adaptive immune system. (From O'Shea *et al*, Science, 2010 [32]).

Naïve CD4 T cells can develop into either Th1, Th2, Tregs, Th17 or Tfh helper cells (Figure 1.3). Activation of Th cell subset and the type of Th cell differentiation depends on the strength of TCR-MHC II binding (signal 1) and cytokine milieu (signal 3) [33]. The co-stimulatory molecules engaged during binding (signal 2) are also crucial in Th cell activation and differentiation and absence of this signal leads to T cell anergy. Each Th cell subset is characterized by the expression of a unique set of transcription factors coupled with the production of specific cytokines (Figure 3). Mycobacterial

infection classically induces the development of Th1 responses through MHC class II antigen presentation and the expression of the Th1 type cytokines IL-12 and IFN- γ . This commits naïve CD4 T cells to a Th1 immune response. The importance of this CD4 T cell in *M.tb* infection and diseases is discussed in more detail in sections 1.4.3 and 1.4.4.

(i) Th2 cells

IL-4 production is the hallmark of the Th2 response as IFN- γ is to the Th1 response. Th2 cells are important in the immune response to parasites, such as helminths, by promoting B-cell activation through the production of IL-4, IL-5, IL-9 and IL-13 [27]. However, initiation of a Th2 response counteracts a Th1 immune response. IL-4 induces expression of STAT6, the transcription factor required to initiate a Th2 T cell differentiation in B cells and other human cells [34], [35]. In murine macrophages, IL-4 has also been shown to downregulate the antimicrobial iNOS [36], Dwivedi *et al* also described the induction of Th2 differentiation with subsequent T cell production of IL-4, when infecting murine DC with virulent *M.tb* [37]. Interestingly, this was not observed for DC infected with BCG or an RD1 knock-out *M.tb* strain which rather induced a Th1 response as measured by IFN- γ production. Nonetheless, various groups have observed an association between increased Th2 activity and TB disease [38]. This is of course of great concern in the development of post-exposure TB vaccines.

(ii) Th17 cells

Th17 cells constitute the third major subset of effector CD4 T cells and require both TGF- β and IL-6, among other cytokines including IL-1 β and TNF- α , for T cell differentiation. Further expansion and effector function of Th17 requires IL-23. Th17 cells are characterized by the expression of the transcription factor, ROR γ t, and cytokines IL-17 and IL-22, and granulocyte macrophage colony-stimulating factor (GM-CSF) [39]. Their ability to produce IL-17 makes Th17 cells important to control the early stages of infection through the induction of acute inflammation. At the site of infection, surrounding cells bind IL-17 which leads to the production of chemokines for the robust recruitment of neutrophils. In a murine TB vaccination study, IL17 induced production of chemokines specific for the recruitment of antigen-

specific Th1 cells to the lung, after aerosal *M.tb* challenge [40]. In addition to the requirement of IL-23 for Th17 persistence, this study also described a role for IL-23 in restricting mycobacterial growth. Thus, Th17 cells may be important in early *M.tb* infection. Furthermore, Wozniak *et al* described a BCG-specific Th17 cells that, under the modulation of IFN- γ , have a protective role in *M.tb* infection [41].

(iii) Treg cells

Regulatory T cells (Treg) mediate suppression and prevention of alloreactive immune responses and pathogen-induced immunopathology. Two subsets of regulatory T cells have been identified based on origin and mode of action [42]. A naturally occurring subset, nTregs, originates from thymocytes in the thymus and an induced or adaptive subset, iTreg, which differentiate from naïve CD4 T cells in the periphery in the presence of TGF- β alone. Tregs characteristically express the transcription factor FoxP3. However, nTregs induce a suppressive action through surface-bound CTLA-4 and the IL2-receptor, CD25 while iTregs secrete the immunosuppressive cytokines IL-10 and TGF- β . In some infections, the immunosuppressive function of Tregs is exploited to avoid pathogen clearance and to establish chronic infection [43]. In the context of *M.tb* infection, murine studies have shown that Tregs hinder efficient clearance of the pathogen by inhibiting Th proliferation [44]. Furthermore, Shafiani *et al* described the existence of a *M.tb*-specific Treg population which delayed priming and subsequent migration of effector T cells into the lung of infected mice [45]; a mechanism through which *M.tb* could establish infection.

(iv) Tfh cells

T follicular helper (Tfh) cells are localised to follicles of secondary lymphoid organs where they are responsible for recognition of B cells expressing antigen-bound MHC. Interaction with Tfh cells activate B cells to divide and differentiate into antibody-producing plasma cells [27]. Development of Tfh cells requires the presence of IL-6. These cells are characterized by the expression of the transcription factor Bcl-6 and expression of CXCR5 and ICOS; cell-surface molecules required for binding of follicular dendritic cells and B cells, respectively. Tfh cells can also induce antibody class switching

to IgG2a or IgG1, through the expression of IFN- γ and IL-4, respectively [46]. Localisation of Tfh in ectopic lymphoid structures in lung tissue is associated with optimal control of *M.tb* in a non-human primate model of latent TB [47].

1.4.2. Role of CD8 T cells in M.tb infection

CD8 T cells originate from the same double negative thymocytes as CD4 T cells which mature in the thymus and become T cells with cell-surface expression of either CD4 or CD8. CD8 T cell function is associated with cytolytic killing of target cells infected with cytosolic pathogens such as viruses. Cytolytic molecules are produced and maintained within cytotoxic granules within CD8 T cells and are released upon cell-cell contact with an infected cell. Extracellular pathogens and phagosomal mycobacteria can be detected by CD8 cells through the process of cross-presentation [29]. CD8 T cells also produce IFN- γ for activation of MHC I antigen-presenting macrophages.

The role of CD8 T cells in TB has received less attention than CD4 cells because *M.tb* primarily resides in the phagosome of infected cells. However, CD8 T cells may have a critical role in vaccination against TB. Using BCG-vaccinated non-human primates, Chen *et al* showed increased mycobacterial load associated with dissemination of *M.tb* infection when CD8 T cells were depleted prior to BCG vaccination [48]. Interestingly, in addition to CD4 T cell responses, our group has also observed increased CD8 T cell function after vaccination with BCG and novel candidate TB vaccines [49], [50], [51], [52].

A group of T cells bearing unconventional TCRs are also important in immunity against *M.tb* [53] [54] [55]. These include $\gamma\delta$ T cells and natural killer T cells (NK T cells) which recognize lipids presented on CD1-molecules. Similar to CD4 and CD8 T cells, $\gamma\delta$ and NK T cells also display cytolytic activity in addition to the production of Th1 and Th2 cytokine [56].

1.4.3. Cytokine effector function of CD4 Th1 and CD8 T cell subsets

The importance of CD4 and CD8 T cells lies in their capacity to recognize antigen presented by APCs and to mount a specific reaction in order to

irradiate the pathogen. This specific response involves a cascade of events regulated, in the case of *M.tb*, by Th1 cytokines such as IFN- γ , IL-2 and TNF- α . IFN- γ -production by CD4 T cells plays an important role in the activation of anti-microbial action by APCs [57]. IFN- γ -knockout mice are highly susceptible to *M.tb*-infection due to defective macrophage activation and reduced iNOS expression. The importance of this cytokine in humans is emphasized in individuals with defective IFN- γ or IFN- γ receptor expression; these individuals are predisposed to severe mycobacterial infections [58]. In addition to these innate antimicrobial effects, IFN- γ also enhances antigen processing and presentation. IL-2 is required for T-cell expansion and differentiation [59], a crucial component for establishing immunological memory against the specific pathogen. TNF- α has also been shown to have a pivotal role in granuloma formation and chemokine attraction to the site of infection [60], [61]. The importance of TNF- α is further emphasized by studies reporting an increased risk of TB disease in patients with rheumatoid arthritis who are on anti-TNF therapy [62]. *M.tb* infection can induce the production of Th2 cytokines such as IL-4 which has been associated with poor outcome in TB disease [63], [64]. Furthermore, blocking of this cytokine leads to reduced mycobacterial burden after challenge in mice [65].

Immunity against tuberculosis clearly relies on both CD4 Th1 and CD8 T cell function as illustrated by numerous studies conducted in animal models. CD4 T cells induce macrophage activation through the production of IFN- γ , IL-2 and TNF [66]. IFN- γ specifically from CD4 T cells enhances CD8 T cell effector and cytotoxic function in response to *M.tb* infection and also inhibits *M.tb* growth in mice [67]. Murine CD4 T cells appear to control *M.tb* infection through direct recognition of MHCII; *M.tb* growth in the absence of MHCII expression is comparable to growth in the absence of CD4 T cells [68]. Mogues *et al*, elegantly confirmed the importance of CD4 and CD8 T cells in a series of gene-knockout mouse models of *M.tb* infection [69]: CD4 knockout and MHCII knockout mice showed dramatically reduced survival time associated with increased mycobacterial growth in the lung, liver and spleen after H37Rv infection compared to CD8 and MHCI knockout mice. CD8 and MHCI knockout mice had reduced survival and increased

mycobacterial growth compared to wildtype, although not to the extent of the CD4 and MHCII knockout mice. Reduced expression of NOS2 and IFN- γ was also observed in CD4 and MHCII knockout mice compared to wildtype, CD8 and MHCI knockout mice. Similarly, In a study of MHCI-knockout mice, Flynn *et al* illustrated that CD4 cells alone were not sufficient to control *M.tb* growth after challenge [70].

1.4.4. Cytotoxic effector function of CD4 and CD8 T cell subsets

Classically, cytotoxic function has been associated with CD8 T cells, natural killer (NK) cells and NK T cells. Cytotoxic potential is detectable in both effector as well as memory CD8 T cells in viral infection studies of mice [71]. Cytotoxic activity of CD4 T cells has also been implicated in various viral infections and cancers [72]. More interesting is the demonstration of the ability of a cytotoxic CD4 T cell subset, expressing cytotoxic molecules, to kill *M.tb* [73]. Thus, in addition to Th cytokine production, CD4 together with CD8 T cells can mount a cytolytic response against intracellular pathogens, involving the cytotoxic molecules granulysin, perforin and granzyme B, among others [74]. Appay *et al.* described cytotoxic CD4 T cells, derived from HIV infected patients, as highly differentiated antigen-experienced cells that had lost expression of CD28, CD27, CD45RO and CCR7, possibly due to chronic activation [74]. Interestingly, these cells also expressed the Th1 cytokines, TNF- α and IFN- γ , but not IL-2. However, the involvement of cytotoxic CD4 T cells may be pathogen-specific as cytolytic potential of these cells have not been detected in murine LCMV-infection [71].

Cytotoxic CD8 T cells have been shown to lyse *M.tb*-infected M ϕ and also reduce mycobacterial growth [75]. The recombinant form of granulysin, a saposin-like protease, has been shown to directly kill various bacteria, including *M.tb*, as well as fungi and parasites [76]. Decreased perforin production which translates into reduced lysis of mycobacterial antigen-pulsed M ϕ , has been observed in peripheral blood mononuclear cells (PBMCs) from TB patients, compared to healthy controls [77], [78]. BCG also induces expression of granzyme B and perforin by antigen-specific CD8 T cells in healthy 10-week-old infants vaccinated at birth [49].

1.4.5. Mechanism of cytotoxic killing

In addition to the induction of innate mechanisms involving pathogen killing through activation of APCs, infected cells can also be detected and killed by cytotoxic T cells (CTLs). CTLs have the ability to induce apoptosis of target cells through either cytotoxic degranulation (the release of cytotoxic granules) or the Fas-FasL pathway. Fas-FasL death pathway appears to be involved in homeostatic control of T cells and defects in this pathway are associated with lymphoproliferative disorders in mice and humans [79]. Cytotoxic degranulation is associated with killing of pathogen-infected cells by CTLs. CTLs produce cytotoxic molecules which are contained within lysosomal structures, termed cytotoxic granules. When these effector CTLs come into contact with an infected cell, the granules migrate toward the interface between the effector and the target cell, called the immunological synapse, and the preformed cytotoxic molecules are released. Three types of cytotoxic molecules have been identified i.e. perforin, granzymes and granulysin.

1.4.6. Cytotoxic molecules

Perforin is part of the membrane attack complex protein family (MACPF) and, in a calcium-dependent manner, binds lipids and polymerizes to form pores in the cell membrane [80]. Perforin and granzymes synergistically initiate apoptosis of target cells; perforin forms pores in the cell membrane of the target cell which allows entry of granzymes into the cell [81].

Granzymes are a family of serine proteases consisting of thirteen isoforms which have been identified, to date, in mice [82]. Humans express only five isoforms, granzyme A, B, H, K and M, of which granzyme A and B have been extensively studied. Granzyme B induces apoptosis through cleavage of BID, a pro-apoptotic protein, which leads to the release of mitochondrial cytochrome c and subsequent DNA fragmentation [83].

Granulysin is a lytic protein with saposin-like protease activity which binds membrane lipids and activates lipid-degrading enzymes of target cells leading to cell death through apoptosis [84]. In order to prevent autolysis of CTLs, granulysin is expressed as a 15kDa inactive precursor which, under

conditions of acidification, is reduced to a 9kDa cytolytic protein. Granulysin has been shown to have direct antimicrobial activity against an array of bacteria, fungi and parasites, as well as *M.tb* [76]. Ernst et al successfully illustrated the ability of granulysin to permeabilize the cell membrane of *M.tb* [85]. Granulysin formed pores in the cell wall causing the detachment of the inner and outer membranes and leading to the ultimate release of the cytosol.

1.5. The BCG-induced immune response

Following WHO guidelines, BCG is administered as a percutaneous or intradermal injection into the deltoid muscle. At the site of inoculation, BCG is likely to follow a similar fate as described for *M.tb*. Briefly, at the site of delivery, BCG induces an inflammatory response leading to the recruitment and maturation of monocytes and immature DCs. M ϕ internalize BCG and initiate innate responses to kill the pathogen. BCG is also taken up by Langerhans cells (immature DCs in the skin) and migrate to regional lymph nodes. BCG induces DC maturation characterized by increased surface expression of MHC molecules and co-stimulatory molecules [86]. Mature DCs also lose their phagocytic function. Naïve CD8 T cells are primed to become cytotoxic T cells whereas naïve CD4 T cells can develop in an array of helper T cells depending on the cytokine milieu present during priming [27]. BCG-pulsed DCs induce the production of Th1 cytokines by naïve CD4 and CD8 T cells mediated by IL-12 [87]. Within the DC, BCG is taken up into phagosomes that allow antigen presentation through MHC class II molecules to CD4 T cells [88]. BCG can also induce apoptosis of infected cells leading to the release of apoptotic vesicles that contain mycobacterial antigens. These are then taken up by bystander DCs and processed through the endosomal pathway for cross-priming of CD8 T cells through MHC class I antigen presentation [29]. BCG has also been shown to prime CD8 T cells through a MHC-independent pathway through CD1 molecules [88]. More over, Kawashima *et al* also showed preferential recognition of live and dead BCG by CD8 and CD4 T cells, respectively. Clearly the role of CD8 T cells is underestimated.

In addition to a Th1 response, BCG vaccination induces effector responses of other Th subsets. Burl *et al* reported increased expression of Treg, Th17 as well as Th2 specific cytokines in 4^{1/2} month old newborn vaccinees compared to unvaccinated controls [89]. We have also observed detectable Treg and Th2 function in 10-week old newborn vaccines, measured by IL-10 and IL4 expression, respectively. However expression of these cytokines was at notably low levels compared to Th1 cytokines [90].

Newborn BCG vaccination can induce expression of cytotoxic molecules by both CD4 and CD8 T cells as measured in infant blood [49] [91]. In work done in our laboratory, Soares *et al* showed a peak cytotoxic CD4 response induced by BCG at 6 weeks after vaccination, measured by intracellular expression of perforin, granulysin and granzyme B [91]. Cytotoxic molecules remained detectable at 10 weeks post-vaccination in both CD4 and CD8 T cells [49], [91], [92].

1.6. Vaccine-induced immunity

Immunity against disease is dependent on host ability to mount an immune response, which clears the pathogen by eliminating the infection, and ideally prevents disease recurrence. In the absence of such natural immunity against a specific pathogen, a protective immune response can be induced by vaccination. Characteristics of the immune response elicited through vaccination depend on route of administration, i.e. oral or injected, and the type and formulation of vaccine. Generally, vaccines are killed or live, attenuated forms of the pathogen (e.g. smallpox, yellow fever, measles, mumps, rubella and chicken pox) or subunit vaccines comprising pathogen product such as proteins, polysaccharides or DNA, often formulated in adjuvant (e.g. hepatitis B, HiB, pneumococcal vaccines). Live attenuated vaccines are typically the most successful vaccines and can confer lifelong protection by eliciting a humoral immune response and establishing B cell memory. For example, vaccination against smallpox induces vaccinia virus-specific B cells and IgG antibody titers which persisted up to 60 years after vaccination [93]. Similarly, neutralising antibodies could be detected up to 40 years after yellow fever vaccination in a group of US WWII veterans [94].

In addition to induction of B cell memory, various viral vaccines also induce long-lived T cell memory [95].

BCG is a live attenuated strain of the bovine TB pathogen, *Mycobacterium bovis*. BCG has been in use as the only vaccine against TB disease since 1921, more than 90 years. BCG vaccination appears to protect against *M.tb* infection as shown by various groups using BCG-scarring as proxy [96], [97]. Despite its variable efficacy of 0-80% in protection against TB disease [98], [99], BCG protects against severe forms of childhood TB such as miliary and TB meningitis [100]. Thus, the immune response which confers protection against TB in some, but not in others, needs to be elucidated. This will aid in the development of safer and better vaccines to either boost the BCG primed response or to replace BCG.

Several studies conducted in our group and by others have described the characteristics of BCG-induced immune responses in infants [49], [90], [91], [101], [102]. BCG is given at birth in many TB endemic countries and appears to have an adjuvant effect, enhancing protective immunity induced by subsequent EPI vaccinations. This observation was illustrated immunologically by Ota *et al*, who showed enhanced proliferation and cytokine production (IFN- γ , IL-5 and IL-13) in response to vaccine antigens in BCG-vaccinated infants who also received HBV, DPT and OPV compared to BCG-unvaccinated infants [103]. Investigation of infant mortality also supports a non-specific beneficial role for newborn BCG vaccination in addition to protecting against TB [104]–[107]. Moulton *et al*, reported reduced mortality in the first six months of life in infants who were BCG vaccinated, compared with unvaccinated infants [107].

Considerable effort and progress is being put into global development of new TB vaccines, even in the absence of correlates of protection, or risk of TB disease. Th1 cytokines are thought to be important in maintenance of latency and resistance to disease progression [61] [108]. However, a study of disseminated TB pediatric patients determined no difference in the levels of IFN- γ and TNF- α between kids who received BCG compared to those who did not [109]. Similarly, our group reported no differences in BCG-specific Th1 responses in infants who developed TB compared to those who

remained healthy. Using intracellular cytokine staining of whole blood collected 10 weeks after BCG vaccination, similar levels of expression of IFN- γ , TNF- α and IL-2 was detected between infants who ultimately developed TB disease, and those who remained healthy [50]. Further analysis of these proteins on a soluble level in the same cohort also revealed no differences (data not published). Clearly other factors are at play.

1.7. Methods of measuring immune responses

T cell responses are thought to be essential for protective immunity against TB. As such, new TB vaccines are designed to induce a T cell-mediated immune against *M.tb* infection. The development of a flow cytometry-based whole blood assay (WBA) to detect antigen-specific CD4 and CD8 T cells allows assessment of vaccine-induced responses [110]. This assay is ideal for infant studies in which blood volumes are limited. Flow cytometry assays based on small volumes of whole blood allow measurement of many different parameters, at phenotypic (cell-surface) and functional levels (intracellular cytokine stain; ICS). A more direct measure of antigen- or vaccine-specific T cells makes use of tetramers i.e. fluorescently labeled MHC molecules conjugated to antigenic peptides from the relevant pathogen [111] [112]. However, this approach is limited by the requirement for prior knowledge of the restricting HLA element, which makes tetramer work expensive and only feasible in a subset of subjects who possess the relevant HLA alleles.

