Investigations of mycobacteria-specific memory/effector T cell responses in HIV-infected children receiving antiretroviral therapy

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DEDICATION

THANKS TO LIFE

(by Violeta Parra a Chilean poetess and singer, 1917-1967)

Thanks to life that has given me so much
It has given me two eyes, that when I open them
I clearly distinguish the black from the white
And in the infinite sky, its starry depths,
And from the crowds of people, the friend that I love.

Thanks to life that has given me so much
It has given me hearing that in all its breadth
Night and day records crickets and canaries,
Hammers, turbines, barking, splashes,
And the tender voice of my loved ones.

Thanks to life that has given me so much
It has given me sound and the alphabet
And with it the words to think and speak
Mother, friend, sister, and the light that brightens
The path of the soul of my loved ones.

Thanks to life that has given me so much
It has kept my tired feet walking
With them I walked through the cities and the puddles,
Beaches and deserts, mountains and plains
And to your house, your street and your courtyard.

Thanks to life that has given me so much
It has given me laughter and it has given me tears
With them I can tell the difference between joy and pain,
The two things that make up my song,
And your song that is everyone's song that is my own song.
Thanks to life.....

To my parents

Mama and Tata Tena

DECLARATION

I Nontobeko Gwendoline Tena-Coki hereby

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I am now presenting this thesis for examination for the degree of PhD.

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ABBREVIATIONS

ADC	albumin D-glucose catalase
ADAM	a disintegrin and metalloprotease
Ag85B	antigen 85B
AIDS	acquired immunodeficiency syndrome
[1]APC	antigen presenting cells
[2]APC	allophycocyanin
ART	antiretroviral therapy
AVP	avidin peroxidase
BCG	bacillus Calmette-Guérin
BSA	bovine serum albumin
BSL-3	biosafety level 3
BP	band pass
CCR	chemokine receptor
CDC	Centers for Disease Control and Prevention
CD	cluster designation
CFU	colony forming units
CFP-10	culture filtrate protein 10
CLIP	class II associated Ii chain peptide
CR	complement receptor
CMI	cell-mediated immune response
CRP	C-reactive protein
CST	cytometer setting and tracking
CV	coefficient of variation
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin
DCLP	dichroic long pass
dH20	distilled water
DNA	deoxyribonucleic acid
DOTS	directly observed treatment strategy
DTH	delayed type hypersensitivity
E	ethambutol

EDTA	ethylenediaminetetraacetic acid
E_m	maximal emission
ELISA	enzyme linked immunosorbent assay
EPI	Expanded Programme for Immunization
ER	endoplasmic reticulum
ESAT-6	early secretory antigenic target 6
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward scatter
GR	growth ratio
H	isoniazid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HNP	human neutrophil α-defensins
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
IL	interleukin
Ii	invariant chain
IQR	interquartile range
IRIS	immune reconstitution syndrome
IUATLD	International Union against Tuberculosis and Lung Disease
LAM	lipoarabinomannan
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
LTA	lipoteichoic acids
lux	bacterial luciferase
Mø	macrophages
Man-LAM	mannose-capped lipoarabinomannan
MIP-lα	macrophage inflammatory protein-lα
M. africanum	*Mycobacterium africanum*
M. bovis	*Mycobacterium bovis*

M. canetti	*Mycobacterium canetti*
M. fortuitum	*Mycobacterium fortuitum*
M. microti	*Mycobacterium microti*
M. tuberculosis	*Mycobacterium tuberculosis*
MAbs	monoclonal antibodies
MHC	major histocompatibility complex
MDR	multidrug resistant
MFI	median fluorescence intensity
N/A	not available
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NK	natural killer
NO	nitric oxide
NOS2	nitric oxide synthase 2
NLR	nucleotide-binding oligomerization domain (NOD)–like receptors
OADC	oleic acid albumin D-glucose catalase
O_2	oxygen
p	plasmid
PE	R-phycoerythrin
PE-Cy7	R-phycoerythrin cyanine 7
PerCP-Cy5.5	peridinin-chlorophyll-protein complex cyanine 5.5
PPD	purified protein derivative
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PBL	peripheral blood lymphocytes
PFC	polychromatic flow cytometry
PMT	photomultiplier tubes
PRR	pattern recognition receptors
QC	quality control
QD	quantum dot
r	recombinant
R	rifampicin

RD1	region of difference 1
Rev	revolutions
RLU	relative light units
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
S	streptomycin
SEB	staphylococcal enterotoxin B
SD	standard deviation
SSC	side scatter
SI	staining index
S/N	signal-to-noise
TAP	transporters associated with antigen processing
TACE	TNF-α converting enzyme
TB	Tuberculosis
TCR	T cell receptor
T_{CM}	central memory T cells
T_{EM}	effector memory T cells
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
T_{reg}	T regulatory cells
TREC	thymic rearrangement excision circle
TST	tuberculin skin test
USA	United States of America
V. harveyi	*Vibrio harveyi*
WHO	World Health Organization
XDR	extensively drug resistant
Z	pyrazinamide

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ABSTRACT

Human immunodeficiency virus (HIV) infected children are at greater risk of developing tuberculosis disease, and might benefit from vaccination with novel TB vaccines. However, little is known about the effect of HIV-infection on function and phenotype of T cell responses to mycobacterial antigens in children. This study compares both CD4 and CD8 T cell cytokine expression and memory phenotype in children, following in vitro stimulation with mycobacterial antigens, also contained in novel anti-TB vaccines that are currently undergoing clinical trials.

Blood was collected from 3 groups of children previously vaccinated with bacille Calmette-Guérin (BCG): HIV-unexposed healthy children (n=30), HIV-seronegative children diagnosed with TB - who had been receiving standard anti-TB therapy (n=30) and HIV-infected children not on TB treatment (n=30). Blood was stimulated with Antigen 85B and TB10.4, purified protein derivative or early secretory antigenic target-6. Cytokine production and the memory phenotype of antigen-specific CD4 and CD8 T cells were determined by flow cytometry. Longitudinal analysis was performed on functional and memory phenotypes of HIV-infected children on antiretroviral therapy. Mycobacterial growth inhibition was assessed in whole blood from the HIV-infected group using a reporter gene tagged strain of *Mycobacterium tuberculosis*.

Mycobacteria-specific CD4 and CD8 T cell responses were detectable in all 3 groups. Children receiving TB treatment had significantly higher frequencies of antigen-specific CD4 T cells, compared with HIV-infected children ($p=0.0176$). We observed higher frequencies of CD4 T cells, expressing interferon-γ (IFN-γ), interleukin-2 (IL-2) or both, in children with TB, but no differences in memory phenotype between children with and without HIV-infection. No significant differences in magnitude, function or phenotype of antigen-specific T cells were observed in HIV-infected children, compared with healthy controls. CD4 T cells expressing IFN-γ, IL-2 or both were of an effector memory phenotype. Mycobacteria-specific CD8 T cells expressed

mostly IFN-γ in all groups; these cells also expressed an effector memory phenotype. Longitudinal follow-up of the HIV-infected children during the first 12 months of antiretroviral therapy (ART) showed early immune restoration in total CD4 T cell percentages and naïve T cells. The proportion of effector memory cells declined significantly within 3 months of ART. Similarly, mycobacteria-specific IFN- γ responses decreased within the first 3 months of ART. Peripheral blood mycobacterial growth inhibition increased over time in HIV-infected children on ART.

Our data provide a detailed functional and phenotypic analysis of mycobacteria-specific T cell memory responses in HIV-infected and uninfected children, and the impact of ART.

CHAPTER 1

Introduction and literature review

1.1 Introduction

Tuberculosis (TB) is an infectious disease, caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). The current TB vaccine, bacillus Calmette-Guérin (BCG), does not provide adequate protection against TB disease in children. Further, safer more efficacious TB vaccines are needed for children with immunodeficiencies such as human immunodeficiency virus (HIV) infection – this population is at highest risk of disease. These factors contribute to the need for intensified research in the field of TB vaccine development. This chapter summarises a comprehensive review of published literature.