Lymphoproliferation assays allows for the expansion and detection of antigen-specific T cells which are present in low levels in blood samples. Whole blood or isolated PBMC are incubated with antigen for up to 6 or 7 days and antigen specific cells are detected as cells undergoing division. These proliferating cells are then flow cytometrically detected based on BrdU incorporation, dye dilution of Oregon Green (or CFSE), or high Ki67 expression [113]. This offers the added benefit of facilitating measurement of other markers by addition of other fluorescently labeled antibodies to define function and phenotype of antigen-specific proliferating cells.

In addition to intracellular detection, cytokine levels can also be quantified as secreted soluble molecules in serum, plasma or culture supernatants. Thus, the use of a single blood volume can be expanded measure multiple T cell outcomes. The enzyme-linked immunosorbent assay (ELISA), can detect soluble molecules by using protein-specific capture and detection antibodies, which binds different epitopes on the particular protein. This assay is restrictive in the volume of sample required and the number of proteins that can be detected in one sample. Multiplex assays, such as the commercially available flow cytometry-based bead assay, the Luminex bead array, allows simultaneous detection of up to 42 different markers. In brief, micron-sized beads are fluorescently stained and conjugated with capture antibodies specific to a protein which is quantified by the binding of a fluorescent secondary antibody [114]. This technology, referred to as a 42-plex bead array, has received favourable use in infant study due to minute sample volume required [115] [116] [102]. Although a hundred different fluorescent beads are available, at this point only 42 distinct cytokines and chemokines can be measured in a single assay.

Vaccine-induced cytotoxic potential, conventionally associated with CD8 T cells, can also be assessed by flow cytometry [49]. Cytotoxic potential is described as the ability of cytotoxic cells to produce cytotoxic molecules and their subsequent release through degranulation. PBMC are incubated with antigen for three days after which fluorescent-labeled antibodies specific for cytotoxic molecules like granzymes, granulysin and perforin can be detected intracellularly. Degranulation is determined by detection of CD107, which becomes exposed on the cell surface after granule release. Cytotoxic function, however, is measured as the ability of antigen-specific cytotoxic T cells to kill target cells presenting antigen via cell surface MHC. Traditionally, this was measured as chromium (^{51}Cr) release, in which cytoplasm of target cells are labeled with ^{51}Cr and co-cultured with cytotoxic effector cells [117]. The amount of ^{51}Cr released is directly associated with degree of target cell killing. Other killing assays, in which cells are labelled with non-hazardous fluorochromes or dyes that allow

detection of the reduction in fluorescent cells by flow cytometry, are now in use [118] [119].

In essence, flow cytometry technology provides a powerful tool for extensive exploration of vaccine- or infection-induced immune response. This technology has contributed immensely to our current understanding of immune cells and their functions and interactions.

1.8. Immune correlates of risk of TB disease

BCG-induced immunity against TB disease is suboptimal. This, together with the complications of intercurrent HIV-infection, has urged the re-evaluation and development of TB vaccines. However, rational vaccine design is hampered by the lack of correlates of protection against TB disease. The success of many EPI vaccines can be ascribed to their induction of defined antibody responses that correlate with protection against the disease [120]. As TB disease is thought to be associated with cell-mediated immune responses, the role of antibody responses has traditionally received limited attention [121].

In contrast, Th1 cytokines, particularly IFN- γ , have been widely explored as possible biomarkers of protection. However, despite strong evidence that such Th1 cytokines are necessary for immunity against TB, to date, identifying a correlate of risk of TB has been unsuccessful. New approaches are required to identify a measurable marker which indicate resistance to or risk of TB disease.

1.9. Current Study

In this study, we aimed to investigate BCG-specific cytotoxic T cell function following routine BCG vaccination at birth, as a biomarker of risk of TB disease. First, we determined whether BCG-specific cytotoxic potential translates into cytotoxic killing of BCG-infected target cells and describe the optimisation of a flow cytometry based assay to investigate BCG-induced target-specific killing. Furthermore, we compared BCG-induced production of cytotoxic molecules by CD4 and CD8 T cells at 10 weeks of age, in infants at risk or not at risk of TB disease. Finally, we optimised a bead array

assay to measure release of cytotoxic molecules by flow cytometry. In essence, we uncovered an immune profile that may correlate with risk of developing TB disease.

1.9.1. Objective 1 (Chapter 2)

To determine if BCG-induced T cell production of cytotoxic molecules is associated with killing of monocytes infected with mycobacteria.

Specific aims: (i) To determine optimal BCG inoculating dose and monocyte infection time to measure T cell-mediated killing of infected cells. (ii) To determine the effect of IL-2 mediated T cell expansion on cytotoxic molecule production and killing capacity of mycobacteria-specific T cells.

1.9.2. Objective 2 (Chapter 3)

To investigate cytotoxic capacity, cytokine production and lymphoproliferation as candidate biomarkers of risk of tuberculosis, following BCG vaccination at birth.

Specific aims: (i) To compare BCG-induced T cell expression of cytotoxic molecules and cytotoxic function of CD4 and CD8 T cells at 10 weeks of age in infants at risk or not at risk of TB disease. (ii) To compare BCG-induced proliferation and cytokine expression of CD4 and CD8 T cells at 10 weeks of age in infants at risk and infants not at risk of TB disease.

1.9.3. Objective 3 (Chapter 4)

To establish methodology for optimising custom bead array assays for detection of soluble molecules by flow cytometry

Specific aims: (i) To establish methodology for optimisation of a custom bead array assay for detection of soluble IFN- γ by flow cytometry.

Chapter 2: Optimisation of a flow cytometry assay of mycobacteria-specific killing

2.1. Background

This chapter aims to describe the optimisation of a flow cytometry based assay to measure killing of BCG-infected monocytes by mycobacteria-specific T cells. Development of this assay may be useful in our understanding of the role of cytotoxic T cells in TB disease development in our cohort of BCG-vaccinated infants.

Traditionally, cytotoxic killing was assessed in a chromium (^{51}Cr) release assay involving preincubation of target cells with radioactive sodium chromate ($\text{Na}_2^{51}\text{CrO}_4$) [117]. ^{51}Cr is taken up into the cytosol of the cells and upon killing of the targets by effector cells, is released into the culture supernatant. Released ^{51}Cr can then be measured with a gamma counter. However, the use of radioactive isotopes off course holds many health hazards and thus advances have been made in the development of non-hazardous alternatives to radioactive assays.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is one such alternative and has been widely used in both *in vivo* and *in vitro* assay systems [118]. CFSE-based assays enables the assessment of various T cell responses and processes such as cell migration, tolerance, proliferation and cell death by flow cytometric analysis. Briefly, cells of interest are stained with the fluorescent dye which is taken up in the cell cytosol. Processes such as proliferation can then be quantified as a dye dilution as the fluorescent dye is halved between daughter cells.

A second alternative is the use of the chloromethyl derivative, CellTracker™ Orange CMTMR (CTO) to assess cytolytic killing as described in the VITAL assay [122]. Assessment of killing of target cells by effectors using this approach has shown good correlation with the chromium release assay [119]. This compound is able to stain cells fluorescent orange by reacting with intracellular thiols in the cytoplasm.

We have available in our laboratory, an adapted version of the VITAL assay to assess killing of PPD-pulsed monocytes by antigen-specific T cells. As

described below, this assay makes use of the two fluorescent dyes: CTO and the CFSE-derivative Oregon Green[®] (OG). Infected and uninfected, control monocytes are stained with OG and CTO, respectively. Equal amounts of infected and control monocytes are cultured with effector T cells and killing is then determined as a ratio of the amount of OG⁺ infected cells to CTO⁺ control cells as quantified by flow cytometry.

We wanted to determine whether BCG-induced T cell production of cytotoxic molecules was associated with killing of monocytes infected with mycobacterial antigens. Studies suggest that mycobacteria can kill APC directly [17], [123]. We thus assessed the viability of monocytes after incubation with live BCG at the dose used in the 3-day cytotoxic marker assay i.e. MOI 0.1. We also compared viability of monocytes after infection with live and heat-killed BCG at this dose. Infection efficiency of the dose as well as optimal time required to infect monocytes with minimal killing of monocytes was determined. The addition of IL-2 to expand mycobacteria-specific T cell populations was also assessed for possible direct effects on the production of cytotoxic molecule production and killing of infected monocytes.

2.2. Methods

2.2.1. Blood collection

Informed consent was obtained from healthy adult volunteers by using a protocol approved by the University of Cape Town Research Ethics Committee. Heparinised whole blood was collected from each participant for the expansion of mycobacteria-specific T cells and the isolation of autologous monocytes.

2.2.2. Expansion of mycobacteria-specific effector cells

On day 1 of the assay, PBMC were isolated from fresh whole blood by density gradient centrifugation. PBMC were incubated at 2×10^6 PBMC in 1mL culture medium (12.5% v/v AB serum in RPMI) at 37°C in 5% CO₂ in medium alone, 2µg/mL purified protein derivative (PPD Tuberculin RT50; Staten Serum Institute) or BCG MOI 0.1 (BCG Vaccine SSI, Danish 1331, Staten Serum Institute). On day 3, 100µL cells from each condition were

collected before 50 units of IL-2 (eBioscience) were added and incubated further. Another 100 μ L cells were collected on day 6 and cultures were replenished with 500 μ L fresh culture medium and incubated overnight. On day 7, cells were harvested with repeated wash steps with cold RPMI followed by centrifugation, resuspension in fresh culture medium and resting overnight at 37°C in 5% CO₂.

2.2.3. Preparation of mycobacteria-pulsed target cells

On day 7, autologous monocytes were isolated from freshly prepared PBMC using anti-CD14 microbeads (MACS Miltenyi Biotec) following the manufacturer's recommendations. Isolated monocytes were resuspended and divided among three conditions: one part of the cells were left unpulsed (control target cells), one part was pulsed with 20 μ g/mL PPD and the remaining cells were pulsed with BCG at MOI 0.1. Cells were incubated at 37°C in 5% CO₂ overnight in culture medium. Next, pulsed monocytes were washed and stained with 0.5 μ g/mL OG or 50 μ M CTO. For co-culture of target cells with effector T cells, antigen-pulsed monocytes were stained with OG and control target cells were stained with CTO.

2.2.4. Co-culture of target and effector T cells

Cell counts were determined and effector and target cells were co-cultured at the following ratios, with target cells consisting of equal numbers of control and antigen-pulsed cells: 50:1, 30:1, 10:1, 5:1, 1:1 and 0.5:1 in FACS tubes. Cells were incubated overnight at 37°C in 5% CO₂, harvested, stained with 1 μ g/mL LIVE/DEAD[®] Fixable Violet Dead Cell Stain (Invitrogen) and acquired fresh on a LSRII flow cytometer to assess killing of target cells by mycobacteria-specific effector cells (Figure 2.1 and Figure 2.5). Anti-CD3 PacBlue, anti-perforin FITC and anti-IL4 PE were used to set compensation for the violet viability dye (ViViD), OG and CTO, respectively. Data was analyzed using Flowjo 8.8.4 (Treestar).

2.2.5. Flow-cytometric analysis of cytotoxic molecule production

Cultured PBMC collected on day 3 and day 6 were washed and stained with 1 μ g/ml ViViD. Cells were fixed with FACS lysing solution (BD Biosciences) and cryopreserved. Later, cryopreserved, fixed cells were thawed, washed

Expansion of mycobacteria-specific cytotoxic T cells

Generation of Target cells

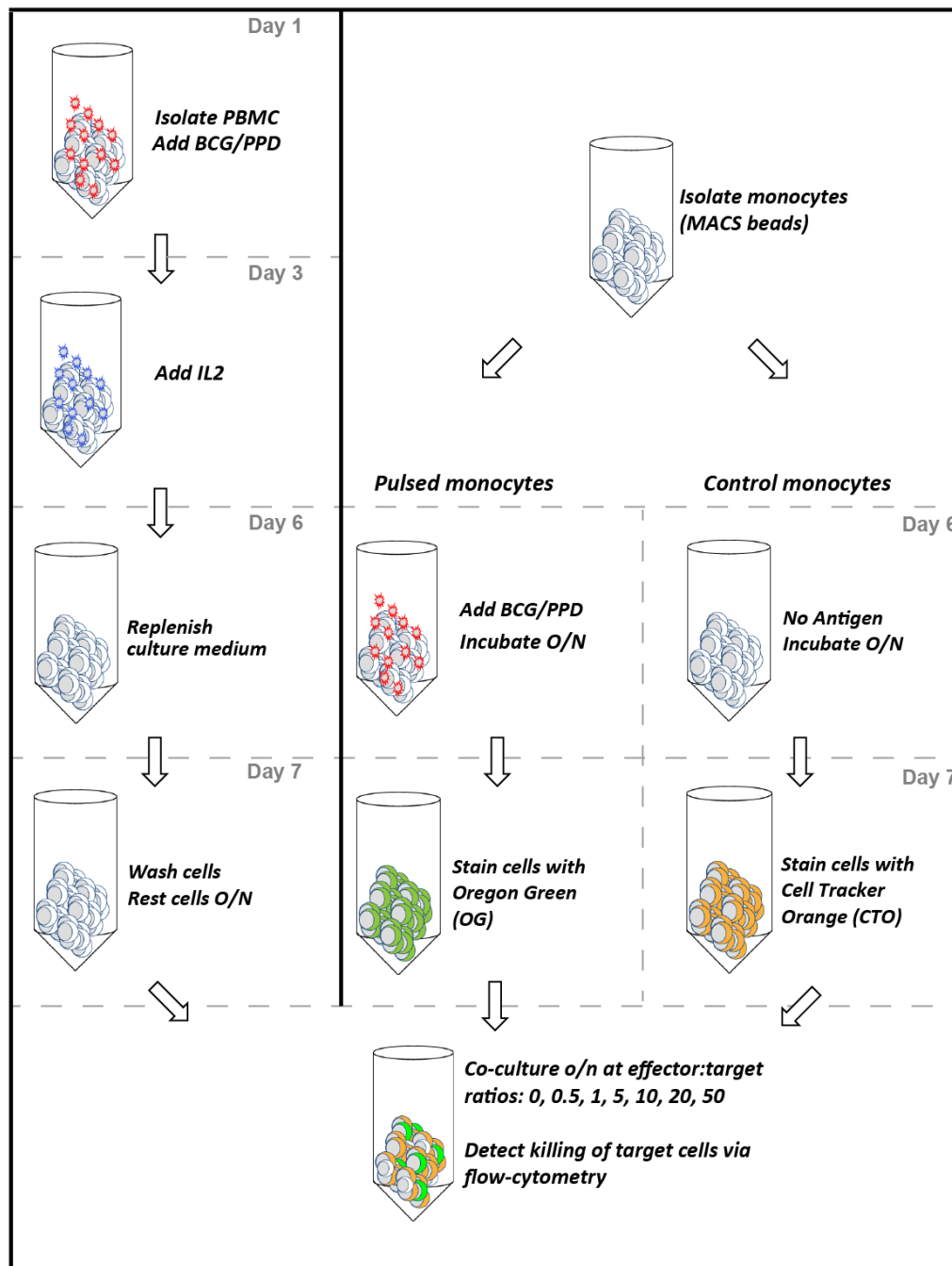


Figure 2.1. Flow-diagram depicting the flow-cytometric based killing assay.

and permeabilized with Perm/Wash solution (BD Biosciences), and stained with the following antibodies for 1 hour at 4°C: anti- CD3 Qdot605 (clone UCHT1), anti-CD8 Cy5.5PerCP (Sk1), anti-granzyme B Alexa 700 (GB11), anti-perforin FITC (δG9), anti-Ki67 (B56; all from BD Biosciences) and anti-granulysin PE (eBioDH3, eBioscience). Cells were then washed and acquired on a LSRII flow cytometer, using FACS Diva 6.1 software. Anti-

mouse kappa beads (BD Biosciences), stained with the respective fluorescent-labeled antibody, were used to configure compensation settings. Anti-CD3 PacBlue (UCHT1) was used to set compensation for the violet viability stain. Data was analyzed using Flowjo 8.8.4.

2.3. Results

2.3.1. Monocyte viability after BCG co-culture

To determine how many monocytes remained viable at BCG MOI 0.1 at different incubation periods, we pulsed monocytes with BCG for 2, 4 and 18 hours.

Monocytes were selected based on their side scatter/forward scatter distribution and viable cells were identified as CTO⁺Vivid^{low} (Figure 2.2. A). Viability of monocytes were comparable between 2 hours and 4 hours with medians of 99.8% at each timepoint. A decrease in viable monocytes at 18 hours was observed compared with 2 and 4 hours of culture (Figure 2.2. B). At 18 hours 99.1% monocytes were viable, however, no statistical significance could be detected between the timepoints. An accumulation of dead cells as Vivid^{high} cells was observed when comparing 2hrs and 18hrs incubation of monocytes with BCG (Figure 2.2. C). Data collected from three donors indicated that higher proportions of monocytes die at the later timepoints.

We concluded that a sufficient number of monocytes do remain viable up to 18 hours of incubation with BCG at an MOI 0.1.

2.3.2. Monocyte viability after infection with live or heat-killed BCG

We also explored the viability of the monocytes after infection with heat-killed BCG. BCG was resuspended in 250µL RPMI and incubated at 60°C for 30 minutes in a waterbath. Freshly isolated monocytes were incubated without BCG or in the presence of live or heat-killed BCG MOI 0.1 for 18 hours.

Data was collected from four adult donors (Figure 2.3. A). Low numbers of cells were detected for two donors (DN025 and DN053). Monocyte survival in the absence of BCG was variable with only two donors (DN009 and

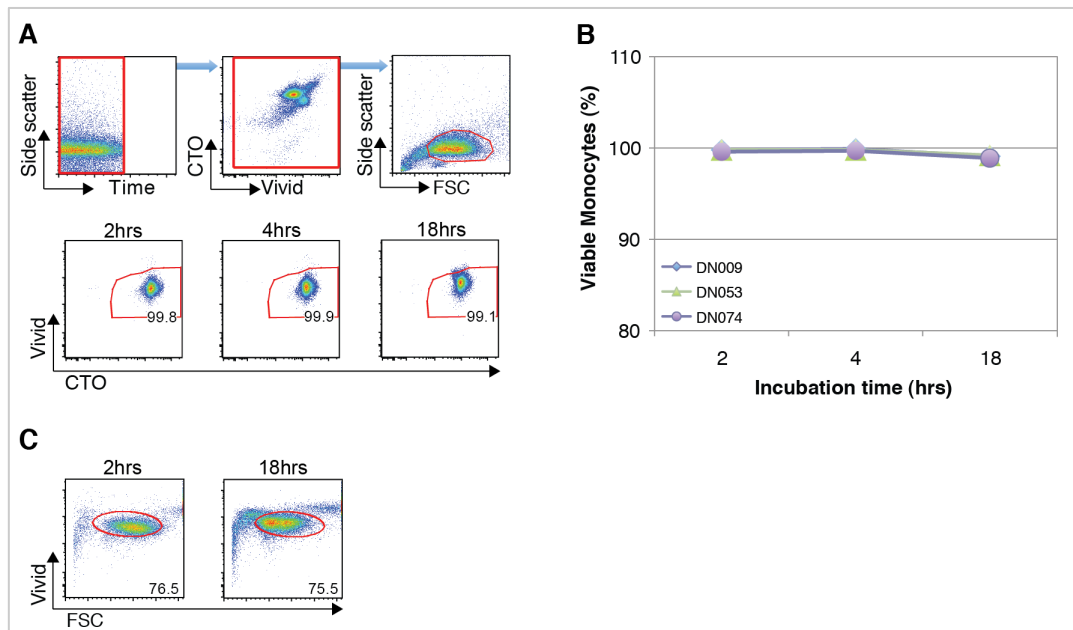


Figure 2.2. Monocyte infection with BCG over time. Monocytes were freshly isolated from healthy adult donor whole blood, stained with CTO and co-cultured with BCG for 2, 4 and 18 hours at 40 000 monocytes per condition. After co-culture with BCG for 2, 4 and 18 hours, monocytes were stained with the violet viability dye and acquired on the flow-cytometer. (A) Viable monocytes were detected as CTO⁺Vivid^{low}. (B) Monocyte viability was measured in four adult donors. (C) The accumulation of Vivid^{high} cells over time during incubation of monocytes with BCG MOI 0.1.