1.2 Epidemiology of tuberculosis

1.2.1 Global burden of tuberculosis

TB causes the deaths of almost two million people globally per annum. It is especially endemic in developing countries (**Figure 1.1 A**), where HIV infection is also most prevalent (**Figure 1.1 B**). The World Health Organisation (WHO) estimated that in 2009 there were 9.4 million incident cases of TB in the world (WHO, 2010a). HIV-infection was estimated to occur in 12% of the incident cases; 80% of these HIV-positive cases were living in Sub-Saharan Africa (WHO, 2010a). The two epidemics fuel each other and significantly contribute to mortality from infectious diseases worldwide (Sharma *et al.*, 2005). TB is the leading cause of death among people who are HIV-positive (Harries *et al.*, 2001). HIV is the single most important factor contributing to the increase in incidence of TB in Africa (**Figure 1.1 B**) (WHO, 2010a). Sonnenberg *et al.* showed that the risk of TB doubles within a year of infection with HIV in a cohort study in South African gold miners (Sonnenberg *et al.*, 2005).

The development of *M. tuberculosis* strains with drug resistance in the population heightens concerns of future control of the TB epidemic. In 2008, the WHO reported over 440,000 cases of multidrug resistant TB (MDR-TB) (WHO, 2010a). MDR strains of *M. tuberculosis* are resistant to at least the two main first-line anti-TB drugs, rifampicin and isoniazid (Palmero *et al.*, 2003) Resistance to anti-TB drugs is primarily the consequence of suboptimal TB care and control programmes, including weak health systems. The contributing factors to this problem include incorrect drug prescribing practices by providers (e.g. choice of drugs, dosage, duration of treatment), programme factors (e.g. irregular supply of drugs), and most importantly, patient factors (e.g. patient non-adherence to drug regimens) (Chaulet *et al.*, 1995). Emergence of Extensively Drug Resistant TB (XDR-TB), poses a serious threat to TB control (Gandhi *et al.*, 2006). XDR-TB strains are resistant to any fluoroquinolone, and at least one of three injectable second-line drugs, capreomycin, kanamycin, and amikacin, in addition to rifampicin and isoniazid (Shah *et al.*, 2007). These forms of the disease have high mortality, particularly in HIV co-infection and are challenging and expensive to treat (Gandhi *et al.*, 2006; Shah *et al.*, 2007).

1.2.2 Tuberculosis in South Africa

TB continues to be a scourge in South Africa, with extremely high disease burden, incidence rates and HIV co-infection rates (WHO, 2010a). The current epidemic is exacerbated by the growing epidemics of MDR-TB and XDR-TB (Cohen *et al.*, 2010). South Africa is ranked 3rd in the world for incident cases, out of 22 high-burden TB countries. The WHO registered an incidence of 970 cases per 100,000 population in 2009, and 60% of these new TB cases were HIV-positive (WHO, 2010a). The problem is compounded by the legacy of fragmented health services, which are still very poor in many areas, in both cities and rural areas. The co-existence of TB and HIV infection has led to escalating TB rates in the South African province of KwaZulu-Natal, which has overtaken the Western Cape province for the highest rates of TB (Abdool Karim *et al.*, 2009; Cohen *et al.*, 2010). Increased drug resistance threatens the achievements of TB directly observed treatment strategy

(DOTS) programmes and antiretroviral therapy (ART) distribution programmes (Abdool Karim *et al.*, 2009). Despite improvement in the TB control programme since its introduction in 1996, stringent measures must be taken in its implementation to ensure continued improvement in overall TB control including, diagnosis, treatment and case finding.

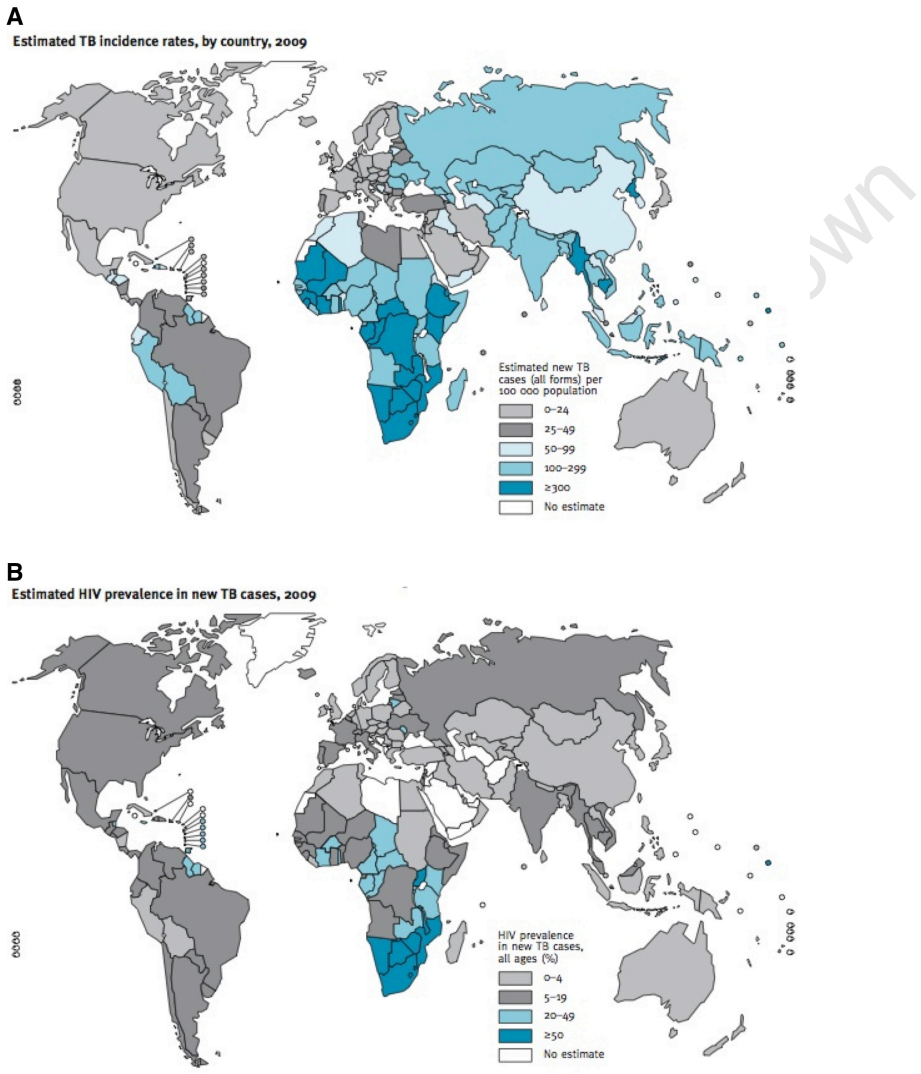

Figure 1.1: Tuberculosis burden

(A) Global picture of tuberculosis – New TB cases per 100, 000 people in 2009. (B) Prevalence of HIV in Tuberculosis. Adapted from (WHO, 2010a).

1.3 Mycobacterium tuberculosis

More than a century ago Robert Koch identified *M. tuberculosis* as the causative agent of human TB (Koch, 1982). *M. tuberculosis* can infect a wide variety of animals but man is the principal host. Other disease causing mycobacteria within the *M. tuberculosis* complex includes: *M. bovis, M. africanum, M. canetti* and *M. microti* (Chimara *et al.*, 2004). However, these species are less common in the human host. *M. tuberculosis* is a highly aerobic, rod-like, acid-fast bacterium. The high lipid content of the bacterial cell wall and the waxy coating of the cell surface (primarily consisting of mycolic acid) makes the cell resistant to Gram staining, and acid-fast techniques are used instead (Shapiro and Perlmutter, 2008). Figure 1.2 shows that bacteria retain a bright red colour after staining with carbolfuchsin, and are resistant to decolourisation with acid alcohol (hydrochloric acid and ethanol), which is known as a Ziehl-Neelsen stain (CDC, 1979).

The mycobacterial genome is a single chromosome in a closed loop. The complete genome sequence of the virulent strain, H37Rv, was published in 1998 (Cole *et al.*, 1998). It consists of 4,411,529 base pairs encoding around 4000 genes. Several factors make *M. tuberculosis* a difficult organism to study in the laboratory, hindering TB research: the bacteria multiply very slowly, only once every 18 to 24 hours, and can take more than a month to form colonies in culture. The tubercle bacilli tend to form clumps, making it difficult to enumerate numbers of bacilli. Most discouraging, *M. tuberculosis* is a dangerous, airborne organism and can only be studied in laboratories that have specialized biosafety level 3 (BSL3), safety equipment and facilities.

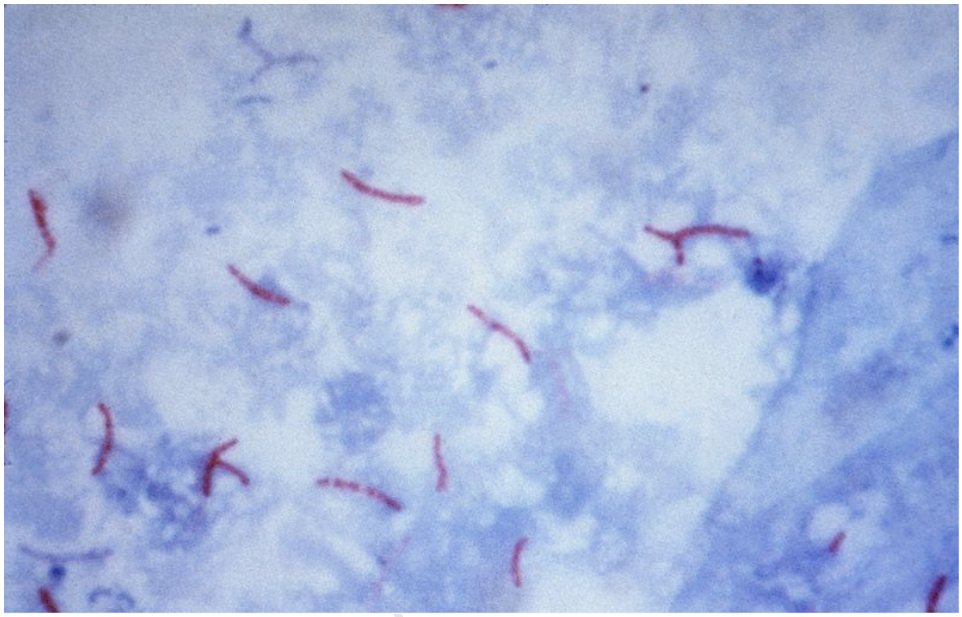

Figure 1.2: *Mycobacterium tuberculosis* Ziehl-Neelsen stain
Sputum smear after Ziehl-Neelsen staining showing acid-fast bacilli (red rods) retain bright red dye after washing with acid alcohol. Downloaded from (CDC, 1979).

1.4 Pathogenesis

TB is transmitted in the form of aerosol droplets inhaled into the lungs. When a person with active lung TB (smear-positive TB) coughs, sneezes, talks or even spits, they propel mycobacterium into the air (WHO, 2010d). Pulmonary or lung TB is the most common form of the disease and is characterised by a cough (with bloody sputum in the advanced stage) lasting more than 3 weeks, accompanied by weakness or fatigue, chest pain, weight loss, fever and/or night sweats (WHO, 2010b). The infection can also spread via the bloodstream, becoming miliary or disseminated TB, and affecting many organs including the meninges, the bones, or the internal organs. Once inside the respiratory tract, complex interactions occur between the pathogen and the host immune system (Schluger and Rom, 1998). The virulence of the organism together with the non-specific and specific immune responses of the host, determine the clinical outcome.

Infection with the organism can lead to 1) immediate elimination, 2) immediate disease (primary TB), 3) indefinite dormancy inside the host (latent infection), or 4) disease many years later (secondary TB), often in the context of relative or overt immunosuppression (Kaufmann and McMichael, 2005). Figure 1.3 illustrates the possible outcomes after exposure to *M. tuberculosis* (Kaufmann and McMichael, 2005). The majority of immunocompetent people (90%) will not develop TB disease after exposure. These individuals can mount an effective immune response to the initial encounter, which limits multiplication of the bacilli. Long lasting, albeit partial, immunity is achieved, both to control further infection and reactivation of latent bacilli. Only 10% will go on to develop pulmonary or non-pulmonary forms of TB. Secondary TB (reactivation disease) is usually due to the reactivation of old lesions (Lillebaek *et al.*, 2002; Manabe and Bishai, 2000). *M. tuberculosis* microfoci that have remained dormant after primary infection (latent TB) may undergo reactivation at a later time leading to secondary TB. Recent evidence suggests that exogenous re-infection is responsible for a significant proportion of TB observed in high incidence countries. (Caminero *et al.*, 2001). Latent TB has recently been described as a broad spectrum of conditions from low

grade TB, non-progressing percolating infection, to dormant infection (Barry *et al.*, 2009; Lin and Flynn, 2010).

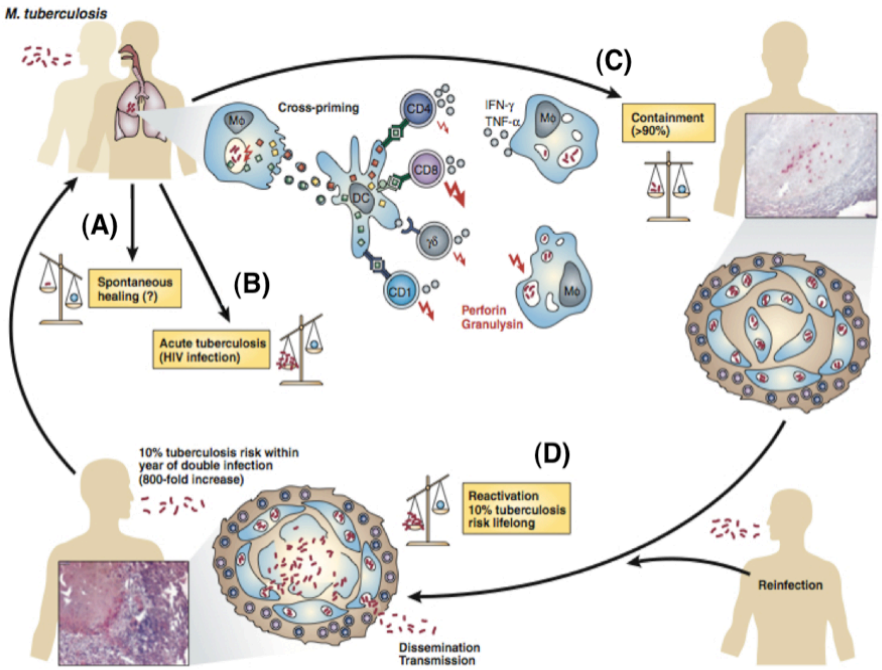

Figure 1.3: Schematic diagram showing possible outcomes after *M. tuberculosis* exposure

(A) Spontaneous healing is rare to absent. (B) Primary disease occurs most notably in the presence of immunodeficiency (e.g. HIV-infection). (C) Containment of infection inside granulomas occurs in 90% of infected individuals. Granulomas consist of infected macrophages and dendritic cells, which present mycobacterial antigens to CD4 T cells, CD8 T cells, gamma delta (γδ) T cells and CD1-restricted T cells. *M. tuberculosis* is killed by IFN-γ and tumour necrosis factor-α (TNF-α) activated macrophages or via perforin and granulysin. (D) Secondary disease due to reactivation or reinfection may occur at a later time. Adapted from (Kaufmann and McMichael, 2005).