DN074) showing increased viability compared to incubation with live or heat-killed BCG. In all donors, there was a lower number of viable monocytes when cells were cultured with heat-killed BCG compared to live BCG (Figure 2.3. A and B). However, no significant difference was detected between the viability of monocytes cultured with live or heat-killed BCG.

We concluded that culture of monocytes with heat-killed BCG does not improve the viability of the cells.

2.3.3. Low monocyte death detected at BCG MOI 0.1

BCG has the capacity to kill infected monocytes directly. In our established cytotoxic marker assay, BCG is used at MOI 0.1 which induced detectable levels of T cell proliferation, IFN- γ expression and CD8 degranulation [10]. To determine a link between production of cytotoxic marker by BCG-specific T cells and killing of BCG-infected monocytes, we assessed killing capacity at MOI 0.1 compared to higher doses (MOI 1 and 5), lower doses (MOI 0.01 and 0.001) and no BCG.

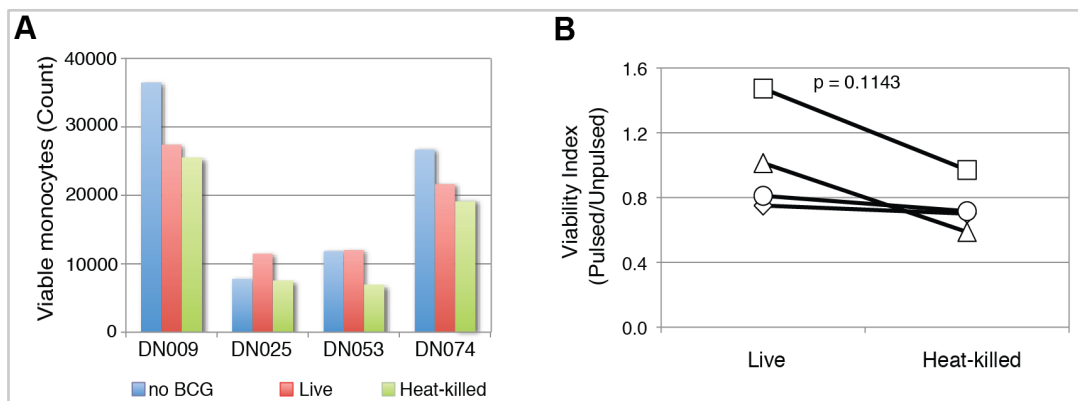


Figure 2.3. Infection of monocytes with live and heat-killed BCG MOI 0.1. Monocytes were freshly isolated from healthy adult donor whole blood, stained with CTO and co-cultured with BCG, live or heat-killed for 18 hours at 40 000 monocytes per condition. After co-culture with BCG, monocytes were stained with the violet viability dye and acquired on the flow-cytometer. Data collected from four adult donors is shown. (A) Infected monocytes were detected as CTO⁺Vivid^{low} and the number of viable monocytes was determined. (B) A viability index was calculated for each donor as the number of viable monocytes under pulsed conditions divided by the number of viable monocytes under unpulsed conditions.

High doses of BCG were associated with the lowest viability compared to cultures with no BCG added after 18hours incubation (Figure 2.4. A). Low doses BCG i.e. MOI 0.001, 0.01 and 0.1, showed levels of viability comparable to uninfected monocytes. In four adult donors tested, an average of 99.1% monocytes remained viable after 18hours incubation with MOI 0.01. Decreasing the dose further did not improve the viability of monocytes (Figure 2.4. B and C).

We concluded that BCG MOI 0.1 is an optimal dose which does not lead to marked killing of monocytes.

2.3.4. Effect of IL-2 on cytotoxic molecule production and killing by cytotoxic T cells

On day 3 of our killing assay protocol, IL-2 was added to cultures to promote survival and to expand the number of specific effector T cells [119]. We wanted to assess whether the addition of IL-2 had an effect on production of cytotoxic molecules as well as the killing capacity of antigen-specific cytotoxic cells. PBMC were cultured with or without the addition of IL-2 on day 3, in the presence of either PPD or BCG or in medium alone. Cells were collected after 6 days of culture. Intracellular expression of granzyme B,

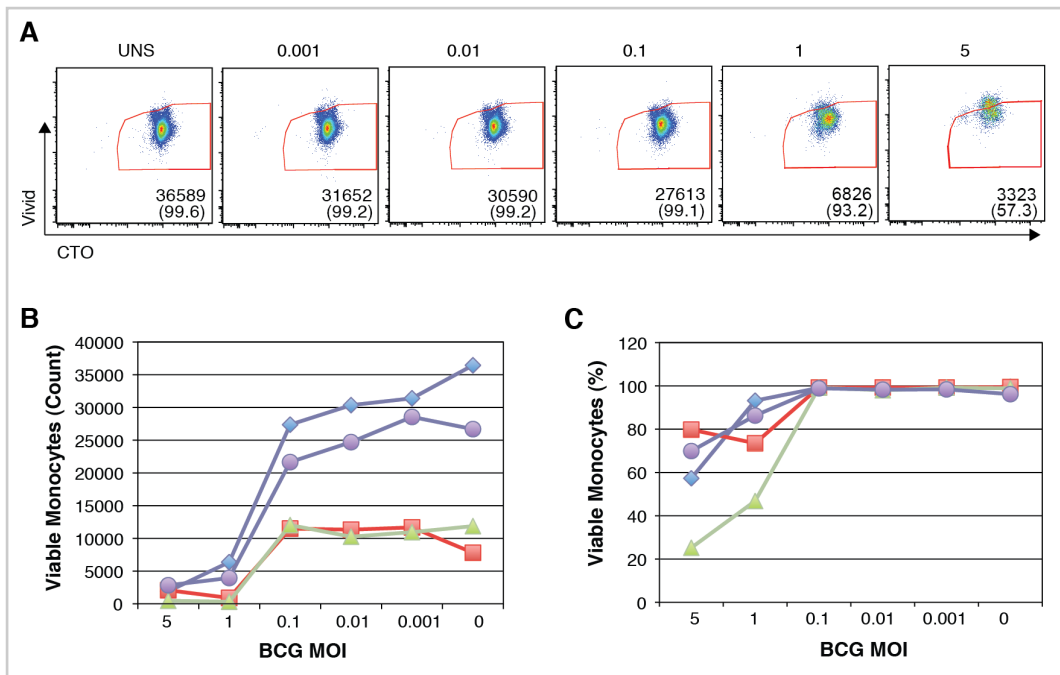


Figure 2.4. Monocyte viability after infection with BCG at different doses. Monocytes were freshly isolated from whole blood collected from healthy adult donors and stained with CTO. Cells were cultured without BCG (UNS) or with BCG MOI ranging from 5 to 0.001, for 18 hours at 40 000 monocytes per condition. After culture, monocytes were stained for viability and acquired on the flow-cytometer. (A) Viable monocytes were detected as CTO⁺VIVID^{low}. Numbers indicate the viable monocyte count with the relative proportion of live monocytes in parenthesis. The viable monocyte count (B) and as a percentage of the total monocyte population (C) from four donors is shown.

granulysin, perforin and Ki67 by CD4 and CD8 T cells was determined on day 3 and day 6 using flow-cytometry. The ability of expanded cells to kill infected target cells was also determined by flow-cytometric analysis (Figure 2.1. and 2.5.).

Data collected from four adult donors, indicated increased production of Ki67, perforin and granzyme B on days 3 and 6 upon BCG-stimulation in the presence of IL-2 compared to expansion without IL-2 (Figure 2.6. A). Similarly, granulysin and granzyme B production of PPD-specific cells were increased in the presence of IL-2 (Figure 2.6. A). However, IL-2 appeared to decrease perforin production by BCG- and PPD-specific cells. None of these differences were significant.

BCG-specific effectors expanded in the presence of IL-2 appeared to have higher killing capacity to those expanded without IL-2 (Figure 2.6. B). However, again this was not significant. Similarly, the killing capacity of PPD-specific effectors expanded in the presence or absence of IL-2 were not

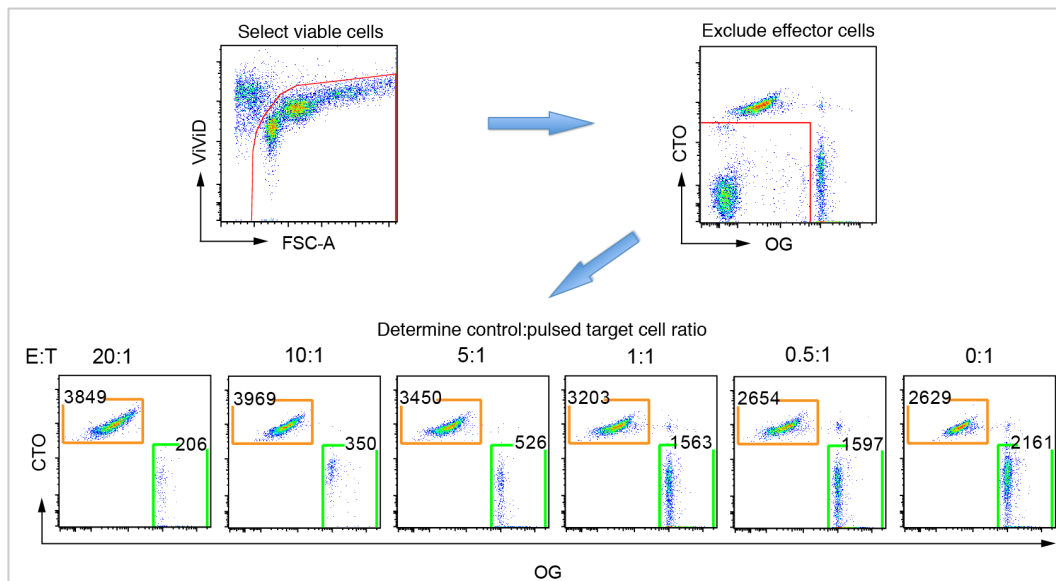


Figure 2.5. Flow-diagram illustrating the flow-cytometric analysis to determine killing by cytotoxic T cells. Prior to effector:target co-culture, BCG-pulsed target and control (unpulsed) target cells were stained with OG and CTO, respectively. All cells were stained with ViViD after co-culture to exclude dead cells (ViViD^{high}). Effector cells (CTO^{low}OG^{low}) were also excluded. BCG-pulsed targets (OG^{high}) and control targets (CTO^{high}) were selected and killing capacity determined as a ratio of the number of control and pulsed target cells.

different (Figure 2.6. C). Specific killing by effectors expanded with IL-2 addition in the absence of antigen could not be detected (Figure 2.6. B and C). A significant difference could only be detected in the killing capacity of effectors expanded with BCG and IL-2 compared with no antigen and IL-2 ($p=0.032$). Similarly, PPD-specific effectors expanded with or without IL-2 addition showed significantly higher killing compared to effectors expanded in the absence of PPD with IL-2 addition ($p=0.008$).

We concluded that the addition of IL-2 to expand the effector cell population, had no effect on the production of cytotoxic molecules and killing capacity of mycobacteria-specific CD4 and CD8 T cells.

2.4. Discussion

Our laboratory has developed a flow-cytometric assay to measure killing of PPD-pulsed monocytes by PPD-specific cytotoxic T cells. In this optimisation exercise we aimed to expand the use of this assay to measure killing of mycobacteria-infected monocytes by BCG-specific cytotoxic T cells. Ultimately, we wanted to investigate cytotoxic killing of BCG-infected

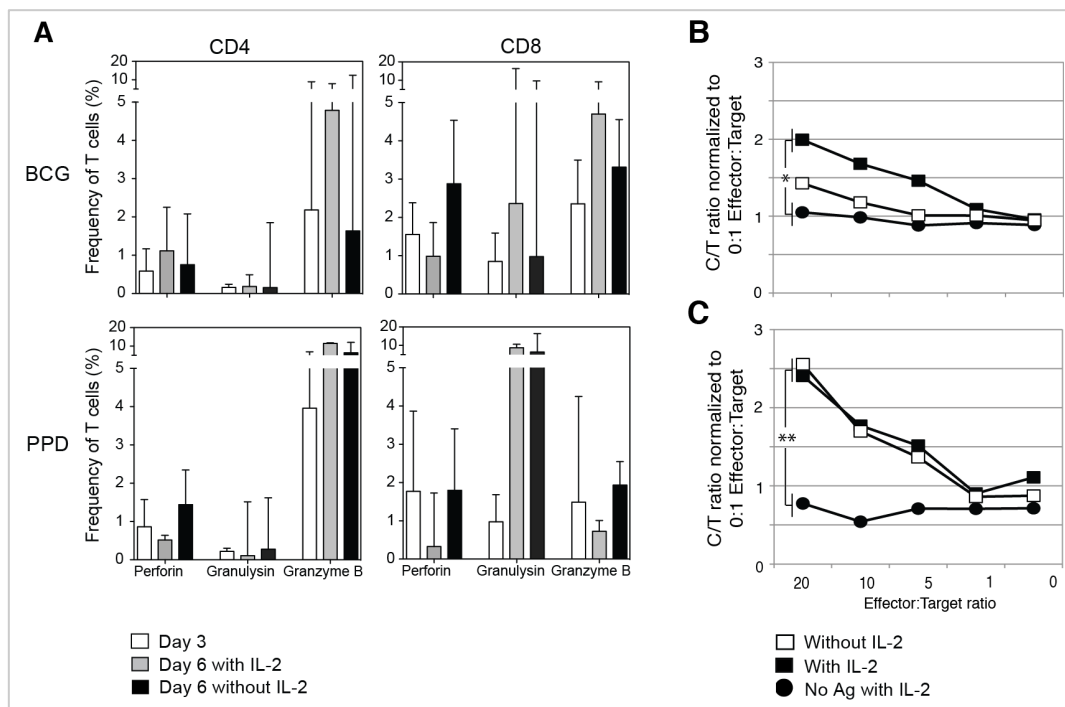


Figure 2.6. The effect of IL-2 addition on cytotoxic T cell function. (A) Freshly isolated adult PBMC were cultured with BCG or PPD. Production of perforin, granulysin and granzyme B by Ki67+ CD4 (grey) and CD8 (white) T cells was assessed by flow-cytometry on day 3 (clear bars) and on day 6 with (grey bars) and without (black bars) IL-2. Data shown is the median with interquartile ranges calculated from four adult donors. Killing of BCG-infected (B) and PPD-pulsed (C) monocytes by the antigen-specific effectors expanded with (black squares) or without IL-2 (white squares) was also assessed. Effectors were also expanded with IL-2 in the absence of antigen (black circles).

monocytes by antigen-specific effectors in infants. However, due to the bloodvolume required to obtain sufficient cell numbers for this assay, we opted to pursue our investigation in adults. Sufficient cell yields were obtainable using adult whole blood.

Our established killing assay protocol requires pulsing of monocytes with PPD for 18 hours. However, infection of monocytes with live BCG for 18 hours may lead to increased cell death. We compared viability of monocytes pulsed with live BCG for 2, 4 and 18 hours. Viability of the monocytes decreased over time, however, this decrease was not significant. In these experiments, a BCG MOI 0.1 was used. Which is consistent with our cytotoxic marker assay [49]. We found this dose to be optimal for infecting monocytes. In addition, infection with heat-killed BCG did not improve monocyte viability in the four donors assessed. This observation should,

however, be interpreted with caution due to the low sample size. Interestingly, both live and heat-killed BCG have also been shown to prevent apoptosis through the upregulation of anti-apoptotic proteins [124].

IL-2 has been shown to be a master regulator of proliferation of hemapoietic cells [125] and thus have long been used in cytotoxicity assays to expand antigen-specific effectors. IL-2 has also been shown to play a role in the development of cytotoxic T cells and NK cells. Following suit, our cytotoxic killing assay uses IL-2 to expand antigen-specific T cells [119]. Thus, we wanted to determine that our assay is specific and not driven by the recombinant IL-2 added to induce proliferation. Indeed, we did not find differences in the production of cytotoxic molecules by specific T cells cultured in the absence or presence of IL-2. Similarly, no difference could be detected between the killing capacity of these cells. Moreover, cells expanded in the presence of IL-2 in medium alone showed significantly lower killing capacity compared to mycobacteria-specific cytotoxic T cells.

In essence, we have expanded the use of our flow-cytometry based cytotoxicity assay to determine killing of BCG infected target cells by mycobacteria-specific T cells. Firstly, we have identified an optimal dose of BCG to ensure that killing is driven by cytotoxic T cells and not by the bacteria itself. Secondly, we determined that IL-2 does not contribute to the cytotoxic potential and function of the T cells.

2.5. Contributions

Alana Keyser designed and conducted the experiments, analysed the data and wrote this chapter under supervision of Prof. W.A. Hanekom, Prof. T. Scriba and Dr. A. Penn-Nicholson.

Chapter 3: T cell capacity to produce cytotoxic molecules may be a correlate of risk of TB disease, following newborn vaccination with BCG.

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3.1. Abstract

Our aim was to identify biomarkers of protection against TB disease in infants, following routine vaccination with Bacille Calmette-Guerin (BCG) at birth. 5,675 infants were enrolled at 10 weeks of age, when blood was collected and stored. During 2 years of follow-up, 29 infants developed culture-positive pulmonary TB: they were regarded as being at risk of TB disease. Two groups of healthy infants were regarded as not at risk of TB disease: 55 infants from households where adults had TB, and 55 infants randomly selected from the cohort.

PBMC from the infants at risk and those not at risk of TB disease, stored at 10 weeks of age, were retrieved and mycobacteria-specific responses compared. We found no difference in proliferation of CD4 and CD8 T-cells following incubation of PBMC with BCG for 6 days, between the 4 groups. However, these infants' CD4 and CD8 T-cells had greater capacity to produce granzyme B, perforin and granulysin, following incubation of PBMC with BCG for 3 days.

To validate findings, we repeated assays in another group of 29 infants at risk of TB disease and 55 infants not at risk of TB disease, from the same cohort. The only finding that could be validated was a greater frequency of CD4 T-cells

producing granzyme B among infants at risk of TB disease; other findings from the discovery cohort did not validate.

We conclude that a commonly used outcome of mycobacteria-specific immunity, the proliferative response and Th1 cytokine production, did not correlate with ultimate prevention of TB disease progression. Rather, our results suggest that capacity to produce cytotoxic molecules might be a correlate of risk of TB disease, and should be studied further.

3.2. Introduction

BCG has been in use as the only vaccine against TB disease for the past 90 years. Despite high vaccination coverage in TB endemic countries, the disease still remains very common. Clearly, BCG-induced protection against TB disease is suboptimal. However, BCG does protect against severe forms of TB in children, and is therefore used extensively.

A considerable number of new TB vaccines, aimed at either replacing BCG or boosting the BCG-induced immune response, are at various stages of clinical trials [126]. Development of these vaccines is hampered by the lack of defined biomarkers of protection against TB disease. Correlates of protection may be identified in the setting of a clinical trial of an effective vaccine, which included a placebo group. In the meantime, we are focusing on correlates of risk of TB disease, with the hypothesis that knowledge gained would inform about protection.

Establishment of memory T cell pools through vaccination is thought to be essential in protection against disease. IL-2 is required for the expansion and maintenance of such memory T cell pools [127], [128]. IFN- γ produced by both CD8 and CD4 T cells is thought to have an essential role for M.tb control due to the multiple antimicrobial functions [57] [129] [130]. IFN- γ activates macrophages to produce the antimicrobial molecules, RNI and ROI to control the intracellular pathogen, and the cytokine induces upregulation of MHC class II molecules on the surface of infected macrophages [131]. In addition, IFN- γ induces autophagy, another mycobacteria [23]. TNF- α has been shown to be important in the induction

of chemokine expression for the recruitment of macrophages, granuloma formation and RNI production [60] [132]. The importance of TNF- α in the host response against tuberculosis is further emphasized by the association between TB disease progression and anti-TNF therapy of autoimmune patients [133].