1.5 Tuberculosis treatment

Tuberculosis treatment involves daily combination therapy for the duration of 6 to 12 months, depending on the clinical manifestations of TB. The treatment is usually divided into 2 phases: 2 months of 3-4 drugs to rapidly decrease bacillary load and then followed by a maintenance phase with 2 drugs for the remainder to kill off remaining organisms. The routinely used first line drugs include isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E) and streptomycin (S). These drugs have high microbicidal effect and an acceptable safety profile. The advantages of using combination therapy are (1) to prevent emergence of resistant bacilli, (2) drugs like H and R may act synergistically, (3) Z is most effective when bacteria are actively replicating in the initial stages of therapy, (4) duration of treatment is reduced, and (5) to act simultaneously on all subpopulations of *M. tuberculosis* (faster and slower growing organisms) (Marais *et al.*, 2006). Second line drugs are only used in instances of drug resistance to the first line drugs, which have lower anti-TB effect, higher toxicity or both. The WHO recommends the same regimen and duration of treatment in HIV-infected individuals as in HIV negative individuals. Drug susceptibility testing is now recommended at initiation of therapy in previously treated TB patients as well as TB/HIV co-infected patients (WHO, 2010c).

1.6 Childhood tuberculosis

1.6.1 Tuberculosis in the immunocompetent child

Approximately one million children are estimated to develop TB globally every year (Nelson and Wells, 2004; Walls and Shingadia, 2004; Newton *et al.*, 2008). The source of transmission of *M. tuberculosis* to children most often is a household source case, usually a parent or other adult family member (Shah *et al.*, 2006). Other adults in the community may also be a source of childhood TB (Schaaf *et al.*, 2003). Children rarely transmit bacilli to each other because of lower bacterial loads. Infants and young children are at much higher risk of developing disseminated infection after primary

tuberculosis and particularly tuberculosis meningitis, which has the highest mortality (Marais *et al.*, 2006). HIV infection markedly increases this risk (Lawn and Churchyard, 2009) and infected children have increased mortality (Madhi *et al.*, 2000) compared with HIV-negative children (Swaminathan, 2004).

The diagnosis of childhood TB is more difficult than adult TB. Children have nonspecific symptoms, chest x rays are less specific, it is difficult to obtain sputum samples from children and their lower bacterial loads in sputum makes smear and culture less effective (Marais *et al.*, 2006). Improved diagnostic methods are urgently required for childhood disease. Development of drug resistance is less common in children because of their low bacterial load (Schaaf *et al.*, 2000). Multidrug resistant tuberculosis is therefore almost always acquired from adults (Schaaf, 2007). The continued increase in childhood cases of TB represents ongoing transmission of *M. tuberculosis* in the community.

1.6.2 Tuberculosis in the HIV-infected child

Immune suppression, most commonly due to HIV infection, greatly enhances the risk of primary or latent infection progressing to active TB (Cantwell and Binkin, 1997; Jeena *et al.*, 2002; Lawn and Churchyard, 2009). Although most HIV-related TB is due to reactivation of latent infection in adults, primary disease of all clinical types also occurs, particularly in children (Mukadi *et al.*, 1997). HIV-related TB differs from the classical types in that, as immune responses are suppressed, there is limited walling-off of the lesions. This encourages local spread as well as widespread dissemination of disease. More specifically, HIV-infection results in the loss of CD4 T cells. This includes mycobacteria-specific CD4-T cells, and comprehensive disturbances of all areas of immune function, from antigen-presentation to adaptive responses, driven by T cells. Increases in HIV viral load in serum and macrophages (Mø) following *M. tuberculosis* infection has been observed, as well as a switch from Th1 to Th2 dominance via alterations in IL-10, regulatory

T cells, IL-12, IL-4, and TNF, leading to loss of granuloma integrity, and progression to active TB (Djoba Siawaya *et al.*, 2007).

The hallmark of HIV-infection is the ongoing loss of CD4 T lymphocytes. If untreated, this ultimately promotes the development of Acquired Immunodeficiency Syndrome (AIDS) and increased susceptibility to opportunistic infections. HIV infection and AIDS also complicate diagnosis of TB markedly. The Mantoux test, which measures sensitisation to mycobacterial antigens and is considered one of the mainstays of TB diagnosis in childhood, is significantly less sensitive in HIV infection. A retrospective study by Jeeana *et al.* showed Mantoux positivity in 18% of TB/HIV co-infected children compared with 48% in a group of non-HIV-infected TB infected children (Jeena *et al.*, 2002). Both groups of children were recruited from the same public hospital that receives referrals from a population of 3–4 million children. Hence, it has been proposed that 5 mm induration on Mantoux testing instead of 10 mm can be used as the cut-off for positive result in HIV-infected individuals and AIDS patients. HIV-infected individuals and AIDS patients receive the same TB treatment as HIV negative TB patients. Strict follow-up with chest radiographs and follow-up cultures for *M. tuberculosis* is highly recommended.

1.7 Immunity to tuberculosis

1.7.1 Host response to Mycobacterium tuberculosis

After inhalation of circulating aerosol droplets containing infective mycobacteria, initial infection is established in resting Mø lining the alveolar spaces of the lungs (Henderson *et al.*, 1963). Recognition and uptake of microbial products is facilitated by pattern recognition receptors (PRR) (Akira *et al.*, 2006). These receptors recognise conserved pathogen associated molecular patterns (PAMPs) on microbes, such as lipoarabinomannan (LAM), lipopolysaccharide (LPS) and bacterial lipoteichoic acid (Medzhitov and Janeway, 2000; Medzhitov, 2007). Individual PRRs have distinct expression patterns, bind to specific ligands (PAMPs), activate signaling pathways and trigger anti-pathogen host responses, such as activation of proinflammatory cytokines (e.g. TNF, and interleukin IL-12, co-stimulatory molecules, or type I IFN) (Akira *et al.*, 2006). PRRs are surface or cytosolic molecules, and include complement receptors, mannose receptors (Schlesinger, 1993; Ernst, 1998), scavenger receptors (Ernst, 1998), Toll-like receptors (TLRs) (Thoma-Uszynski *et al.*, 2001) and nucleotide-binding oligomerisation domain (NOD)–like receptors (NLRs) (Ferwerda *et al.*, 2005; Fritz *et al.*, 2006).

The first line of defence after inhalation of *M. tuberculosis* is represented by Mø, dendritic cells (DC) and possibly neutrophils, which may destroy the intracellular bacteria using microbicidal properties (van Crevel *et al.*, 2002; Kisich *et al.*, 2002; Eum *et al.*). Mø are equipped with machinery to kill and degrade ingested microorganisms. In the mouse macrophage the oxidative burst leads to production of highly microbicidal reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Ding *et al.*, 1988; Akaki *et al.*, 2000). Lysosomal enzymes may also degrade pathogens in an acidic environment after phagolysosomal fusion (Schaible *et al.*, 1998). However, *M. tuberculosis* has co-evolved with the human host and has developed multiple strategies to evade destruction by Mø. Described mechanisms include the prevention of acidification of the phagosome and prevention of phagosome/lysosome fusion (Deretic *et al.*, 2006; Vandal *et al.*, 2009).

Armstrong and Hart cultured mouse macrophage monolayers containing Ferritin-labelled lysozymes with a viable or gamma irradiated (inactivated), virulent strain of *M. tuberculosis* (H37Rv) (Armstrong and Hart, 1971). Electron microscopy revealed a marked tendency for the phagosomes containing viable bacilli not to have fused with ferritin-marked lysosomes compared with the inactivated bacilli. This allowed the viable bacilli to survive and multiply within the infected cell. Phagolysosomes are characteristically acidic (below pH 5.5) and are rich in hydrolytic enzymes. The low pH enhances host defenses by inhibiting microbial growth and enhancing the activity of degrading enzymes. Thus by preventing phagosome/lysosome fusion the bacilli avoid direct exposure to the toxic contents of these organelles.

When Mø fail to destroy the mycobacteria, the host's immune system second line of defence is to limit mycobacterial growth inside granulomas (**Figure 1.4**), the hallmark of TB pathology (Ulrichs and Kaufmann, 2006). The host's innate immune response is crucial for the initial stages of defence (van Crevel *et al.*, 2002; Bhatt and Salgame, 2007). However, it is the adaptive immune response that is required when infection persists. Monocytes, Mø, epitheloid cells, neutrophils, T cells and natural killer cells are recruited to the site of infection. These cells form a granulomatous wall around the infected Mø (**Figure 1.4**) (Ulrichs and Kaufmann, 2006). Infected epitheloid cells fuse to form multinucleated Langerhans cells. The centre of the granuloma is sometimes characterized by caseation necrosis, more especially in progressive disease. IFN-γ producing antigen-specific CD4 T cells activate, infected Mø to kill mycobacteria (Chan *et al.*, 1992). CD8 T cells are also important in chronic infection (van Pinxteren *et al.*, 2000). A less than optimal cell-mediated host immune response leads to poor granuloma formation, characterized by uncontrolled *M. tuberculosis* replication within phagocytic cells and subsequent development of TB disease. The exact immune factors and mechanisms that lead to the successful or unsuccessful immune response to *M. tuberculosis* infection are poorly understood. Comprehension of these factors and mechanisms would greatly accelerate the process of developing more effective TB vaccines.

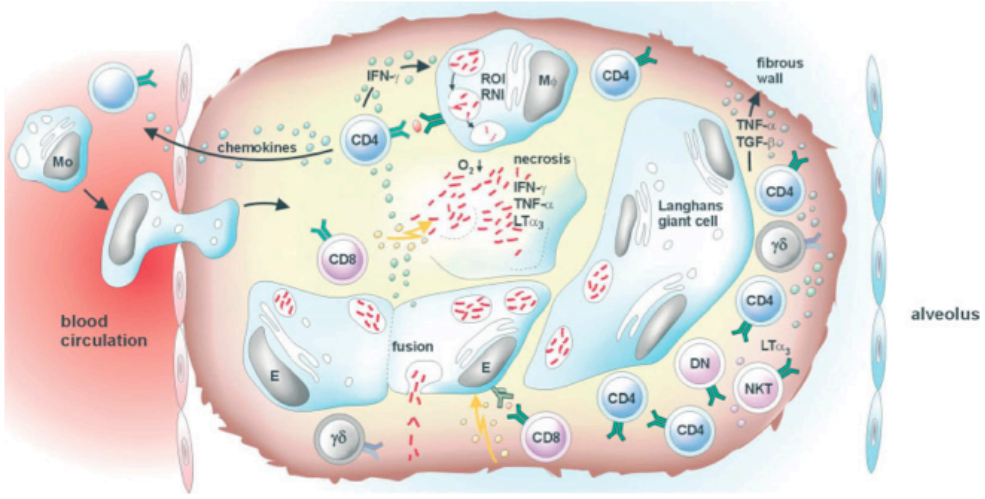

Figure 1.4: Host response and granuloma formation

Macrophages (Mø) and epitheloid cells are at the centre of the granuloma. The latter fuse to form giant Langerhans cells. These infected cells present antigens to T cells. CD4 T cells are activated to produce cytokines such as IFN-γ and chemokines. IFN-γ activated antigen-presenting cells kill the intracellular bacteria via reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). Lymphocytes are recruited from blood and migrate to the site of infection in response to chemokines CD4+ T cells and Mø produce TNF-α and lymphotoxin-α 3 (LTα 3), which are required for formation of the wall surrounding the granuloma. In the centre of the granuloma, low oxygen forms a hostile environment for mycobacteria. Activated CD8+ T cells kill mycobacteria by means of granulysin and perforin. Adapted from (Ulrichs and Kaufmann, 2006).

1.7.2 Innate responses against mycobacteria

Innate immunity is described as the first step of host responses following antigen exposure. It is described as rapid, lacks antigen-specific qualities and does not create immunological memory. The contribution of the innate response to TB is beyond the scope of this literature review and is only briefly addressed. *M. tuberculosis* first encounters obstacles of innate immunity such as antimicrobial peptides (e.g. defensins, cathelicidins) and proteins (e.g. lactoferrin, lysozyme) in airway secretions with both bactericidal and

immunomodulatory effects (Ganz, 2002). These peptides and proteins are produced by multiple cell types; including neutrophils, monocytes, Mø, T cells and epithelial cells (Ganz, 2002; Yang *et al.*, 2001). They are present in the airways from the early neonatal period, however their role in paediatric TB is yet to be established (Yoshio *et al.*, 2004; Zasloff, 2003).

M. tuberculosis infects human alveolar epithelial cells (Bermudez and Goodman, 1996) and induces production of β-defensins, which participate in bacillary killing (Rivas-Santiago *et al.*, 2006). Mø acquire human neutrophil α-defensins (HNPs) released from apoptotic neutrophils upon ingestion of these cells, which results in killing of the intracellular bacilli (Tan *et al.*, 2006). TNF-α secreted by Mø, in turn, can stimulate neutrophil antimicrobial activity (Kisich *et al.*, 2002). Neutrophils are abundant in the BAL fluid of adults with pulmonary tuberculosis and frequently contain mycobacteria in an active state of division (Eum *et al.*, 2010). When stimulated with mycobacterial antigens, neutrophils produce cytokines and chemokines, such as IL-8 and macrophage inflammatory protein-lα (MIP-lα) (Kasahara *et al.*, 1998). IL-8 attracts more neutrophils and lymphocytes, and (MIP-lα) attracts and activates monocytes/Mø. Thus, neutrophils can potentially influence adaptive immune responses. These observations suggest a substantial role for neutrophils in the control and/or pathogenesis of *M. tuberculosis* infection.

1.7.3 Initiation of adaptive immunity through antigen processing and presentation

Having survived the first obstacles of innate effector mechanisms, *M. tuberculosis* is readily phagocytosed by resident alveolar Mø and DCs (van Crevel *et al.*, 2002). DCs are extremely proficient antigen presenting cells (APC). They have a crucial role in the initiation of the antigen-specific T cell response to pathogen (Banchereau and Steinman, 1998; Henderson *et al.*, 1997; Giacomini *et al.*, 2001).

Researchers have identified DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) as the major receptor for *M. tuberculosis* entry into human DCs, in addition to CR3 and mannose receptor (Geijtenbeek *et al.*, 2003; Tailleux *et al.*, 2003; Kaufmann and Schaible, 2003). Internalisation of *M. tuberculosis* is initiated by binding of DC-SIGN to mannose-capped lipoarabinomannan (Man-LAM), a major component of the mycobacterial cell wall. This process results in activation and maturation of the DC and is characterised by upregulation of major histocompatability complex (MHC) class I and II molecules, co-stimulatory molecules, CD54, CD40, and B7.1 (CD80) and secretion of IL-1, IL-12 and TNF-α (Henderson *et al.*, 1997; Giacomini *et al.*, 2001). Activated DCs migrate to the draining lymph nodes where they present processed *M. tuberculosis* antigens on surface MHC class II to naïve CD4 T cells and MHC class I to naïve CD8 T cells. Cytokines such as IL-12 stimulate the proliferation and differentiation of naïve T cells, thereby inducing the adaptive immune response (Hamza T, *et al.*, 2010). In addition to the recruitment of CD4 T cells by the action of IL-12 from Mø and DCs, this cytokine recruits multiple other cell types to the locality, such as natural killer (NK) cells and $\gamma\delta$ T cells, which also secrete cytokines to activate infected Mø.

Each MHC class II molecule is composed of a heterodimer of two transmembrane glycoproteins, the α and β chains (van Niel *et al.*, 2008). This molecule is synthesized in the endoplasmic reticulum (ER) and binds with trimers of the invariant chain (Ii). Association of MHC II with the Ii prevents MHC-peptide binding in the ER (Roche and Cresswell, 1990; Stumptner and Benaroch, 1997). The Ii then facilitates the transport of MHC class II molecules from the ER to acidic endosomal compartments (Lamb *et al.*, 1991). Once in the endosome, lysosomal proteases degrade the Ii chain into a smaller fragment known as the class II associated Ii chain peptide (CLIP), which remains attached to the MHC-peptide groove (Villadangos, 2001). Binding of the MHC-CLIP complex to the human leukocyte antigen (HLA)-DM frees the CLIP and allows loading of peptides onto the MHC class II molecule (Roche, 1995; Kropshofer *et al.*, 1997). MHC-peptide complexes are then

transported to the cell membrane for presentation of these exogenously derived antigens to naïve CD4 T cells.