Thus, together, these cytokines potentially presents a united front in the host response against TB disease progression. Moreover, proliferative capacity and concomitant production of these Th1 cytokines, IL-2, IFN- γ and TNF- α in response to *M. bovis*-challenge have been associated with protection in BCG-vaccinated mice [134].

In addition to the Th1 response, host responses against TB infection also involve killing of infected APC and the pathogen by cytotoxic T cells. This cytolytic response involves production of cytotoxic molecules such as granzyme B, perforin and granulysin by CD4 and CD8 T cells [73]. These molecules have the ability to penetrate infected macrophages and dendritic cells and induce killing mechanisms either directly or through the induction of caspases [135] [136]. BCG vaccination has been shown to induce the expression of these molecules, and reduced expression of granulysin and perforin have been implicated in TB disease progression [49] [78] [137] [138].

Anti-inflammatory Th2 cytokines have been shown to associate with poor outcome in TB infection. Mice vaccinated with a *M.tb*-culture filtrate in the presence of the Th2-inducing adjuvant aluminum, showed increased expression of IL-4 and IL10 associating with increased mycobacterial growth [139]. Moreover, IL-4 has been shown to be expressed at high levels in TB cases compared to controls in various studies [38] [140]. A pre-existing or induced Th2 immune response may thus lead to predisposition to develop TB disease.

In this study we aimed to identify biomarkers of risk of TB disease in BCG-vaccinated infants who went on to develop TB within the first two years of life. We compared proliferative capacity and cytokines produced by proliferating cells as well as production of cytotoxic molecules by T cells in

response to BCG between infants who went on to develop TB and those who remained healthy at 10 weeks after vaccination when infants were healthy. We hypothesized that reduced proliferative capacity and concomitant Th1 cytokine production as well as reduced production of cytotoxic molecules by T cells in response of BCG, will associate with risk of developing TB disease. In addition, we hypothesized that a measurable Th2 immune response in terms of IL4 expression will associate with disease development.

3.3. Materials and Methods

3.3.1. Participant recruitment, enrollment, blood collection and follow-up

Study participants were recruited at the South African Tuberculosis Vaccine Initiative (SATVI) field site in the Worcester area, near Cape Town, South Africa. Infants, 10 weeks of age, who received Japanese BCG within 24 hours of birth, were recruited and enrolled into the study. Infants were excluded when BCG had not been administered within 24 hours after birth, when significant perinatal complications had been present, when any acute or chronic disease at the time of enrollment, including clinically apparent anaemia, when infants had been born to HIV+ mothers, or when the infant was from a household where any person had TB disease or a cough. Informed consent was obtained from the parent or legal guardian according to US Department of Health and Human Services and Good Clinical Practice guidelines. This included protocol approval by the University of Cape Town Research Ethics Committee.

Heparinised blood was collected from each infant, at 10 weeks of age. Study participants were followed up for a period of two years to identify those who developed TB. Infants who had a household TB contact and those who had signs suggestive of TB disease were admitted to a dedicated ward. Evaluation for possible TB included a chest X-ray, 2 induced sputa and 2 gastric washings for *M.tb* smear or culture, a tuberculin skin test and a HIV test. Infants and children with a positive HIV antibody test were excluded from the study. A diagnostic algorithm was used to classify TB

cases as definite, probable and possible. Definite disease (n=29) was defined as culture positive TB, whereas probable disease (n=29) was defined as culture negative presenting with clinical and epidemiological features strongly suggestive of TB disease. Among children who did not have TB disease, household controls were defined as children exposed to a household TB contact (n=55), while community controls were defined as healthy infants with unknown TB exposure (n=55).

At 10 weeks of age when blood was collected and cryopreserved for analysis, all infants were clinically healthy but may have presented different levels of risk of developing TB. TB cases identified during the two-year follow-up period, were considered to be, at the 10 week-bleed, at risk of TB disease. Similarly, control groups were considered as not at risk at 10 weeks of age, as they did not ultimately develop TB disease.

3.3.2. Cytotoxic marker assay

PBMC were isolated from blood collected at 10 weeks of age by density gradient centrifugation, and cryopreserved. Later, PBMC were thawed in culture medium (12.5% v/v AB serum in RPMI) containing 10µg/mL DNase (Sigma-Aldrich). After washes, cells were rested overnight at 37°C in 5% CO₂. Duplicate wells containing 2x10⁵ cells in 200µL culture medium were incubated with BCG, prepared as previously described [49], at an MOI of 0.1, at 37°C in 5% CO₂. Staphylococcus enterotoxin B (SEB, 0.5µg/mL) served as positive control, while medium only served as negative control. On day 4, cells were harvested with EDTA (2mM) and stained with 1µg/ml Fixable Violet Dead Cell Stain (LIVE/DEAD®, Invitrogen). Cells were fixed with FACS lysing solution (BD Biosciences) and cryopreserved. Later, cryopreserved, fixed cells were thawed, washed and permeabilized with Perm/Wash solution (BD Biosciences), and stained with the following antibodies for 1h at 4°C: anti-CD4 Qdot605 (clone UCHT1), anti-CD8 Cy5.5PerCP (Sk1), anti-granzyme B Alexa 700 (GB11), anti-perforin FITC (δG9; all from BD Biosciences) and anti-granulysin PE (eBioDH2, eBioscience). Cells were then washed and acquired on a LSRII flow cytometer (BD Biosciences), using FACS Diva 6.1 software. Anti-mouse kappa beads (BD Biosciences), stained with the respective fluorescent-

labeled antibody, were used to configure compensation settings beforehand. Anti-CD4 PacBlue (UCHT1) was used to set compensation for the violet viability stain. A positive response was regarded as a frequency of granzyme B-expressing CD4 or CD8 T cells, following SEB incubation, that was greater than the median frequency plus 4 median absolute deviations (MAD) of the negative control,

3.3.3. Lymphoproliferation assay

PBMC were stained with 1µg/mL Oregon Green (Molecular Probes) and rested overnight at 37°C in 5% CO₂. PBMC were incubated with BCG, MOI 0.01, at 2x10⁵ in 200µL culture medium for 6 days, at 37°C in 5% CO₂. Controls included SEB (0.05µg/mL) or medium alone. To determine the cytokine producing capacity of specific (proliferating) cells, phorbol 12-myristate 13-acetate (PMA, 50ng/mL, Sigma-Aldrich) and ionomycin (250ng/mL, Sigma-Aldrich) was added on day 6, in the presence of brefeldin A (10µg/mL, Sigma-Aldrich), for 5 hours. Cells were then harvested, stained with the viability stain, fixed and cryopreserved as described above. Later, cells were thawed and washed as described above, and stained with anti-CD4 and anti-CD8 as above, and with anti-IFN γ Alexa 700 (B27), anti-IL2 APC (MQ1-17H12), anti-TNF α PECy7 (MAb11), anti-IL4 PE (8D4-8, all from BD Biosciences). Compensation settings were configured as mentioned above. Anti-perforin FITC (δ G9) was used to set compensation for Oregon Green. A positive proliferative response was regarded as a frequency of proliferating CD4 T cells, following SEB stimulation, that was greater than the median frequency plus 4 MAD of the negative control.

3.3.4. Data analysis and statistical considerations

Flow cytometric data was analyzed using Flowjo 8.8.4 (Treestar). For the cytotoxic marker assay, results from infants who had a frequency of granzyme B-expressing CD4 or CD8 T cells, following SEB incubation, that was greater than the median frequency plus 4 median absolute deviations (MAD) of the negative control, were included. Similarly, only infants who had a frequency of proliferating CD4 T cells, following SEB stimulation, that was greater than the median frequency plus 4 MAD of the negative control, were included in the analysis of the lymphoproliferation assay. Furthermore,

infants with fewer than 25 proliferating cells in response to BCG were excluded from the analysis of the cytokine production by proliferating CD4 T cells. The proliferation index was calculated as the frequency of CD4 and CD8 T cells proliferating in response to BCG divided by the proliferating frequencies in unstimulated samples. Boolean gating was used to identify subsets of CD4 and CD8 T cells expressing combinations of cytotoxic markers.

Assay results were compared between the following groups of infants: a training cohort consisting of infants who ultimately were classified to have definite TB disease, household controls and community controls; a test cohort, to validate observations made in the training cohort, consisting of infants who ultimately were classified to have probable TB, and community controls. Differences between the three groups were assessed by the Kruskal-Wallis test. The Mann-Whitney U test was then used to compare individual groups. P-values were adjusted for multiple comparisons by using the Bonferonni correction. A p-value < 0.05 was considered as significant.

3.3.5. Generation of expanded effector cells

PBMC were isolated from fresh, adult whole blood by density gradient centrifugation. Cells were incubated with 2µg/mL purified protein derivative (PPD Tuberculin RT50; Staten Serum Institute) or medium alone as negative control, at 2×10^6 PBMC in 1mL culture medium at 37°C in 5% CO₂. On day 4, cells were resuspended and 200µL of cells were used to assess cytotoxic capacity as described above. Fifty units of IL-2 was added to the remaining cells and incubated until day 6. Cultures were then replenished with fresh culture medium and incubated overnight. On day 7, cells were harvested with repeated wash steps with cold RPMI followed by centrifugation, resuspension in fresh culture medium and incubation overnight at 37°C in 5% CO₂, for co-culture with target cells (below).

3.3.6. Generation of PPD-pulsed target cells

On day 7 of effector T cell expansion, PBMC were isolated from fresh, adult whole blood by density gradient centrifugation. Autologous monocytes were isolated using anti-CD14 microbeads (MACS Miltenyi Biotec) as per the manufacturer's recommendations. Isolated monocytes were then

resuspended of which half of the cells were left unpulsed (control target cells) and half were pulsed with 20µg/mL PPD at 37°C in 5% CO₂ overnight in culture medium. Next, pulsed monocytes were washed and stained with 0.5µg/mL Oregon Green. Control target cells were stained with 50µM CellTracker Orange CMTMR (Molecular Probes).

3.3.7. Co-culture of effector and target cells

Cell counts were determined and effector and target cells were co-cultured at the following ratios with the target cells consisting of equal numbers of control and pulsed cells: 50:1, 20:1, 10:1, 5:1, 1:1 and 0.5:1. Cells were incubated overnight at 37°C in 5% CO₂, harvested, stained with the violet viability dye and acquired fresh on a LSRII flow cytometer to assess killing by PPD-specific effector cells. Anti-CD4 PacBlue, anti-perforin FITC and anti-IL4 PE were used to set compensation for the violet viability dye, Oregon Green and CellTracker Orange, respectively.

3.4. Results

3.4.1. Study participants

A total of 5,724 infants routinely vaccinated with BCG at birth, were randomly enrolled from a parent cohort of 11,680 infants [141]. Figure 3.1. shows the selection of the 3 groups of infants included in the training cohort (black boxes), to identify markers that associate with risk of TB disease, and 2 groups of infants included in the test cohort (dark grey boxes), to test observations found with the first round of analysis.

3.4.2. Increased expression of cytotoxic molecules by CD4 and CD8

T cells in infants at risk of TB disease, in the training cohort

Cytotoxicity is a critical T cell function allowing the immune system to kill pathogens either directly or after they have been taken up by APC. Cytotoxic molecules are constitutively expressed and maintained within cytotoxic granules in the cytosol. These granules migrate to the cell surface where the contents is released into the immunological synapse to commence killing of the infected cell. We assessed the production of cytotoxic markers by CD4 and CD8 T cells in our 3 day cytotoxic marker assay, which allows for the measurement of newly synthesised molecules

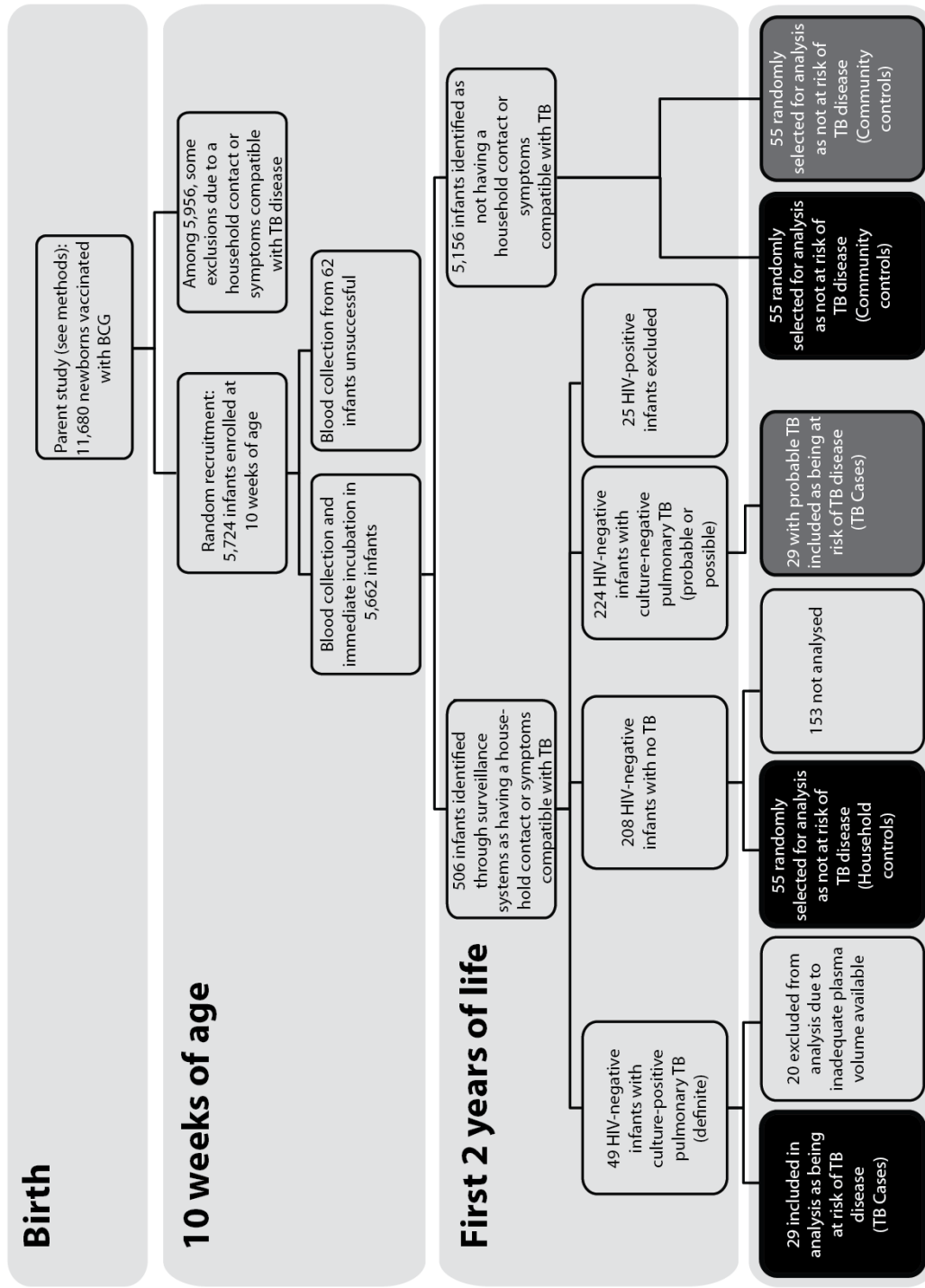


Figure 3.1. Study participants. Recruitment and enrollment of participants into the study. Training and test cohorts are indicated as black and dark grey boxes, respectively. The training cohort included 29 infants who developed definite TB disease as well as 55 household controls and 55 community controls. The test cohort included 29 infants who developed probable TB disease and 55 community controls.

accumulated in the granules. PBMC collected at 10 weeks, when all infants were healthy, were thawed, incubated with BCG and assessed for the production of granzyme B, perforin and granulysin. The frequency of CD4 and CD8 T cells expressing cytotoxic markers was assessed by flow cytometry (Figure 3.2). Incubation of PBMC with BCG induced production of cytotoxic markers granzyme B, granulysin and perforin by both CD4 and CD8 T cells in all three groups of infants (Figure 3.3. A–D). Frequencies of cells expressing cytotoxic markers were similar between CD4 and CD8 T cells.

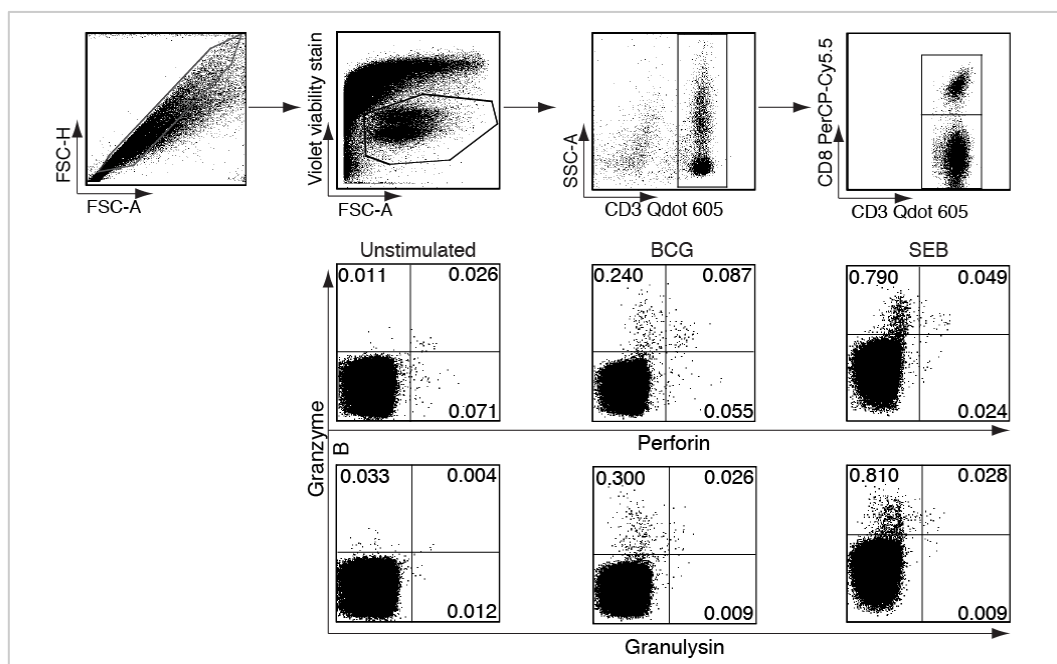


Figure 3.2. Flow-cytometric analysis to assess cytotoxic capacity of CD4 and CD8 T cells. A representative sample illustrating the gating strategy used to identify CD4 and CD8 T cells producing cytotoxic molecules in response to stimuli.