In contrast, MHC class I molecules present peptides to CD8 T cells, generated from predominantly endogenous proteins produced in the cytosol (Rock and Goldberg, 1999; Pamer and Cresswell, 1998). Intracellular organisms such as *M. tuberculosis* are enclosed within phagosomal compartments and this separates bacterial proteins from the cytosol (Deretic and Fratti, 1999; Vergne *et al.*, 2004). Despite this, several researchers have observed CD8 T cell responses to *M. tuberculosis* antigens (Serbina and Flynn, 1999; Smith *et al.*, 2000). Mechanisms of presentation of these mycobacterial peptides to CD8 T cells include cross-presentation and cross-priming (**Figure 1.5**) (Kaufmann and Schaible, 2005).

In cross-presentation, mycobacterial proteins such as ESAT-6 and CFP-10 (with membrane-disrupting properties), escape from the phagosome into the cytosol **(Figure 1.5 A.i)**. Escape into the cytosol leads to degradation of these mycobacterial proteins by the proteasome **(Figure 1.5 A.ii)**: the classical MHC I pathway. Bacterial peptides are then transported back into the phagosome by transporters associated with antigen processing (TAP, **Figure 1.5 A.iii)**, where loading onto MHC class I **(Figure 1.5 A.iv)** occurs via ER-mediated phagocytosis (Gagnon *et al.*, 2002; Guermonprez *et al.*, 2003; Ackerman *et al.*, 2003; Houde *et al.*, 2003). Cross-priming, at least of mycobacterial antigens is proteasome-independent (Schaible *et al.*, 2003). This process requires antigen translocation from infected Mø to bystander DCs (Kaufmann and Schaible, 2005). This may occur when *M. tuberculosis* infected Mø undergo apoptosis, release apoptotic blebs containing bacterial antigens **(Figure 1.5 B.i)** that are then taken up by DCs **(Figure 1.5 B.ii)** (Schaible *et al.*, 2003; Winau *et al.*, 2004; Winau *et al.*, 2005; Winau *et al.*, 2006). Apoptotic blebs fuse with lysosomes, bacterial antigens are degraded and peptides are transported via TAP into the ER **(Figure 1.5 B.iii)**. These peptides are then loaded onto MHC class I **(Figure 1.5 B.iv)**, or CD1 molecules **(Figure 1.5 B.v)**. DC migrate to the lymph nodes and prime CD8 T cells by presenting antigen on MHC class I molecules.

Intact antigen processing and presentation machinery are important for efficient priming of CD4 and CD8 T cell responses to *M. tuberculosis*. Efficient priming of specific T cell populations is thought to be central for new vaccines to be effective.

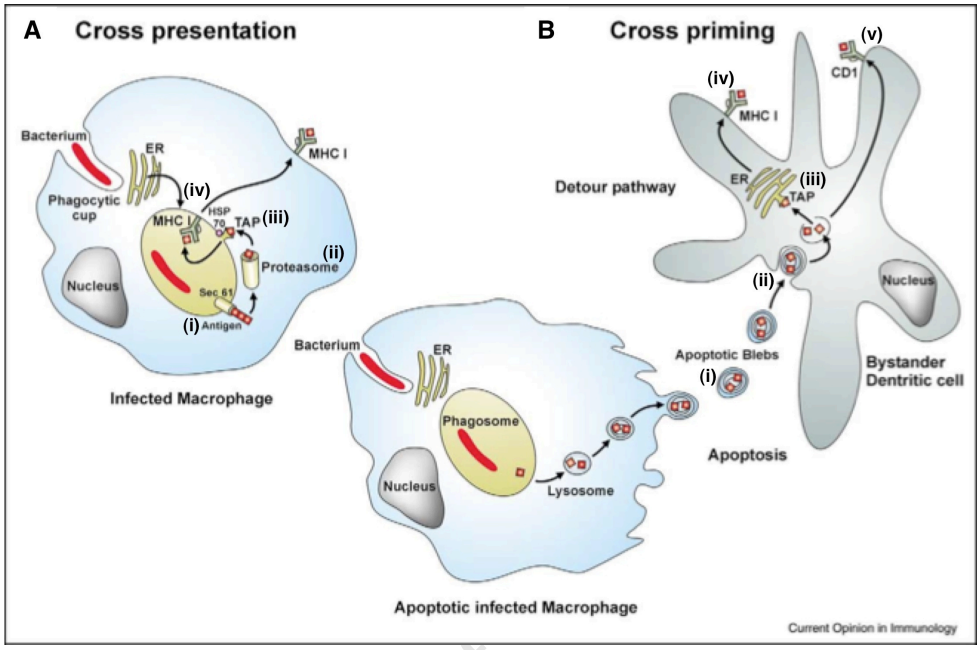

Figure 1.5: Bacterial peptide processing and presentation to CD8 T cells
Priming of CD8 T cells with exogenously derived antigens occurs through mechanisms of (A) Cross-presentation and (B) cross-priming on MCH class I molecules. In cross presentation, antigens are loaded onto MHC class I molecules from the same cell. In cross-priming antigens from infected macrophages are translocated to nearby DCs and loaded onto MHC I molecules. Adapted from (Kaufmann and Schaible, 2005).

Th1 type cytokines such as IFN-γ and TNF-α are critical for effective immunity against *M. tuberculosis* infection (Newport *et al.*, 1996; Keane *et al.*, 2001). IL-17 a Th17 cytokine, may contribute to inflammation induction of antimicrobial peptides and recruitment of Th1 cytokine producing CD4 T cells, resulting in inhibition of *M. tuberculosis* growth in the lungs of mice (Lockhart *et al.*, 2006). This suggests a role for the Th17 response in triggering of Th1 cells and subsequent macrophage and effector T cell activation at the primary site of

TB infection. Th17 cells are a T cell subset with significant proinflammatory functions.

In contrast to a Th1/Th17 response, a Th2 response characterised by IL-4, IL-5 and IL-13 production has been associated with a reduced Th1 response (Lienhardt *et al.*, 2002; Rook, 2007). IL-10, an anti-inflamatory cytokine and transforming growth factor beta (TGF-β) production by T regulatory cells (T_{reg}) inhibit Th1, Th2 and Th17 subsets. TGF-β is associated with increased dissemination of *M. tuberculosis* (Sharma *et al.*, 2009). However, this remains controversial, and may simply reflect a response to the inflammation driven by the pathogen.

1.7.4 Cell mediated adaptive responses against mycobacteria

An effective immune response to TB requires activation of infected Mø by antigen-specific T cells and killing of intracellular tubercle bacilli (Cooper and Flynn, 1995). Murine gene knockout experiments have shown that IFNγ is a crucial activating cytokine (Cooper *et al.*, 1993; Flynn *et al.*, 1993). Human studies have shown that antigen-specific CD4 T cells are critical, since impairment of CD4 T cell responses, as seen during HIV infection, leads to increased susceptibility to TB (Elliott *et al.*, 2004). CD8 T cells may also be important for immunity to TB (Derrick *et al.*, 2004; Billeskov *et al.*, 2007; Bruns *et al.*, 2009; Chen *et al.*, 2009). TB vaccine induced CD4 and CD8 T cell responses have therefore been major read-outs in clinical trials.

1.7.4.1 CD4 T cells

Once primed in the lymph node, MHC class II restricted *M. tuberculosis*-specific CD4 T cells are recruited to the site of infection and produce Th1 type cytokines such as IFNγ, TNF-α and IL-2. IFNγ is a crucial effector molecule and together with TNF-α activates infected Mø to kill or control intracelluar organisms. Both murine and human studies have shown that CD4 T cells have an essential role in protective immunity to *M. tuberculosis*. Mice deficient

in MHC II or CD4 T cells demonstrate an impaired ability to control infection and succumb to TB (Caruso *et al.*, 1999; Mogues *et al.*, 2001). Orme et al. demonstrated that adoptive transfer of CD4 T cells conferred protection against M. tuberculosis in T cell deficient mice (Orme and Collins, 1983). Human studies in purified protein derivative (PPD) positive individuals showed increased recruitment of IFN-γ producing antigen-specific CD4 T cells to the lung after bronchi challenge with recall antigens (Walrath *et al.*, 2005). Although $CD8^+$ T cells also secrete this cytokine, $CD4^+$ T cells constitute the major source of this protein. While it is clear that CD4 T cells and Th1 cytokines are critical in the cell-mediated response to *M. tuberculosis*, it is also apparent that this part of the immune response alone is not enough. Improved laboratory assays have allowed the simultaneous measurement of T cell populations and cytokines in small blood samples. This has led to the discovery that T cell subsets, cytokines, and chemokines may be important in immunity to TB.