Infants at risk who ultimately developed TB disease, had higher frequencies of CD4 T cells producing granulysin, compared with household controls (Figure 3.3. A). This group also had higher frequencies of CD4 T cells co-expressing granulysin, granzyme B and perforin, and CD4 T cells co-expressing granulysin and granzyme B (Figure 3.3. B). Among CD8 T cells, infants at risk of TB disease had higher frequencies of CD8 T cells expressing granzyme B, compared to those who are not at risk of TB disease (Figure 3.3. C). Infants at risk of TB disease also had a higher frequency of CD8 T cells co-expressing granulysin and granzyme B, compared to community controls (Figure 3.3. D). Assessment of cytotoxic

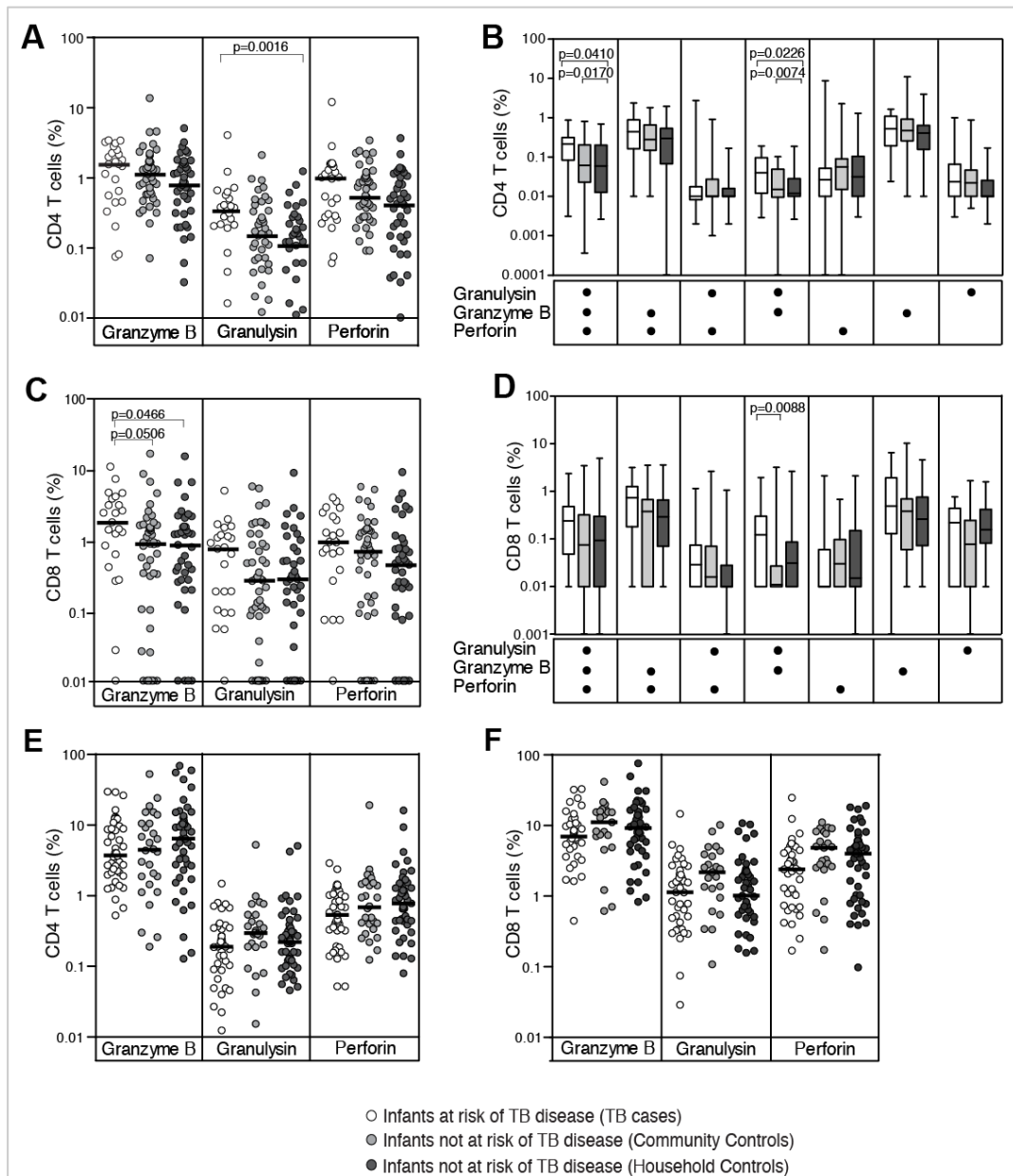


Figure 3.3. Cytotoxic capacity of BCG-specific CD4 and CD8 T cells. PBMCs were stimulated for three days with BCG, SEB or medium alone. (A) Frequency of total granzyme B, granulysin and perforin producing CD4 T cells in response to BCG, as determined by intracellular staining and flow cytometry. (B) Frequency of CD4 T cell subsets producing combinations of the cytotoxic molecules in response to BCG. (C) Frequency of total granzyme B, granulysin and perforin producing CD8 T cells in response to BCG. (D) Frequency of CD8 T cell subsets producing combinations of the cytotoxic molecules in response to BCG. (E) Frequency of total granzyme B, granulysin and perforin producing CD4 T cells in response to SEB. (F) Frequency of total granzyme B, granulysin and perforin producing CD8 T cells in response to SEB. Horizontal lines indicate the median values. The Kruskal-Wallis test was used to determine differences between the three groups. Significance of differences was determined using the Mann-Whitney U test.

molecule expression in response to SEB showed high frequencies of CD4 (Figure 3.3. E) and CD8 (Figure 3.3 F) T cells producing granzyme B, granulysin and perforin at comparable levels between the three groups.

We concluded that the capacity of T cells to express cytotoxic markers, following incubation with PBMC for 3 days with BCG, was greater in infants at risk compared to those not at risk of TB disease.

3.4.3. CD4 and CD8 T cell proliferative response, and cytokine producing potential of specific cells, in the training set

Another important T cell functional characteristic is the ability to proliferate upon encounter of antigen. This allows for the expansion of the antigen-specific memory T cell population for effective activation of the APC to eliminate the pathogen. We assessed proliferation of PBMC following incubation with BCG with a dye dilution assay (Figure 3.4. A). Proliferation of both CD4 and CD8 T cells in response to BCG could be detected after stimulation in most infants (Figure 3.4. B). Interestingly, CD4 and CD8 T cells showed similar levels of proliferation in response to BCG. The capacity to proliferate was similar in the 4 groups of infants (Figure 3.4. B). We conclude that there is no difference in the CD4 and CD8 T cell proliferation in response to BCG, between infants at risk and those not at risk of TB disease.

The capacity of BCG-expanded cells to produce either Th1 or Th2 cytokines, or combinations of Th1 cytokines, was also compared between protected and unprotected infants. Cytokine production was induced by incubating the OG-stained, cultured PBMC with PMA and Ionomycin for 5 hours before cells were harvested. OG-stained PBMCs were fixed and stained intracellularly with fluorescent antibodies for detection of IFN- γ , IL-2, TNF- α and IL-4 production by proliferating cells. Cytokine production was detected by gating on OG^{low} CD4 and CD8 T cell populations (Figure 3.4. A and 3.5. A). A total of 49 infants were excluded due to poor proliferation of CD4 T cells in response to SEB (SEB-induced proliferation < 3MAD + median above the unstimulated condition) and a low number of CD4 proliferating cells (< 25) in response to BCG.

BCG-expanded PBMC produced higher levels of cytokine production compared with PBMC cultured in medium alone (Figure 3.5. A). Dividing

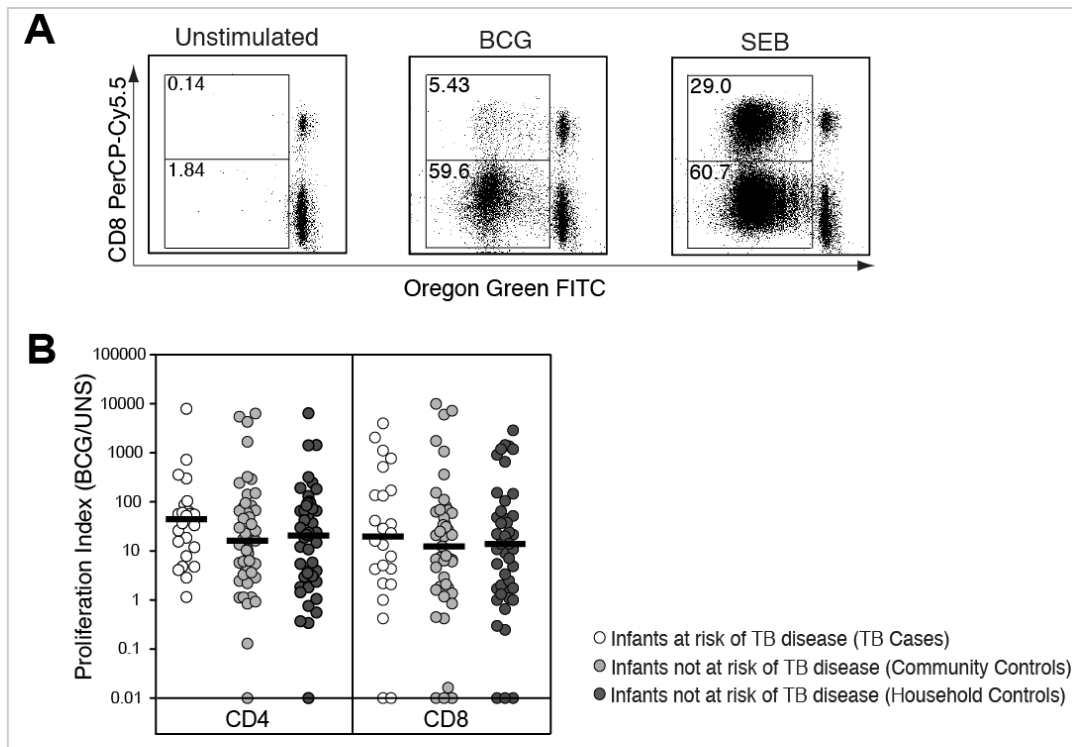


Figure 3.4. Proliferative potential of BCG-specific CD4 and CD8 T cells. PBMCs were stained with Oregon Green and stimulated for six days with BCG, SEB or medium alone. (A) Flow cytometric detection of Oregon green low CD4 and CD8 proliferating T cells. (B) BCG-induced proliferation of CD4 and CD8 T cells expressed as proliferation above background. Bars indicate medians. The Kruskal-Wallis test was used to determine differences between the three groups. Significance of differences was determined using the Mann-Whitney test for non-parametric data. P-values were adjusted for multiple comparisons by using the Bonferroni correction. No significant differences were found between the groups.

CD4. T cells showed comparable expression of IFN- γ , IL-2 and TNF- α in all three groups (Figure 3.5. B) in response to BCG. IL-4 expression by CD4 T cells was detected at similar levels in all groups. However, these levels were much lower compared to CD4 T cells producing the Th1-cytokines. No difference could be detected when comparing Th1 cytokine co-expression by proliferating CD4 T cells between the three groups (Figure 3.5. C). Cytokine production by CD8 proliferating T cells could not be analysed due to a low number of samples meeting our criteria of >25 proliferating cells.

We concluded that there is no difference in the expression of Th1 and Th2 cytokines between the infants at risk and those not at risk of TB disease.

3.4.4. Evaluation of increased BCG-induced CD4 and CD8 cytotoxic T cell function in a infant test cohort.

BCG-induced responses in a second group of 29 infants at risk of TB disease (probable TB cases) and 55 community controls, not at risk of TB disease, were analysed to validate our findings of increased cytotoxic potential, proliferative capacity and IFN- γ production in TB unprotected infants. We repeated the assays described on cryopreserved PBMCs collected at 10 weeks of age from these infants.

CD4 T cells showed quantitatively no difference in cytotoxic marker expression in response to BCG between the groups (Figure 3.6. A). BCG did, however, induce a significantly higher frequency of CD4 T cells producing only granzyme B in infants at risk compared to the group not at risk of TB disease. (Figure 3.6. B) was detected BCG-stimulation induced similar levels of CD4 and CD8 T cell proliferation in both groups. Similar frequencies of CD8 T cells and subsets expressing granzyme B, granulysin and perforin was detected in the two groups (Figure 3.6. C and D).

No difference in BCG-induced proliferation of CD4 and CD8 T cells could be detected between the infants at risk and the groups not at risk of TB disease. A total of 14 and 17 infants showed no CD4 or CD8 T cell proliferation, respectively, above the unstimulated sample in response to BCG. Qualitative analysis of cytokine production showed similar frequencies of proliferating CD4 T cell subsets between the two groups (Figure 3.6. E).

We compared the cytotoxic responses of infants in the training and test cohorts (Figure 3.7). This showed increased production of cytotoxic markers by the community control group of the test cohort compared to the control groups of the training cohort. Community controls in the test cohort showed higher frequencies of CD4 T cells producing granulysin and perforin compared to controls of the training cohort (Figure 3.7. A). Similarly, controls of the test cohort showed increased frequencies of CD8 T cells producing granulysin and granzyme B (Figure 3.7. B). Interestingly, no differences were detected in the CD4 and CD8 T cell cytotoxic responses among the groups of infants at risk of TB disease in the training and test cohorts.

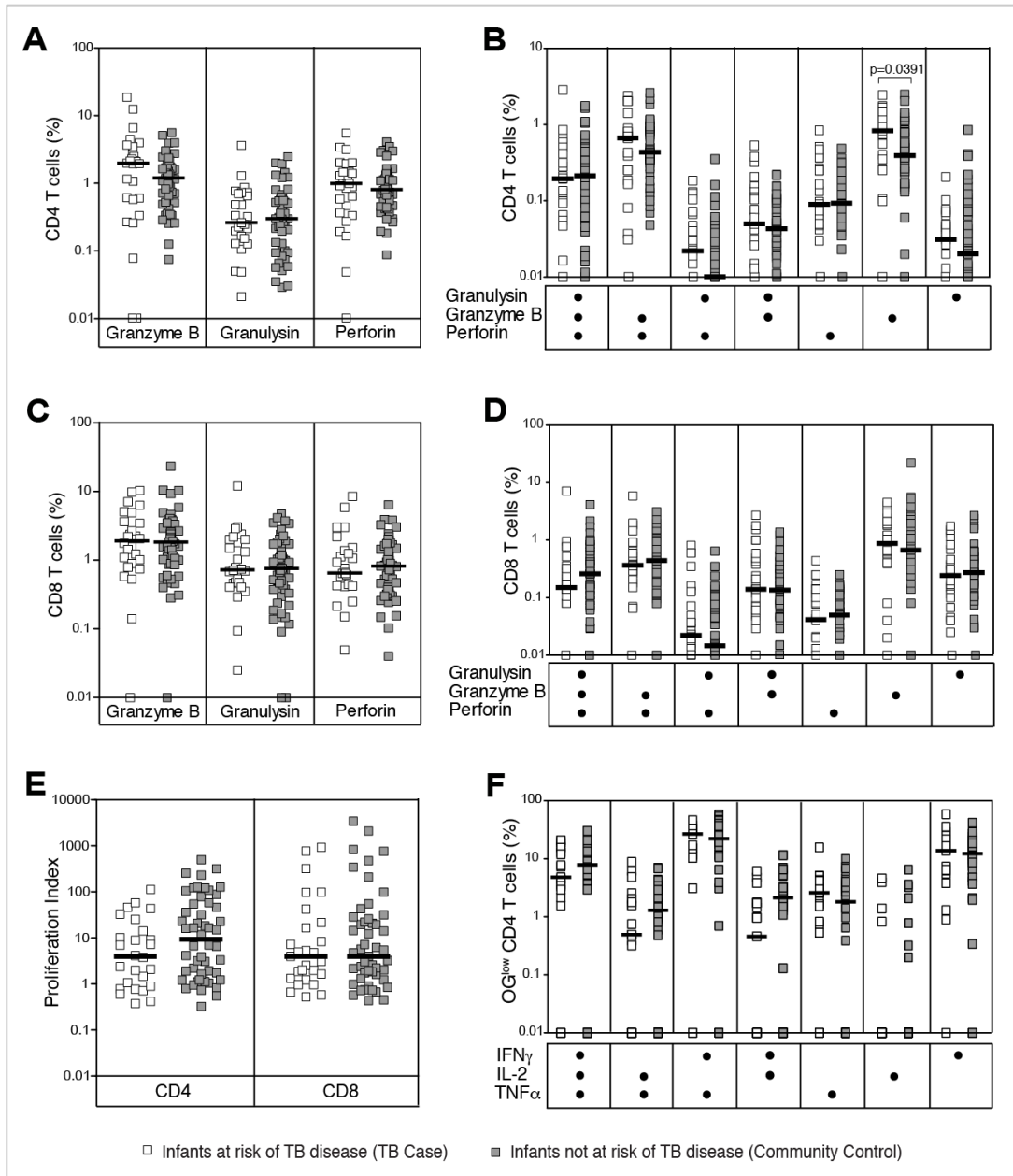


Figure 3.6. Validation of findings in a test cohort. PBMCs from a second group of 29 TB unprotected and 55 TB protected infants were used to validate results from the training group. All assays were repeated and the same gating strategies were used. Frequency of total granzyme B, granulysin and perforin producing CD4 (A) and CD8 T cells (C). Frequency of CD4 (B) and CD8 T cell subsets (D) producing combinations of the cytotoxic molecules. (E) BCG-induced proliferation of CD4 and CD8 T cells expressed as proliferation above background. (F) Frequency of CD4 T cell subsets producing combinations of the cytokines. Horizontal lines indicate the median values. Significant differences were determined using the Mann-Whitney test for non-parametric data. $P < 0.05$ was taken as significant.

We concluded that infants at risk of TB disease have a higher frequency of CD4 T cells producing only granzyme B in response to BCG, compared to infants not at risk of TB disease; however, we could not validate other differences seen in the primary analysis.

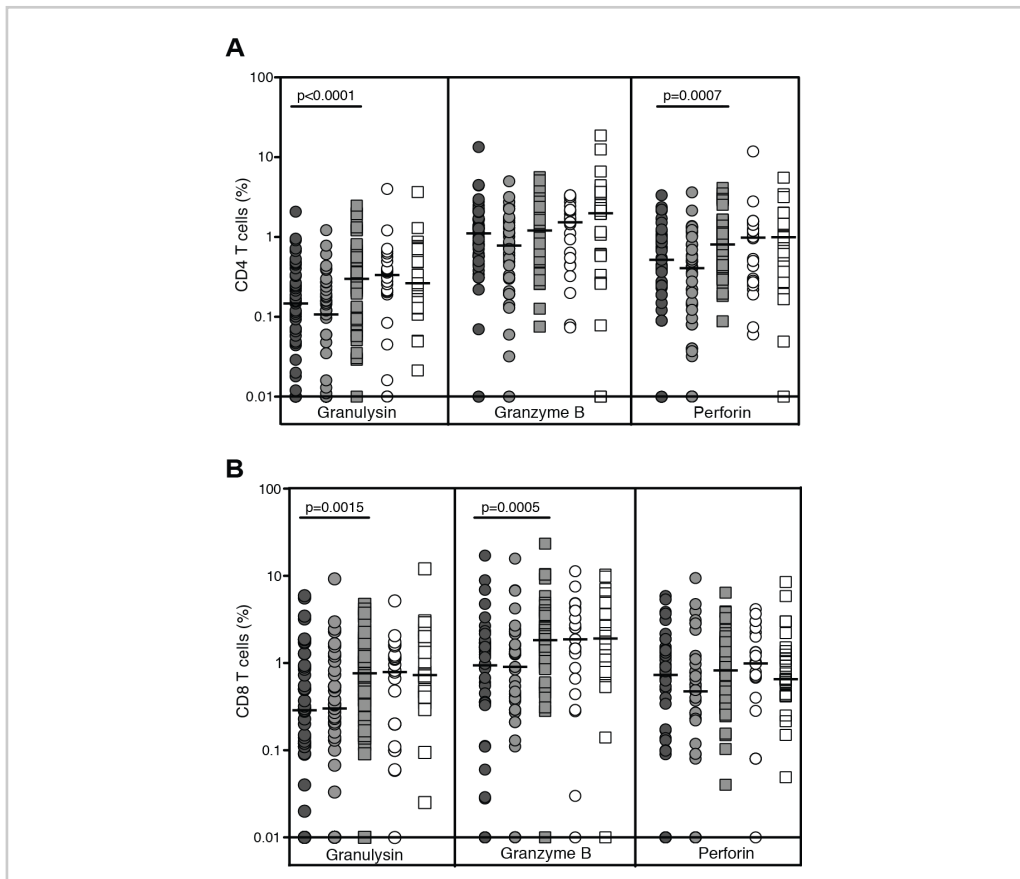


Figure 3.7. Comparison of cytotoxic T cell responses. Frequencies of total granzyme B, granulysin and perforin producing CD4 (A) and CD8 T cells (B) are plotted for comparison of the test (circles) and validation (squares) cohorts. Horizontal lines indicate the median values. Significant differences were determined using the Kruskal-Wallis test for non-parametric data. $P < 0.05$ was taken as significant.

3.4.5. Good correlation between production of cytotoxic molecules and killing capacity of mycobacteria-specific T cells.

Co-culture of PPD-specific T cells with PPD-pulsed monocytes showed a reduction in the percentage of monocytes after 18 hours at various ratios of effector:target ratios (Figure 3.8 A and B). The production of cytotoxic molecules by CD4⁺granzyme B⁺granulysin⁺perforin⁺ as measured at day 3 correlated well with the killing capacity of PPD-specific T cells at a effector:target ratio of 10:1 (Figure 3.8 C). No correlations could be detected between other CD4 subsets and effector:target ratios.

We conclude that the cytotoxic molecules measured after 3 days culture with BCG, have the capacity to kill monocytes infected with mycobacterium.

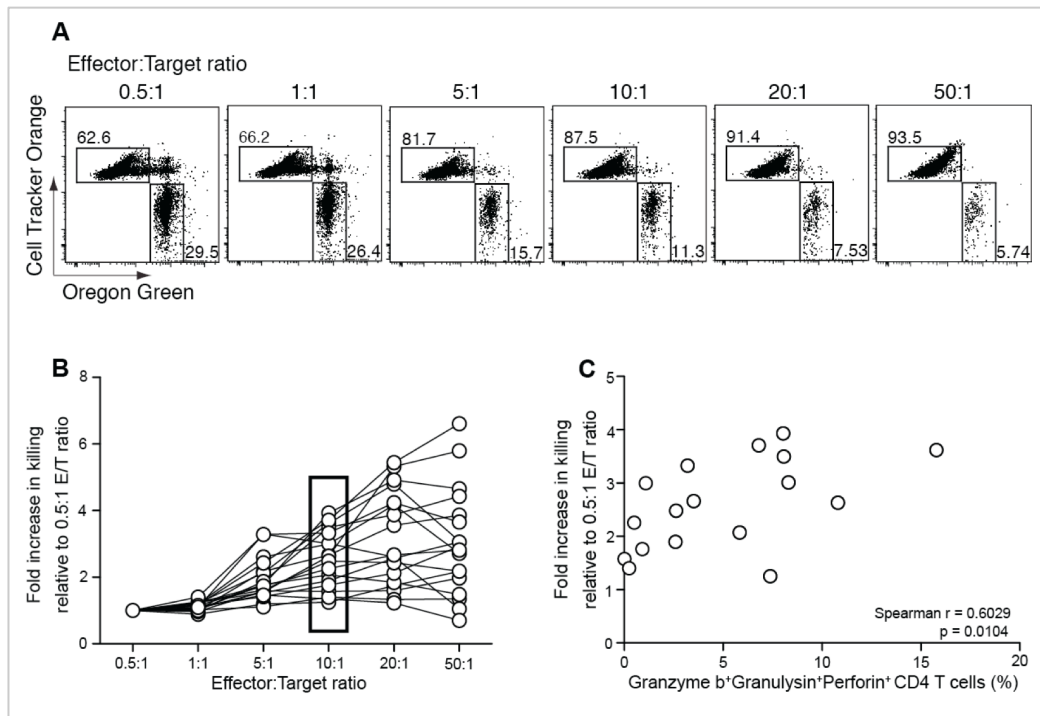


Figure 3.8. Killing capacity of mycobacteria-specific T cells producing cytotoxic molecules measured on day 3. (A) PBMC stimulated for 6 days with PPD were co-cultured with PPD-pulsed monocytes at different effector:target ratios for 18 hours. (B) PPD-specific T cell production of cytotoxic molecules measured at day 3 was assessed for killing capacity measured after 6 days of PPD-expansion. (C) Correlation between infected monocyte killing and cytotoxic CD4 T cells.

3.5. Discussion:

The analysis described in this thesis forms part of a greater study to identify biomarkers which can predict risk of TB disease prospectively [142]. Our group has previously shown that BCG induces a broad immune response, including Th1, Th2 and cytotoxic responses, in infants 10 weeks after vaccination [49] [90].

Contradictory to our hypothesis, analysis of BCG-induced responses in our infant cohorts showed no differences in cytotoxic potential or proliferative capacity between newborn-vaccinated infants at risk and those not at risk of TB disease. Initial analysis of a primary infant cohort showed increased expression of cytotoxic molecules in infants who developed TB compared to those who did not. However, analysis of the test cohort, did not support this observation; with the exception of CD4 T cells expressing only granzyme B, protected and unprotected infants showed similar expression of cytotoxic molecules. We found that the community controls in the test cohort showed similar cytotoxic marker expression to the TB cases, however, this was

significantly higher than the controls in the training cohort. This discrepancy may be due to technical reasons. PBMC were cryopreserved for ± 8 years before this analysis was done and, additionally, analysis of the cohort followed more than 6 months after the analysis of the training cohort. This means that these samples were cryopreserved for a longer period. Quality control data in our laboratory to assess the effect of cryopreservation, shows a decrease of about 10% in PBMC viability after 4 years of cryopreservation. Indeed, inclusion of a viability dye revealed high levels of cell death possibly as a result of cryopreservation which may affect immunological outcomes as observed by others [143]. We did not assess the effect of cryopreservation on proliferation or cytotoxic molecule expression and function. However, good responses in terms of proliferation and production of cytokine and cytotoxic molecules were observed in our study groups, in response to the positive control. In addition, due to the time-delay, a different set of reagents was used for the analysis of the test cohort which may also account for this disparity. These variables need to be controlled for in future studies of such nature. However, it remains unclear why the cytotoxic responses of only the controls in our second cohort increased compared to the other controls.

Granulysin, together with perforin and IFN- γ , have been implicated as a potential biomarker of protection to evaluate bovine BCG-vaccination efficacy [144]. Clinical studies, however, have identified granulysin rather as a possible biomarker of TB disease activity. Mueller *et al*, showed increased CD4⁺ memory populations expressing granulysin by TB cases compared to controls [145]. This is in contradiction to Di Liberto who found decreased plasma granulysin in TB cases compared to controls [137]. Taking this into consideration, it may be possible that the increased cytotoxic activity observed in the unprotected infants may be an indication of *M. bovis* BCG persisting 10 weeks post-vaccination; exposure to environmental mycobacteria at this age is unlikely and *M.tb* infection was controlled for by excluding infants with a cough or household TB contact. However, further analysis is required to establish BCG persistence. Hesseling *et al*, have reported the persistence of BCG in an immunocompetent infant six months after vaccination [146].

Cytotoxic molecules have also been implicated in autoimmune diseases. The protease activity of, primarily, extracellular granzyme B allow for its ability to cleave proteins involved in the extracellular matrix. This in turn, leads to an array of inflammatory responses which manifests in autoimmune diseases of the lung, amongst other, such as chronic obstructive pulmonary disease, asthma and acute respiratory distress syndrome [147]. Thus, it may be informative to explore inflammation further as it is possible that these infants may have an inherent capacity to induce excessive pro-inflammatory response after subsequent infection.

Additional analysis of these samples, not described in this thesis, supports the concept of excessive inflammation in response to mycobacterial infection. BCG-stimulated and unstimulated PBMC from our study participants were analysed at gene-expression level. TB cases showed increased expression of genes associated with inflammation.

The immune response against *M.tb* infection is of a pro-inflammatory nature characterised by increased expression of chemokines for cell recruitment as well as pro-inflammatory cytokines such as IFN- γ and TNF- α for APC activation and granuloma formation. The ability to control this immune response in order to prevent tissue damage and further immunopathology, is thus crucial to resolve the infection. Recently, Horne *et al* described a genetic association between TB disease and inflammation; a single-nucleotide polymorphism in a IL1-TLR inhibitor leading to increased inflammation was identified in TB diseased individuals [148]. Knock-out of this inhibitor lead to increased inflammation and subsequent death of mice after *M.tb* infection [149]. Such an analysis of our study participants at genetic level would be insightful.

Evaluation of proliferative responses and concomitant Th1 cytokine production, the most commonly used outcome of mycobacteria-specific immunity, showed no differences in TB protected and unprotected infants. Our initial analysis of the proliferative response of CD4 T cells of the training set detected significant differences between the groups. However, statistical significance was lost when correcting for multiple comparisons between the groups. No differences were detected in the test cohorts. Together, this

support previous data generated on these samples. Short-term stimulation of whole blood with BCG also showed no differences in the production of IFN- γ , IL-2 and TNF- α between TB protected and unprotected infants [50]. Our data is also consistent with findings by Kori *et al* who reported no difference in proliferative capacity and cytokine production in response to PPD between active TB cases and controls irrespective of BCG vaccination as determined by thymidine incorporation and ELISA, respectively [150].

Furthermore, CD4 production of IL-4 did not associate with disease progression. Literature suggests a strong association between IL-4 and TB-disease progression. Based on this, we restricted our investigation of the role of the Th2 response to IL-4. We can not exclude that other Th2-cytokines may be involved. However, our data is consistent with a two year follow-up study which showed no difference in IL-4 expression between TB cases and their household controls [151]. Hussain *et al*, however, describes IL-4 as a predictor of disease progression in household contacts six months after the diagnosis of the household TB index case in genetically susceptible families [64]. Our study design did not allow for assessment of additional time-points which may have showed a more complete view of the immune response to *M.tb* infection in BCG vaccinated infants. This motivates further genetic evaluation of our study participants.

Our data shows that reduced proliferative potential as well as reduced Th1 cytokine production by BCG-specific T cells, post-vaccination, do not correlate with risk of developing TB disease. Rather, our data eludes to the possibility of an increased cytotoxic response as a risk factor to develop TB disease. Further analysis, at both genetic and protein level, are underway to dissect the underlying mechanisms involved.

3.6. Contributions

Jane Hughes optimised and designed the assays. Alana Keyser conducted the experiments, analysed the data and wrote this chapter under supervision of Prof. W.A. Hanekom and Prof. T. Scriba.

Chapter 4: Optimisation of a flow cytometric bead array assay for quantification of soluble proteins

4.1. Background

Our study which compared immune responses of infants at risk of TB with those who are not at risk implicated frequencies of BCG-specific T cells that produced cytotoxic molecules as a possible risk of TB disease. Higher levels of T cell expressing intracellular cytotoxic molecules were detected in infants who ultimately developed TB, compared to those who remained healthy. Cytotoxic molecules are maintained inside cytotoxic granules within effector cells. Upon activation, the granules migrate to the cell surface distal to the immunological synapse, a highly organized interface, between the effector and target cell [27]. Killing of the target cell requires the release of the contents of the granules into the immunological synapse. Failure to release cytotoxic molecules may lead to increased levels of intracellular cytotoxic molecules and subsequent inability to kill target cells. Soluble levels of granzyme B, perforin and granulysin have been detected in serum or plasma samples and culture supernatants, and high levels have been associated with active TB disease [78] [137] [152].

We sought to determine if soluble levels of granzyme B, perforin and granulysin are correlates of risk of TB in BCG-vaccinated infants. We collected 130 μ L of culture supernatant per condition from the cytotoxic marker assay, described in section 3.3.2 of chapter 3, to compare soluble levels of these molecules in the two groups of infants. Since measurement of analytes by ELISA requires 100 μ L of sample for each individual molecule, we aimed to develop a method that allows measurement of granzyme B, granulysin and perforin in a single sample volume of 100 μ L or less. Cytometric bead-based multiplex technology using the Luminex® platform allows detection of up to 42 analytes from 50 μ L of sample (Figure 4.1). Cytometric bead kits for the detection of granzyme B and perforin are commercially available and can be detected simultaneously using the LEGENDplex™ Luminex xMAP bead-based multiplex assay. Currently no kit is available for detection of granulysin.

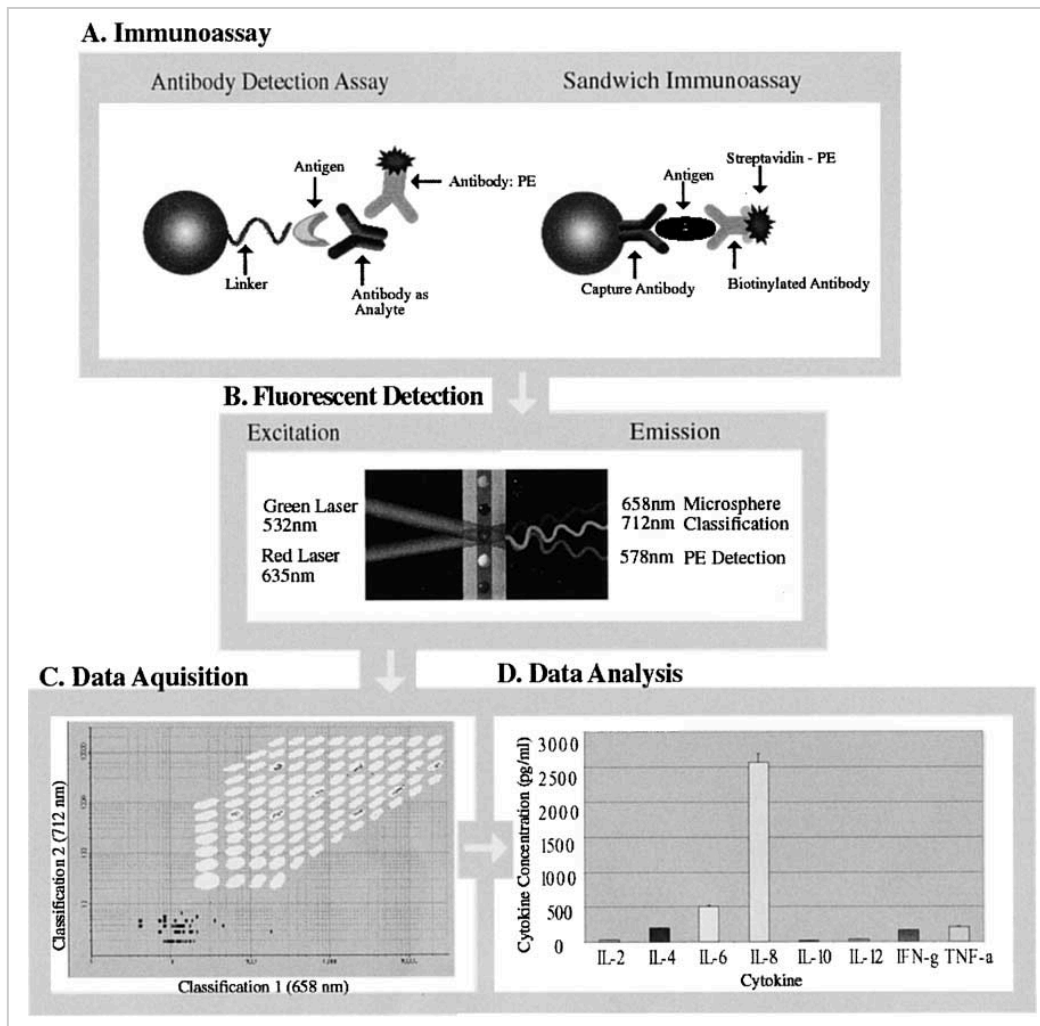


Figure 4.1. The multiplexed microsphere-based flow cytometric immunoassay. Immunoassays using an antigen-antibody format can be performed on microplex beads (A). Microspheres consist of a fluorescent dye dilution excited by the red laser and analytes are measured as PE fluorescence excited by the green laser (B). Each beadset for a particular analyte can be detected at a specific fluorescence intensity (C). Quantification of different analytes of an 8-plex assay is shown (D). (From Kellar et al, *Exp. Hematol.*, 2002 [114])

Luminex kits consist primarily of 5.6µm polystyrene beads containing a fluorescent dye which, upon excitation by a laser emitting red light, yields a specific spectral signature. These beads are also coated with carboxyl groups which can be covalently coupled to amine groups of proteins by using a two-step carbodiimide reaction. Activation of the carboxyl group yields a stable ester which can react with amines of proteins to form a covalent bond. Capture antibodies for a specific analyte can thus be conjugated onto the surface of a bead. A specific secondary biotinylated antibody is then used for detection, followed by binding of a PE-conjugated streptavidin for quantification by flow-cytometry. Using this technology,

carboxylated beads can be custom-conjugated with specific antibodies for any analyte of interest.

Based on these principles, we aimed to design a two-step optimisation protocol to custom-conjugate beads for measurement of granulysin. In the first step, as a proof of principle which is described in this chapter, we optimised conditions for detection of IFN- γ . IFN- γ levels detected by custom-conjugated beads can easily be validated by direct comparison of observed results with those from commercial bead kits or ELISA kits. Once satisfactory performance of the detection system is achieved, the second step would be to apply the validated optimisation protocol for development of reagents to the detection of granulysin. The final custom-conjugate beads can then be incorporated into commercial kits for simultaneous detection of granulysin, granzyme B and perforin. The latter aspects of this process are not addressed in this thesis, because of insufficient time.

4.2. Methods

4.2.1. Conjugation of microplex beads

Microplex beads (xMAP carboxylated microplex beads Region 55 microsphere, Luminex) were conjugated with a mouse anti-human IFN- γ antibody (clone MD-1, Biolegend) by using the protocol for two-step carbodiimide coupling of protein to carboxylated beads, according to the manufacturer's instructions. Briefly, 5×10^6 of resuspended stock microspheres were washed with 80 μ L 100mM monobasic sodium phosphate (Sigma-Aldrich) and vortexed for approximately 20 seconds. Ten μ L of each 50mg/mL Sulfo-NHS (N-hydroxysulfosuccinimide, Thermo Scientific) and 50mg/mL EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) were added to the beads and mixed gently by vortex. Beads were incubated for 20 minutes at room temperature with gentle mixing by vortex at 10-minute intervals. Activated beads were centrifuged at 14,000 x g for 2 minutes and washed twice with 250 μ L 50mM MES (2-[N-Morpholino] ethanesulfonic acid hydrate, Sigma-Aldrich) followed by centrifugation. Pelleted beads were resuspended in 500 μ L of 50mM MES and added at 100 μ L per antibody concentration ranging from 1-50 μ g/mL.

The bead-protein mix was brought up to 500 μ L with 50mM MES, mixed by vortexing and then incubated for 2 hours with mixing by rotation at room temperature. Following incubation, beads were centrifuged, resuspended in 500 μ L PBS-TBN (1% PBS, 0.1% BSA, 0.02% Tween-20, 0.05% Azide, pH 7.4) and again vortexed for 20 seconds. Beads were incubated for 40 minutes with mixing by rotation at room temperature and washed twice with 1mL PBS-TBN. Finally, beads were resuspended in 1mL PBS-TBN and counted under the microscope. Coupled beads were diluted to a final concentration of 100 beads/ μ L in PBS-TBN and stored in the dark at 4°C until use.

4.2.2. Confirmation of conjugation of microplex beads

Binding of anti-IFN- γ antibody to the beads was determined by following the protocol for confirmation of antibody coupling according to the manufacturer's instructions. A two-fold serial dilution, ranging from 4 to 0.0625 μ g/mL, of a PE-labeled anti-mouse IgG detection antibody (R & D Systems) was prepared in PBS-TBN. Fifty μ L of the working microsphere stock was mixed with 50 μ L diluted detection antibody and incubated for 30 minutes at room temperature. The microsphere-antibody mix was washed twice with 1mL PBS-TBN followed by centrifugation after each step. Beads were resuspended in 100 μ L of PBS-TBN and acquired on a LSR Fortessa flow-cytometer using FACS Diva 6.1 software, to confirm conjugation. Data was analyzed using FlowJo 8.8.4.

4.2.3. Detection of IFN- γ concentration with microplex beads

Five-fold serial dilutions of a known recombinant human IFN- γ standard (BD Biosciences) was prepared with PBS-TBN. Reaction mixes were prepared in FACS tubes by adding 50 μ L diluted protein to 50 μ L working bead stock. Samples were incubated for 1 hour at room temperature, followed by two wash steps: in 1mL PBS-TBN, followed by centrifugation at 14,000 x g for 5 minutes and supernatants were decanted. Fifty μ L biotinylated anti-IFN- γ detection antibody (clone 4S.B4, Biolegend) was added. Samples were incubated for 30 minutes at room temperature followed by two wash steps. Fifty μ L Streptavidin PE (Sigma-Aldrich) was added followed by a 30-minute

incubation at room temperature. Beads were washed twice, resuspended in 100µL PBS-TBN and acquired on a LSR Fortessa flow cytometer using FACS Diva 6.1 software. Data was analyzed using Flowjo 8.8.4. Similarly, the IFN- γ concentrations in unknown blood plasma samples were determined by using two-fold serial dilutions.