1.7.4.2 CD8 T cells

CD8 T cells recognise mycobacterial peptides associated with MHC class I via cross-presentation or cross-priming mechanisms (Kaufmann and Schaible, 2005). Although there are conflicting views on the role of CD8 T cells in *M. tuberculosis* immunity, data supporting their importance is increasingly compelling. Chen *et al.* recently demonstrated a role for CD8 T cells in a non-human primate model of TB (Chen *et al.*, 2009). They reported that depletion of CD8 T cells in BCG-vaccinated rhesus macaques led to a significant decrease in vaccine-induced immunity against tuberculosis. Also, depletion of CD8 T cells in macaques previously infected with *M. tuberculosis* and then cured by antibiotic therapy, also resulted in a decrease in control of *M. tuberculosis* re-infection. Work by Bruns *et al.* demonstrated that treatment of autoimmune diseases with anti-TNF antibodies in humans was associated with decreased frequencies of CD8 effector memory T cells as well as decreased *in vitro* antimicrobial activity against *M. tuberculosis*. The *in vitro* antimicrobial activity was recovered when PBMC from patients with anti-TNF

therapy were supplemented with $CD8^{+}CCR^{-}CD45RA^{+}$ effector memory T cells (Bruns *et al.*, 2009).

CD4 and CD8 T cells produce cytokines that activate Mø and lead to granuloma formation (IFNγ and TNFα). Both CD4 and CD8 T cells also have direct-cytotoxic activity and express microbicidal molecules (perforin and granulysin) (Cho *et al.*, 2000; Murray *et al.*, 2006). Granulysin granules are produced by CD8 T cells, NK cells and γδ T cells and can kill extra-and intracellular mycobacteria, in conjunction with perforin (Stenger *et al.*, 1998; Woodworth *et al.*, 2008). *M. tuberculosis* specific CD8 T cells are expanded in adults (Caccamo *et al.*, 2009) and children (Jacobsen *et al.*, 2007) with TB, suggesting that CD8 T cells contribute to the protective immune response against *M. tuberculosis*. Carranza *et al.* have demonstrated that autologous CD8 T cells obtained from healthy household contacts of patients with active pulmonary tuberculosis significantly increased the capability of infected Mø to limit the growth of *M. tuberculosis* (Carranza *et al.*, 2006). However, more research is needed to understand their role in protection against TB more comprehensively.

1.8 Type 1 cytokines

The majority of people exposed to *M. tuberculosis* mount a protective immune response that controls infection. However, our knowledge about biomarkers, which can be used as correlates of protection against tuberculosis remains limited (Mittrucker *et al.*, 2007; Wallis *et al.*, 2010). Although no definite correlate of protection has been identified, the importance of type 1 cytokines such as IFN-γ, TNF-α and IL-12 in immunity against *M. tuberculosis* cannot be disputed (Flynn *et al.*, 1993; Flynn *et al.*, 1995; Cooper and Flynn, 1995; Ottenhoff *et al.*, 1998). IL-2, a T cell growth factor produced by CD4 T cells is required for memory T cell development as well as secondary expansion of memory T cells (Schluns and Lefrancois, 2003; Williams *et al.*, 2006; Dooms *et al.*, 2007). This is important for proliferation of antigen experienced T cells in the defense against *Mycobacterium tuberculosis* (Kamath *et al.*, 2006).

1.8.1 Interferon gamma (IFN-γ)

IFN-γ is the most commonly measured cytokine, since it has been shown to be critical in control of *M. tuberculosis*. (Flynn *et al.*, 1993; Orme *et al.*, 1993). Mice with a disrupted IFN-γ gene are highly susceptible and succumb to *M. tuberculosis* (Cooper *et al.*, 1993; Flynn *et al.*, 1993). These mice are able to form granulomas but are unable to produce RNI and to limit mycobacterial growth. Humans with genetic defects in the IFN-γ (or IL-12) pathways suffer from increased susceptibility to mycobacterial disease, which often manifest in childhood (Newport *et al.*, 1996; Jouanguy *et al.*, 1996; Ottenhoff *et al.*, 1998; Ottenhoff *et al.*, 2000; Ottenhoff *et al.*, 2005). IFNγ is critical for optimal activation of Mø and hence for protection against TB (Flesch and Kaufmann, 1987; Ottenhoff *et al.*, 2000). In mice, IFNγ mediates its protective effect predominantly by the induction of RNI, which are necessary for the killing of the intracellular mycobacteria (Raja, 2004). In contrast, the role of RNI in human immunity to *M. tuberculosis* is not completely understood (Aston *et al.*, 1998). However, nitric oxide synthase 2 (NOS2), expression has been observed in human cells, and mutations in the NOS2 gene are associated

with susceptibility to developing TB (Velez *et al.*, 2009).

1.8.2 Interleukin-12 (IL-12)

IL-12 is a critical promoter of Th1, cells that produce IFNγ (T lymphocytes) and is produced by activated Mø and DC on ingestion of *M. tuberculosis* (Okamura *et al.*, 1998; Fulton *et al.*, 1996; Ladel *et al.*, 1997). In a murine model of TB, treatment of mice with exogenous IL-12 increased resistance to *M. tuberculosis* (Cooper *et al.*, 1997) Treatment with IL-12 increases the accumulation of Mø in infected tissues. IL-12 gene-disrupted mice are unable to control *M. tuberculosis* infection (Cooper *et al.*, 1997) and this is associated to the absence of IFN-γ production. Humans with defects in the IL-12 receptor gene (Altare *et al.*, 1998; de Jong *et al.*, 1998), or the IL-12 gene itself (Altare *et al.*, 1998), are highly susceptible to infection with intracellular pathogens and in particular mycobacteria (Ottenhoff *et al.*, 1998).

1.8.3 Tumour necrosis factor alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine, which activates macrophage antimicrobicidal activity and is essential in granuloma formation (Flesch and Kaufmann, 1990). Although it has long been considered a crucial component of protection in TB, TNF-α also has potent pro-inflammatory activity and acts as a mediator of tissue damage during TB. Its expression by Mø, DC and T cells can be induced by *M. tuberculosis* infection (Ladel *et al.*, 1997; Henderson *et al.*, 1997; Barnes *et al.*, 1993; Serbina and Flynn, 1999) and absence/alteration of TNF-α production or signalling has been shown to impair control of mycobacterial infection (Flynn *et al.*, 1995; Bean *et al.*, 1999). TNF-α-receptor, gene deficient mice are extremely susceptible to tuberculosis (Flynn *et al.*, 1995) and show delayed expression of RNIs. The lack of TNF-α mediated effects also resulted in the lack of epitheloid cells within granulomas, although it did not affect the total number of granulomas formed (Flynn *et al.*, 1995). TNF-α depletion or neutralisation in mice results in decreased killing of mycobacteria by Mø, ultimately leading to tissue necrosis

and granuloma disintegration (Tufariello *et al.*, 2003; Saunders and Britton, 2007). In humans the importance of TNF-α was demonstrated in patients who receive TNF blockers for treatment of rheumatoid arthritis or Crohn's disease (Keane *et al.*, 2001). These patients subsequently had a much higher probability of reactivating latent TB.

TNF-α is first synthesized as a transmembrane precursor protein. The mature soluble TNF-α is cleaved from the plasma membrane by matrix metalloproteinases such as TNF-α converting enzyme (TACE) (Keane *et al.*, 2001; Decoster *et al.*, 1995). TNF is biologically activated by the binding of TNF-α to TNF receptor 1 or 2 (TNFR1 or TNFR2) on the surface of many cells (Vandenabeele *et al.*, 1995). A disintegrin and metalloprotease, (ADAM) family proteins can shed the extracellular domain of both TNF receptors (Crowe *et al.*, 1995; Mullberg *et al.*, 1995) Soluble TNF receptors (sTNFR) are able to bind to both soluble and membrane TNF. Thus they can neutralize TNF-mediated activities.