4.2.4. IFN- γ ELISA

IFN- γ concentrations of unknown blood plasma samples were quantified in parallel using the Human IFN gamma ELISA Ready-SET-Go![®] (eBioscience), following the manufacturer's instructions. Briefly, 96-well plates were coated overnight at 4°C with 100µL/well IFN- γ capture antibody (clone NIB42) in coating buffer. Plates were washed and blocked with assay diluent. Hundred µL two-fold serial dilutions of the IFN- γ standard or blood plasma samples were added to the appropriate wells. Plates were incubated for 2 hours at room temperature. After washing, plates were incubated for 1 hour at room temperature with 100µL/well IFN- γ detection antibody (clone 4S.B4). Plates were washed and incubated with 100µL/well Avidin-HRP for 30 minutes at room temperature. After washing, 100µL/well substrate solution was added. The reaction was developed at room temperature and stopped after 15 minutes by adding 50µL 2N H₂SO₄ to each well. Plates were read at 450nm on a Versamax ELISA plate reader using Softmax Pro software Version 4.7.1. IFN- γ standards ranged from 4-500pg/mL.

4.3. Results

4.3.1. Detection of microplex beads and specific analytes by flow cytometry.

Luminex microplex bead-technology typically makes use of a luminometer for detection of bead fluorescence at a wavelength of approximately 650nm upon red laser excitation (at a wavelength of 635nm) and quantification of specific analytes by detection of fluorescence at approximately 575nm upon excitation by a green laser (560nm). We wanted to optimise this assay to allow acquisition on a BD LSR Fortessa, equipped with red, green, blue and violet lasers.

To determine if microplex beads and specific analytes could be detected on this flow-cytometer, we used three beadsets, with different luminescent dye dilutions, of the Milliplex[®] Map Human cytokine/chemokine kit (Millipore). Each beadset is conjugated to antibody specific for a particular analyte; in this case TNF- α , IFN- γ and IL-2.

As shown in Figure 4.2.A, the fluorochromes on the three beads were excited by the red laser and could be detected at different fluorescence intensities using a 660/20 bandpass filter (typically used to detect APC or AlexaFluor 647). Titration of the kit standards allowed detection of all three beadsets at different fluorescence intensities upon green laser excitation using a 576/26 bandpass filter (typically used to detect PE, Figure 4.2.B).

We concluded that fluorescence of Luminex microplex beads can successfully be detected by using a BD LSR Fortessa flow cytometer.

4.3.2. Custom conjugation of a microplex beadset with a IFN- γ capture antibody.

Commercially available kits for the detection of granzyme B and perforin consist of carboxylated microplex beads manufactured by Luminex. We conjugated a carboxylated microplex beadset with a mouse anti-human IFN- γ antibody following the manufacturers instructions. Four different concentrations of the antibody were used: 5 μ g/ml, 10 μ g/ml, 20 μ g/ml and 50 μ g/ml. Conjugation of the antibody was confirmed by using a two-fold dilution of a PE-conjugated anti-mouse IgG antibody. The beads were then acquired on the LSR Fortessa flow cytometer.

As shown in Figure 4.3.A, the microspheres were detected in the red laser 650nm channel. The anti-mouse IgG was also detected as a dilution of the fluorescence intensity in the green 570nm channel. However, we observed overlap in fluorescence signal of the anti-mouse IgG concentrations, resulting in difficulty to resolve the different IFN- γ concentrations. Further, fluorescence of the highest concentrations fell above the linear range of detection in the green 570nm (PE) channel. This was possibly due to suboptimal photomultiplier tube (PMT) voltage settings, which were likely set

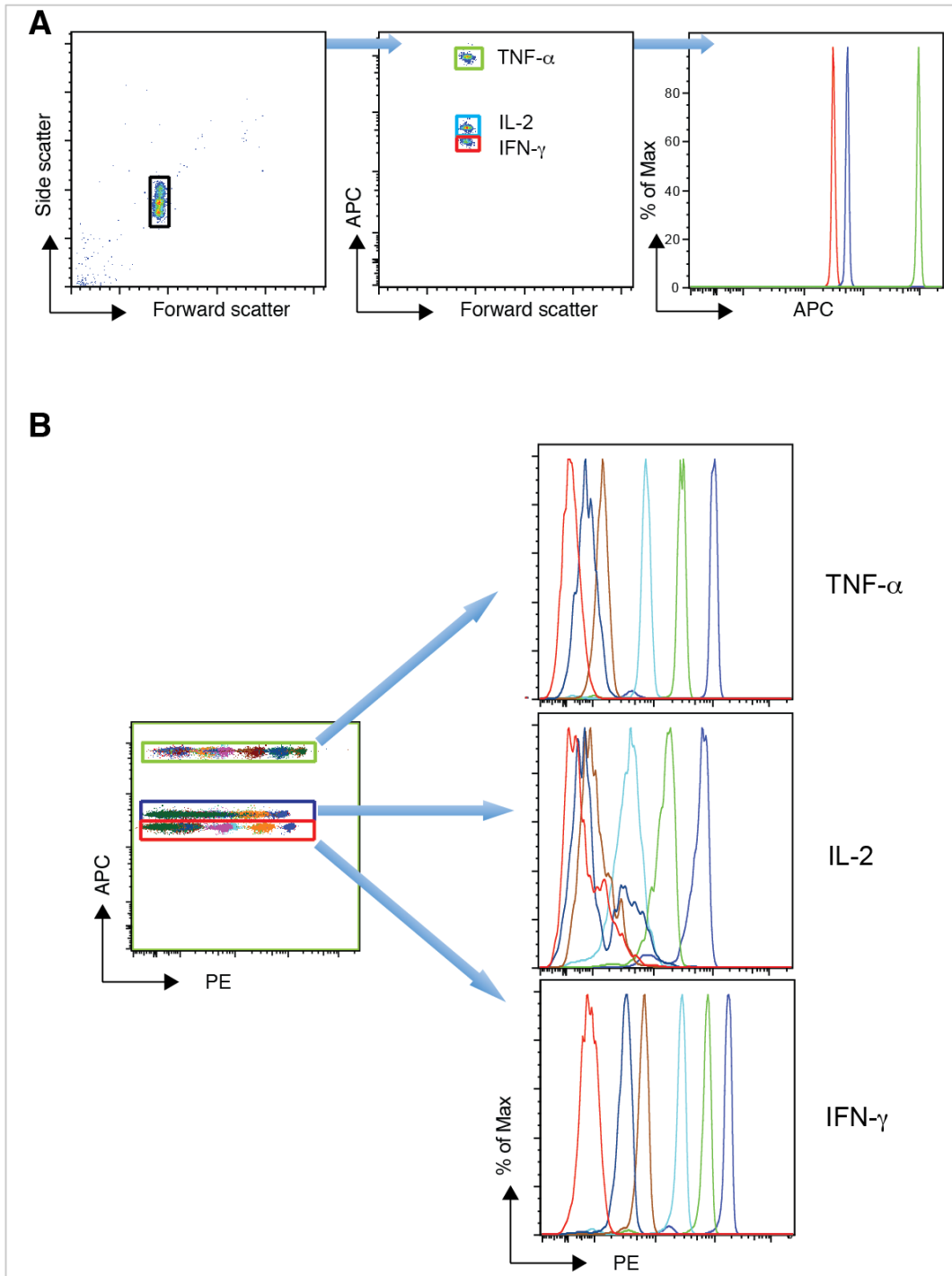


Figure 4.2. Detection of Milliplex beads using a flow-cytometer. Three Milliplex beadsets were acquired on a LSR Fortessa flow cytometer. The beadsets were detectable in the APC channel, as shown in A. Histograms show the beadsets at different fluorescence intensities. Beads were incubated with a concentration range of the kit standards followed by a biotinylated detection antibody and a PE-conjugated streptavidin. Histograms show the dilution in fluorescence intensities in the PE channel for each analyte shown in B.

too high, or suboptimal ratio of biotin and streptavidin concentrations. Fluorescence intensities were plotted against the antibody concentration, Figure 4.3 B, which showed that each of the IFN- γ antibody concentrations used appeared to be efficiently conjugated to the microplex beads.

We concluded that, using the manufacturer's instructions, we could successfully custom-conjugate microplex beads with specific capture antibodies. Further optimisation for optimal detection of IFN- γ , such as finding the optimal PMT voltage would be required.

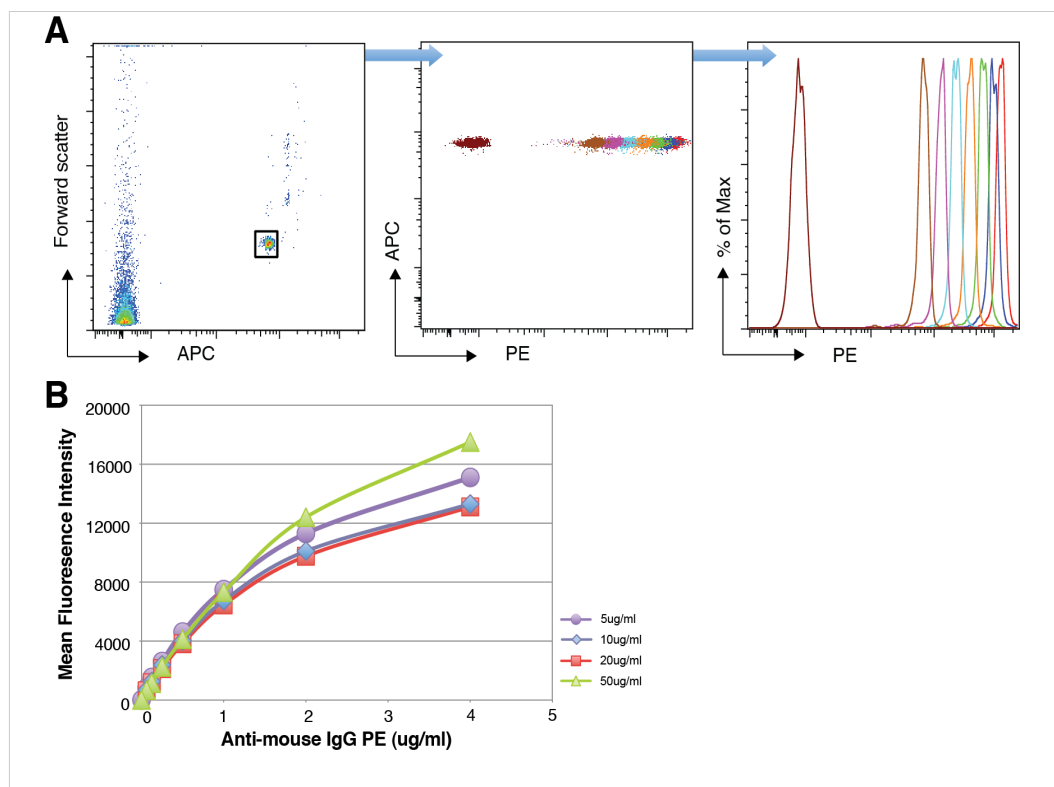


Figure 4.3. Confirmation of custom conjugation. Microplex beads were conjugated with 5, 10, 20 and 50 μ g/ml IFN- γ capture antibody, respectively. Beads were incubated with a range of anti-mouse IgG-PE concentrations. Dot plots and histograms showing the fluorescence intensity of different antibody dilutions in the green 570nm PE channel. Detection of the beads conjugated with 5 μ g/ml IFN- γ detection antibody is shown in A. Fluorescence intensities for beads conjugated with capture antibody is plotted against anti-mouse IgG PE concentrations in B.

4.3.3. Optimal PMT voltage for acquisition in green 570 PE channel

To determine the optimal PMT voltage at which analytes can be detected in the green 570 PE channel, custom conjugated beads (5000 beads per reaction) were incubated with recombinant IFN- γ at a low (3.2pg/ml) and high concentration (2000pg/ml). Beads were also incubated without IFN- γ

standard, to control for background bead fluorescence. Following incubation with biotinylated IFN- γ detection antibody and streptavidin PE, beads were acquired at different PMT voltage settings ranging from 250V to 350V, at 25V increments.

Fluorescence intensities of the background signal as well as the low and high standard concentrations increased when PMT voltage was raised (Figure 4.4. A). Signal:noise ratios were calculated for each voltage setting, where signal was the PE fluorescence intensity of the beads incubated with low or high concentrations of IFN- γ and noise was the fluorescence intensity of the unstained background beads. Using this approach, we observed that the difference in MFI between the high and low IFN- γ concentrations did not change at different PMT voltage settings (Figure 4.4. B). However, different voltage settings did influence the ratio of fluorescence intensities of the background control beads and the low IFN- γ concentration beads (Figure 4.4. C). Acquisition at 250V showed little difference between the background MFI and the MFI of the low IFN- γ concentration. Acquisition at 400V and higher voltages led to increased background MFI and poorer resolution of background from IFN- γ beads. Acquisition at 275V showed the best distinction between the signals of the background beads and low standard concentration. Routine instrument setup and performance standardisation procedures and quality control measures of the LSR Fortessa flow cytometer had identified 280V as the optimal PMT voltage with the lowest background noise for acquisition in the green 570 PE channel.

We concluded that 280V was optimal for detection of cytometric beads and quantification of specific analytes.

4.3.4. Determining optimal concentrations of biotinylated IFN- γ detection antibody and streptavidin-PE.

Luminex microplex technology detects and quantifies specific analytes using a biotinylated detection antibody followed by a PE-conjugated streptavidin. Biotin binds to streptavidin, which, in this case is conjugated to PE. If concentrations of specific analytes are directly proportional to the PE MFI of a known standard, linear quantification can be achieved. However, the

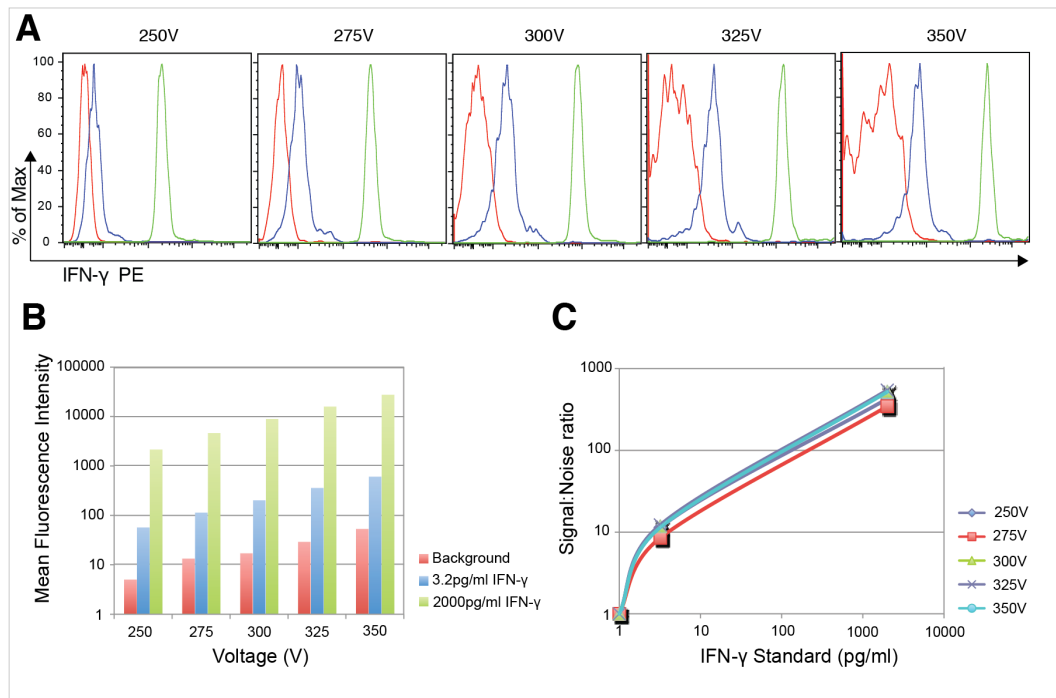


Figure 4.4. Optimal PMT voltage for acquisition in the PE channel. Beads conjugated with 10 μ g/ml IFN- γ capture antibody were incubated with (3.2 and 2000pg/ml IFN- γ standard) or without (background) IFN- γ standard and acquired at different PMT voltages to determine optimal voltage settings for acquisition of the microplex beads. Histograms of the mean fluorescence intensities at each voltage (A) Mean fluorescence intensities were determined (A) and signal:noise ratios at each voltage calculated to identify an optimal voltage (C).

accuracy of detection depends on optimal concentrations of biotin and streptavidin.

Conjugated beads incubated with a five-fold dilution of known IFN- γ standard were used to determine effects of different concentrations of biotinylated detection antibody (biot-dAb) and streptavidin PE (SA-PE) at a constant 5:1 ratio, as described elsewhere [153]. The concentrations tested were as follows: 0.5 μ g/ml biot-dAb with 2.5 μ g/ml SA-PE, 1 μ g/ml biot-dAb with 5 μ g/ml SA-PE and 2 μ g/ml biot-dAb with 10 μ g/ml SA-PE. Signal:noise ratios were calculated as described above.

We observed no difference in signal:noise ratio between the three concentrations used (Figure 4.5. A). PE fluorescence intensity was also not different between the different biotin:streptavidin concentrations. However, an increase in signal spread between the background signal and the lowest standard concentration was observed when biotin and streptavidin concentrations were increased (Figure 4.5. B).

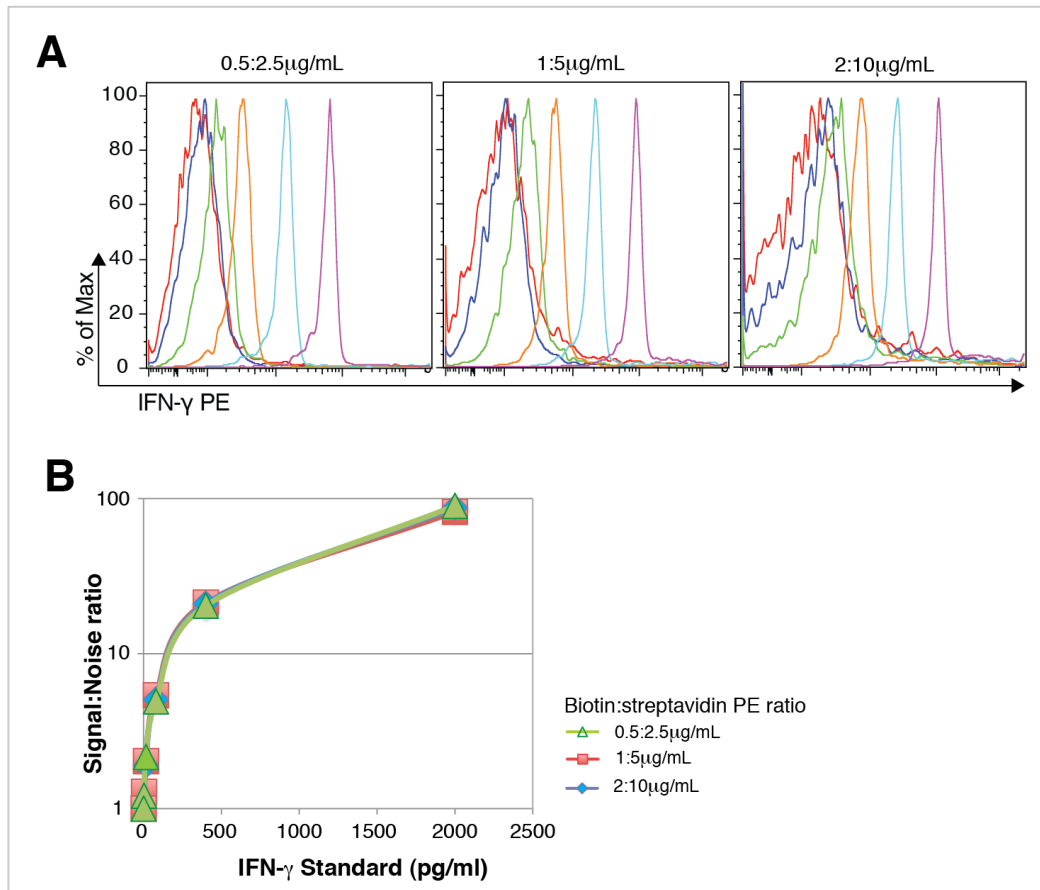


Figure 4.5. Optimising biotin:streptavidin concentrations. Beads conjugated with 10 μg/ml IFN-γ capture antibody were incubated with IFN-γ standard, and a range of Biotin:streptavidin-PE concentrations, and acquired at 280V. Mean fluorescence intensities were determined and signal:noise ratios were calculated (A). Histograms of the mean fluorescence intensities at each voltage were also assessed to determine an optimal concentration (B). Biotin:streptavidin PE concentrations are indicated.

We concluded that an optimal concentration of biot-dAb and SA-PE could not be determined from these experiments. However, using 0.5 μg/ml detection antibody with 2.5 μg/ml streptavidin was sufficient for detection of analyte concentrations covering a wide dynamic range, as there was no difference compared to other concentrations.

4.3.5. Optimising concentrations of capture antibody.

Next, we determined the concentration of IFN-γ capture antibody required to optimally quantify concentrations of soluble IFN-γ. Microplex beads were conjugated with different concentrations of anti-IFN-γ antibody. Beads were incubated with a five-fold titration of a known IFN-γ standard and acquired at 280V to determine the MFI for each concentration.

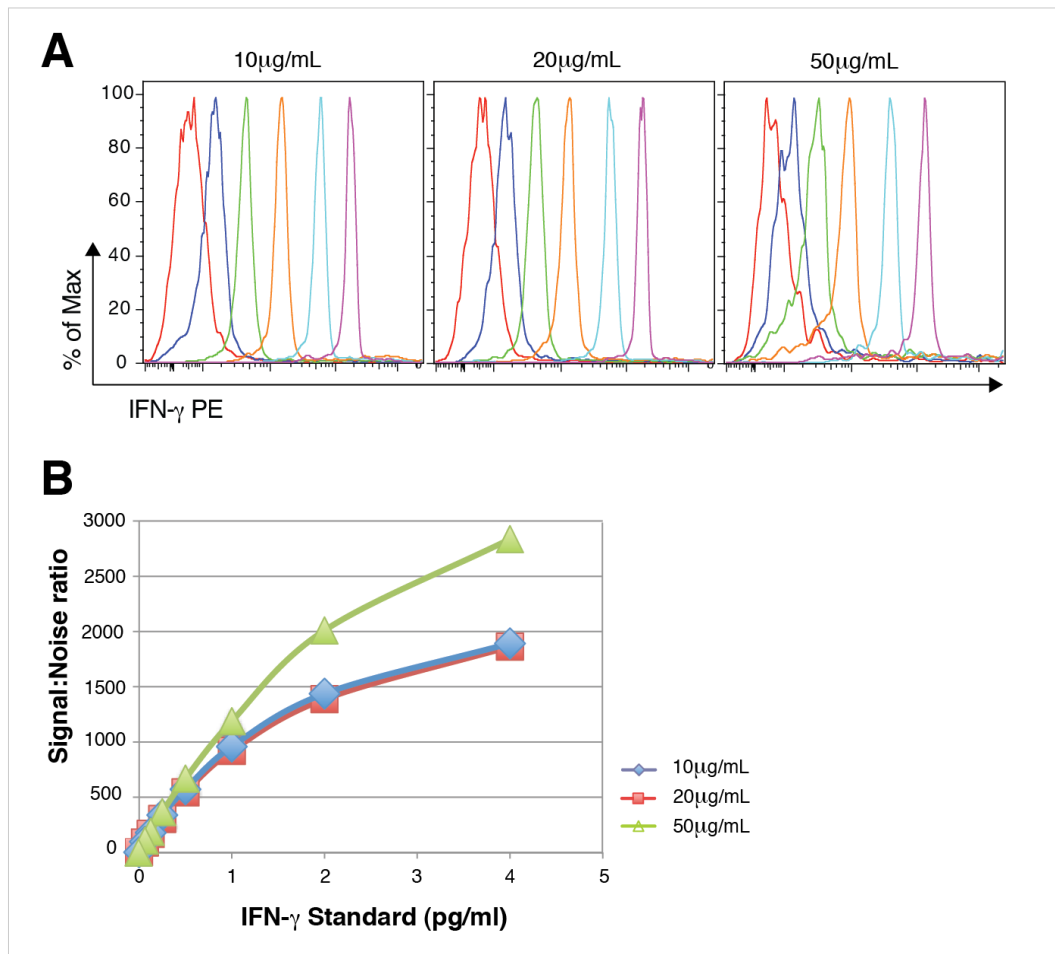


Figure 4.6. Optimising concentrations of IFN- γ capture antibody. Beads were conjugated with 10, 20 and 50µg/ml of IFN- γ capture antibody and incubated with IFN- γ standards at a range of concentrations and acquired at 280V. Mean fluorescence intensities for each beadset were assessed to determine an optimal IFN- γ capture antibody concentration (A). Mean fluorescence intensities were determined and signal:noise ratios were calculated (B).

Signal:noise ratios were highest at a concentration of 50µg/ml capture antibody, compared with the lower concentrations (Figure 4.6 A). There was no difference in signal:noise ratios between 10 and 20µg/mL. However, fluorescence overlap between the background sample and the lowest standard increased when 50µg/ml capture antibody was used (Figure 4.6 B). In essence, all three concentrations of capture antibody could detect the recombinant IFN- γ . We thus explored the use of 10 and 20µg/mL further and directly compared IFN- γ detection between the bead detection method developed here and a commercial IFN- γ ELISA.

Beads were incubated with a two-fold serial dilution of a human plasma sample to allow quantification of IFN- γ . This sample was also quantified by

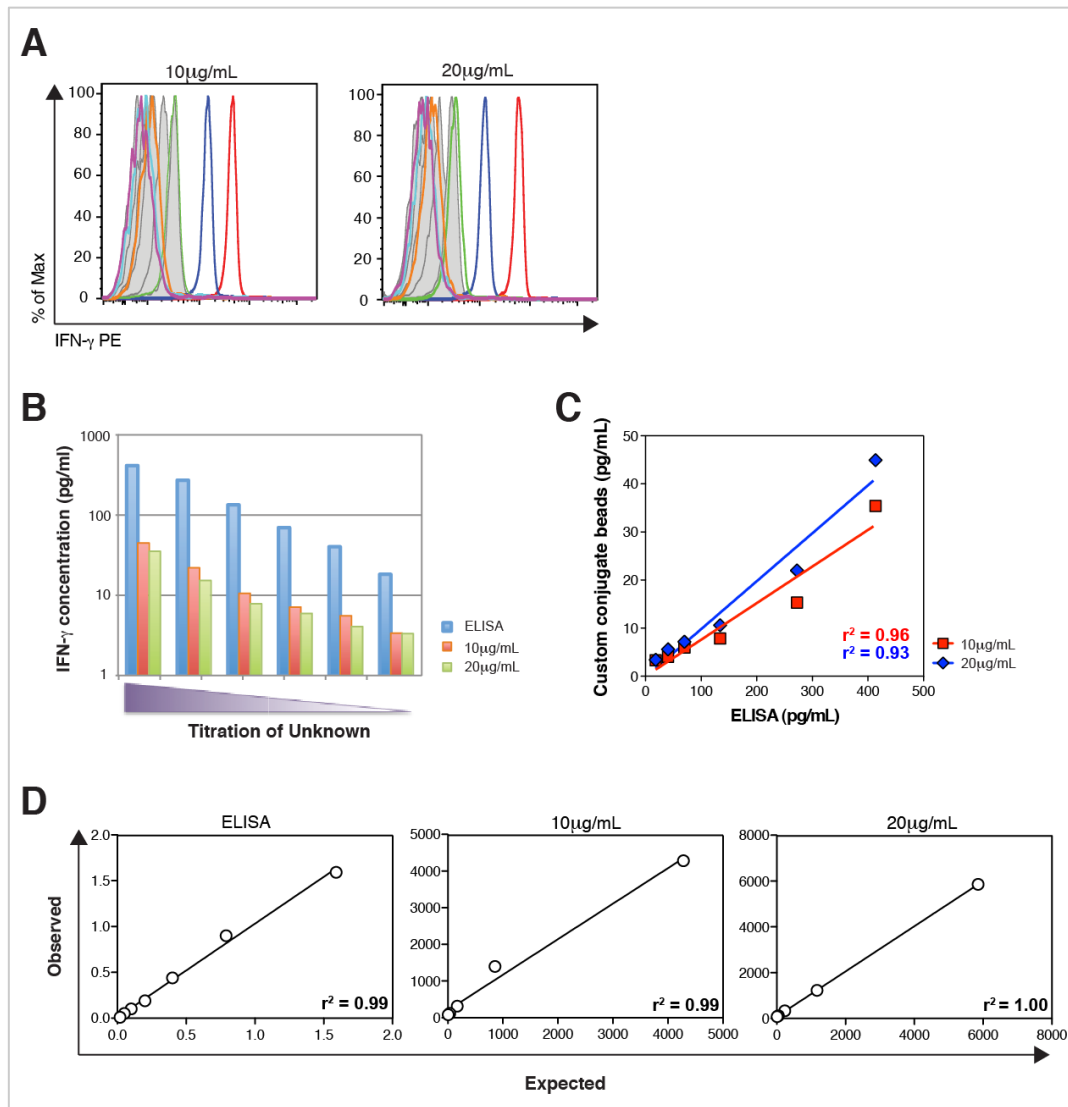


Figure 4.7. Validation of beadsets. Beads conjugated with 10 and 20µg/ml IFN-γ capture antibody were incubated with a two-fold dilution of a human plasma sample with unknown IFN-γ content and a known IFN-γ standard. Mean fluorescence intensities were determined and IFN-γ concentrations were calculated against the standard (A). IFN-γ concentrations of the plasma sample were determined by ELISA and the calculated concentrations were compared to concentrations determined by using the microplex beads (B). Correlation of IFN-γ concentrations of the plasma sample, as determined by ELISA and the custom conjugated bead sets was determined (C). Linear regression analysis of the expected and observed OD and mean fluorescence intensity of the standard was performed for the ELISA and the custom conjugated beads, respectively (D).

using the Human IFN-gamma ELISA Ready-SET-Go! as described above. Concentrations were then compared between the two assay systems. The two beadsets detected soluble IFN-γ at similar levels (Figure 4.7. A and B). However, higher concentrations of IFN-γ were observed using the ELISA assay, compared with the beadsets. This possibly indicated a lower level of sensitivity of the beadsets which required further optimisation. IFN-γ concentrations determined by ELISA and the fluorescent bead sets, showed

good correlation (Figure 4.7. C). In addition, linear regression analysis of the expected and observed OD and mean fluorescence intensity of the standard was performed for the ELISA and the custom conjugated beads, respectively, indicated optimal performance of the two quantification platforms (Figure 4.7. D).

4.4. Discussion

Luminex bead arrays have become a very helpful tool for measuring levels of multiple proteins in low sample volumes owing to their highly multiplexed nature. This property makes bead arrays ideal for infant studies, where sample volumes are generally very small. Commercially available kits have been used to identify correlates of risk of TB disease in infants three months after BCG-vaccination [154]. Various groups have used this technology to determine diagnostic markers of infection and disease progression in TB and HIV [155] [156] [157].

Cytolytic killing of infected target cells requires the release of cytotoxic molecules into the immunological synapse. Failure to release these molecules may associate with poor outcome in M.tb. infection. Differential levels of soluble cytotoxic molecules may associate with risk of TB disease. Ultimately, we wanted to assess soluble levels of granzyme B, perforin and granulysin by comparing concentrations of granzyme B, perforin and granulysin in culture supernatants of PBMC stimulated with BCG of infants at risk and infants not at of TB disease. Here we optimised a flow cytometric bead array assay to quantify soluble levels of cytotoxic molecules. Addressing this aim by applying the optimised bead array assay was not possible in the timeframe of my project.

Our optimisation process involved a number of steps. The first step was to determine optimal conditions for a flow cytometric bead array assay to measure IFN- γ . The performance of this custom IFN- γ bead array assay was then validated against a IFN- γ ELISA as a second quantification platform. We showed that a standard flow cytometer can detect these fluorochromatically labeled beads and identified an optimal acquisition PMT voltage. Using a step-wise approach, we also identified optimal

concentrations of capture and detection antibody for binding of soluble IFN- γ . These optimal conditions were applied to quantify IFN- γ in a human plasma sample using the custom-conjugated beads. However, comparison of calculated values against that of an IFN- γ ELISA for the same sample, showed that IFN- γ levels were detected at higher levels with the ELISA compared to the beads. Regardless, the bead array method yielded highly linear quantification and a high regression co-efficient when compared to a commercial IFN- γ ELISA. Others have reported comparable results between the two platforms with an even higher sensitivity of the beads in some cases [153] [154] [158]. Together, this may indicate reduced sensitivity of our custom-conjugated beads compared to the ELISA. A plausible reason may be that different IFN- γ antibody clone sets were used in the two platforms. Switching to identical IFN- γ antibody clone sets may eliminate this discrepancy.

The Luminex platform remains a robust, yet costly technology to assess multiple analytes in small volume samples. Continued optimisation of a custom-conjugated beadset and conditions was not warranted given limited availability of funds and timelines. However, we developed custom-conjugated beadsets that can quantify soluble IFN- γ and, pending further optimisation and conjugation with an anti-human granulysin antibody, these beads could ultimately be used to quantify soluble cytotoxic molecules. Kits for custom-conjugation of fluorescent beads are also becoming commercially available and may be useful to explore analytes not included in the multiplex kits.

4.5. Contributions

Alana Keyser designed and conducted the experiments, analysed the data and wrote this chapter under supervision of Prof. W.A. Hanekom, Prof. T. Scriba and Dr. A. Penn-Nicholson.

Chapter 5: General Discussion

Identification of biomarkers of protection against TB disease requires a placebo-controlled trial of a successful vaccine, where the vaccine-induced protective immune responses are contrasted with immune responses in unvaccinated individuals [159]. All infants in our cohort had received BCG, at birth as is routine in South Africa due to the high TB prevalence. These infants were followed for two years to identify those who became TB diseased and those who remained healthy. In this context, we are able to explore prospective correlates of risk of TB disease, using blood collected at a time-point prior to *M.tb* infection and TB disease development.

In this thesis we took a novel approach to identify biomarkers of risk of TB disease: we explored BCG-specific cytotoxic T cells as candidate correlates. In order to do this, we first optimised an assay to demonstrate BCG-specific cytotoxic T cell-mediated killing of mycobacteria-infected monocytes. Secondly, we compared BCG-specific cytotoxic T cell responses in infants who are at risk of TB disease to those who remained healthy. We also measured and compared the BCG-specific Th1 and Th2 immune response in these infants. Thirdly, we optimised a fluorescent bead array system to ultimately measure release of cytotoxic molecules in cell-culture supernatants upon mycobacterial stimulation.

We successfully optimised a flow cytometry-based killing assay showing that BCG-expanded cytotoxic T cells can kill monocytes infected with BCG. This optimisation presented two challenges. Firstly, BCG itself has the ability to kill infected cells. Hence, we identified optimal assay conditions in terms of incubation time and dose to infect monocytes to measure BCG-specific T cell-mediated killing. Secondly, only limited volumes of blood are obtainable from infants. However, the killing assay requires more PBMC than the amount harvested from the maximum blood volume accessible at 10 weeks of age. Thus, we proceeded with optimisation using adult blood. We cannot exclude that infant and adult cytotoxic responses may differ. Kollmann *et al* reported similar responsiveness between adult and infant monocytes when stimulated with different TLR agonists [160]. However, differences became

prominent when DC responsiveness and cytokine production by monocytes and DC were assessed. These kinds of differences may also apply between cytotoxic CD4 and CD8 T cells of adults and infants.

In this study, assessment of the CD4 and CD8 T cells revealed responses suggestive of increased cytotoxic potential in BCG-vaccinated infants who were at risk of TB, compared with those who were not at risk of TB disease. Our training cohort showed statistical differences in cytotoxic responses of infants who ultimately developed culture positive TB when compared with healthy infants. However these findings could not be validated in a second cohort of infants at risk, and not at risk, of TB disease. Our initial investigation of a correlate of risk of TB disease did not include validation of findings as described by Qin *et al* [159]. Validation studies usually require identical clinical case definition as discover studies. However, due to limited sample sizes, there was a slight difference in the case definition between our training and validation cohorts. The first ultimately developed culture confirmed TB, while the latter did not have culture confirmed disease, but strong clinical, radiological and epidemiological evidence of TB disease (described in chapter 3). We cannot exclude that this small difference in phenotype could have contributed to the failure of validation.

Assessment of Th1 effector function showed no differences between infants at risk and those who were not at risk of TB disease. BCG-specific CD4 and CD8 T cell effector function of these infants were also assessed in a complementary study using direct *ex vivo* short-term stimulation of whole blood [101]. Interestingly, in the latter study, patterns of BCG-specific IFN- γ , IL-2 and/or TNF- α expression in polyfunctional CD4 and CD8 T cells were similar to those shown with our longer term stimulation; in the current study, no differences were detectable between the different groups. Although these effector T cell populations are important in control of *M.tb* growth and disease progression, it appears that BCG-specific effector function does not correlate with protection or risk of TB disease. We cannot exclude that use of a different mycobacterial antigen, or another time point of evaluation, could have revealed differences between the infants at risk and those not at risk of TB disease. However, results from the historical MVA85A vaccine

trial have been informative in these respects [161]. In this trial infants were vaccinated in a BCG prime-MVA85A boost strategy. Direct *ex vivo*, short-term stimulation of whole blood with Ag85A showed increased antigen-specific Th1 responses in MVA85A-vaccinated infants compared to the placebo group. However, these responses did not confer protection against *M.tb* infection or TB disease in the vaccinated group. During the three-year follow-up period, equal numbers of infants in both groups became *M.tb* infected and similar numbers of infants also develop TB disease. These results appear to confirm that effector T cell responses do not correlate with risk of TB disease.

Recent advances in our understanding of the evolution of BCG-induced memory responses have also provided insight into our observations. In a one-year follow-up study of newborn BCG vaccinees, Soares *et al* showed peak CD4 T cell responses between 6 and 10 weeks of age [91]. Furthermore, the proportion of central memory BCG-specific CD4 T cell population was greater at 27 weeks compared with 6 weeks post-vaccination. Also, BCG has been shown to persist for up to 6 months after vaccination in an immunocompetent infant [146]. Together, this information may suggest that our time-point of 10 weeks post-vaccination may have been too early for clearance of BCG and for establishment of memory populations. Evaluation of infant responses after 27 weeks post-vaccination could have provided complementary information in terms of the cytotoxic and Th1 potential of BCG-specific memory T cells.

We also attempted to uncover differences in the release of cytotoxic molecules between infants at risk and those not at risk of TB disease, using cytometric bead array technology. This presented challenges in that cytotoxic molecules are not included in the commercially available Luminex kits, which measures cytokine related to CD4 helper T cell function, plus additional chemokines and growth factors. Data from the current study seem to confirm other published data that there are no differences in the frequency of Th-related cytokine producing CD4 T cell populations between infants at risk and those not at risk of TB disease. Expansion of commercially available kits to assess beyond the current 42 analytes may

be a helpful tool in determining biomarkers correlating to risk of or protection against TB disease. Our optimisation of a custom bead array lays the groundwork for assessment of novel analytes, if so required, for future studies.

In summary, comparison of BCG-induced responses in infants who were at risk of TB disease and those not at risk of TB disease showed no differences in the conventionally protection-associated Th1 cytokines. We also showed that BCG can induce specific cytotoxic T cell-mediated killing of infected cells. We were unable to validate increased production of cytotoxic molecules by BCG-specific T cells in infants at risk of TB disease in a secondary cohort. Identifying a biomarker of risk of TB disease may require an unbiased systems biology approach to explore global gene expression in this cohort.

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