1.8.4 Interleukin 2 (IL-2)

The induction of immunological memory is the primary goal of vaccination. Although T cells primed in the absence of IL-2 are able to undergo clonal expansion, upon re-stimulation these cells are incapable of effective recall responses. Blattman *et al.* demonstrated that IL-2 therapy administered during the contraction phase in a lymphocytic choriomeningitis virus (LCMV) murine infection model, resulted in proliferation and prolonged survival of virus-specific CD8 and CD4 T cells and decreased viral burden (Blattman *et al.*, 2003). Several studies suggest that administration of IL-2 therapy to patients with mycobacterial disease, including TB, may be useful in limiting disease (Akuffo *et al.*, 1990; Kaplan *et al.*, 1991; Johnson *et al.*, 1997). Millington *et al.* report an increase of *M. tuberculosis*-specific IL-2 and IL-2/IFN-γ secreting T cells in effectively treated TB patients, compared with frequencies of these cells in these patients before treatment (Millington *et al.*, 2007). These researchers suggest that IL-2 production might be a marker of reduced antigen load and could therefore serve as a marker of clinical disease state

(Sargentini *et al.*, 2009). However, a clinical trial in patients with TB receiving recombinant IL-2 immunotherapy together with standard TB chemotherapy did not result in better outcome (Johnson *et al.*, 2003).

1.8.5 Polyfunctional T cells

Polyfunctional T cells are defined as T cells which co-express cytokines such as IFN-γ, TNF-α and IL-2. Such polyfunctional cells have been associated with more effective control of murine intracellular infections (Darrah *et al.*, 2007), including *M. tuberculosis* (Forbes *et al.*, 2008; Lindenstrom *et al.*, 2009). Studies of chronic viral infections in humans associate polyfunctional T cells with protective antiviral immunity (Harari *et al.*, 2006). HIV-1-specific T cells have been shown to shift from a predominantly single IFN-γ producing phenotype before treatment to a polyfunctional phenotype after commencing ART (Harari *et al.*, 2004). Similar to work first carried out by Betts *et al.*, Owen *et al.* observed polyfunctional responses in individuals who naturally control HIV-1 to low levels throughout the course of infection (elite controllers) (Betts *et al.*, 2006; Owen *et al.*, 2010).

Since there is no single cytokine that correlates with protection from TB, more recently, studies of polyfunctional T cells have been carried out to assess if polyfunctionality is associated with increased protection. However, the dynamics of these responses are complex. Induction of polyfunctional T cell responses by novel vaccines in both mice and humans is currently perceived as a desirable outcome (Forbes *et al.*, 2008; Lindenstrom *et al.*, 2009). Polyfunctional T cell responses induced by viral-vectored novel TB vaccines have been observed in adults (Beveridge *et al.*, 2007; Abel *et al.*, 2010) as well as adolescents and children (Scriba *et al.*, 2010).

However, a recent paper from our group demonstrated that there was no difference in the percentages of polyfunctional T cells in infants protected from TB following BCG vaccination compared with infants who were not protected, as they developed TB. This calls into question the association of

polyfunctional responses with protection from TB (Kagina *et al.*, 2010). Young *et al.* report increased polyfunctional CD4 and CD8 T cell responses in patients with active TB prior to treatment compared to after treatment (Young *et al.*, 2002; Young *et al.*). This is possibly a reflection of antigen load as opposed to protective immunity (Millington *et al.*, 2007). Other researchers have reported similar observations in TB patients (Caccamo *et al.*, 2010) and HIV-infected patients (Sutherland *et al.*, 2010) before and after ART. Further studies regarding the mechanism of induction of these polyfunctional T cells and their role in immunity to TB are required.

1.9 Immune memory

Immunological memory leads to a more rapid and quantitatively greater immune response of the host to a repeat exposure to pathogen (**Figure 1.6**) (Kaech and Wherry, 2007). Primary (1°) infection or vaccination induces naïve T cells to undergo clonal expansion resulting in increased numbers of antigen-specific T cells. These expanding cells differentiate into effector T cells with more rapid effector function. After antigen clearance, most of the effector T cells contract or undergo apoptosis (programmed cell death) and memory cells remain. The memory T cells expand faster and with an enhanced response to secondary (2°) challenge with pathogen (Kaech *et al.*, 2002; Seder and Ahmed, 2003). Several models of memory T cell differentiation have been proposed from studies of viral infections in both mice and humans (Kaech *et al.*, 2002; Seder and Ahmed, 2003; Appay and Rowland-Jones, 2004).

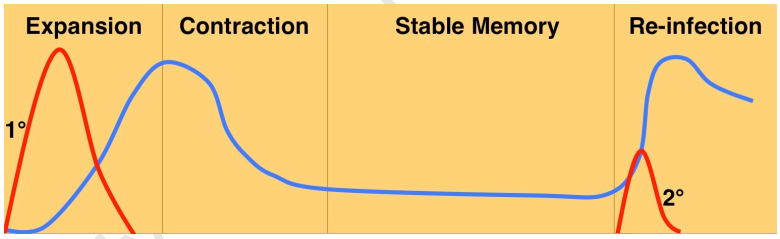

Figure 1.6: Memory T Cell Development
T cells (blue line) expand on pathogen (red line) encounter and differentiate into effector T cells, of which most die during a contraction phase, but some persist to become long-lived memory T cells. Upon re-infection, memory T cells re-expand and control infection faster than with initial encounter with pathogen. Adapted from (Kaech *et al.*, 2002). 1°; primary pathogen challenge, 2°; secondary pathogen challenge.

Memory cells have considerable heterogeneity in phenotype and function. CD4 and CD8 T cell cytokine expression has been linked with cell surface expression of markers of memory phenotype in viral infections in mice

(Sallusto *et al.*, 1999) and humans (Harari *et al.*, 2005). In order to study T cell differentiation and key T cell memory populations, it is useful to analyse these surface markers (Sallusto *et al.*, 1999). CD45RA (a leukocyte common antigen isoform) is lost by T cells upon priming, and can thus be used to differentiate naïve from memory (antigen experienced) T cells (Akbar *et al.*, 1988; Kolber, 2004). However, in some models highly differentiated antigen experienced cells can re-express this marker (Hamann *et al.*, 1997; Seder and Ahmed, 2003). Dunne *et al.* suggest that $CD45RA^{-}CD45RO^{+}$ may better represent primed cells (Dunne *et al.*, 2002).

Expression of the chemokine receptor, CCR7 on antigen-experienced cells, defines central memory T cells (T_{CM}), which express IL-2 and are able to home to lymphoid organs. Although these cells do not express immediate effector function, they are long lived and proliferate rapidly upon antigen reencounter. By contrast, effector memory T cells (T_{EM}), which lack CCR7 expression, predominantly express effector cytokines, such as IFN-γ (Sallusto *et al.*, 1999; Harari *et al.*, 2005). These populations can be further dissected according to their degree of differentiation, based on the expression level of CD27. This co-stimulatory marker is sequentially lost during T cell differentiation (Fritsch *et al.*, 2005).

The differential expression of cell surface markers such as CD45RA, CCR7, and CD27 on CD4 and CD8 T cells characterise the heterogeneity of the memory T cell response (**Figure 1.7 A and B**) (Appay *et al.*, 2002; Sallusto *et al.*, 2004; Appay *et al.*, 2008). Different effector functions associate with distinct intracellular molecules and surface phenotypes. For example antigen experienced $CCR7^{-}CD45RA^{+}CD27^{-}$ CD8 T cells express immediate effector function when presented with viral antigen and are pre-dominantly IFN-γ producers (Hamann *et al.*, 1997). IL-2 expression by $CCR7^{+}CD45RA^{-}CD27^{+}$ CD8 T cells can be low to high (**Figure 1.7 A**). Similarly CD4 T cells can also be subdivided into T cell populations showing differential expression of these phenotypes with distinct effector functions (**Figure 1.7 B**).

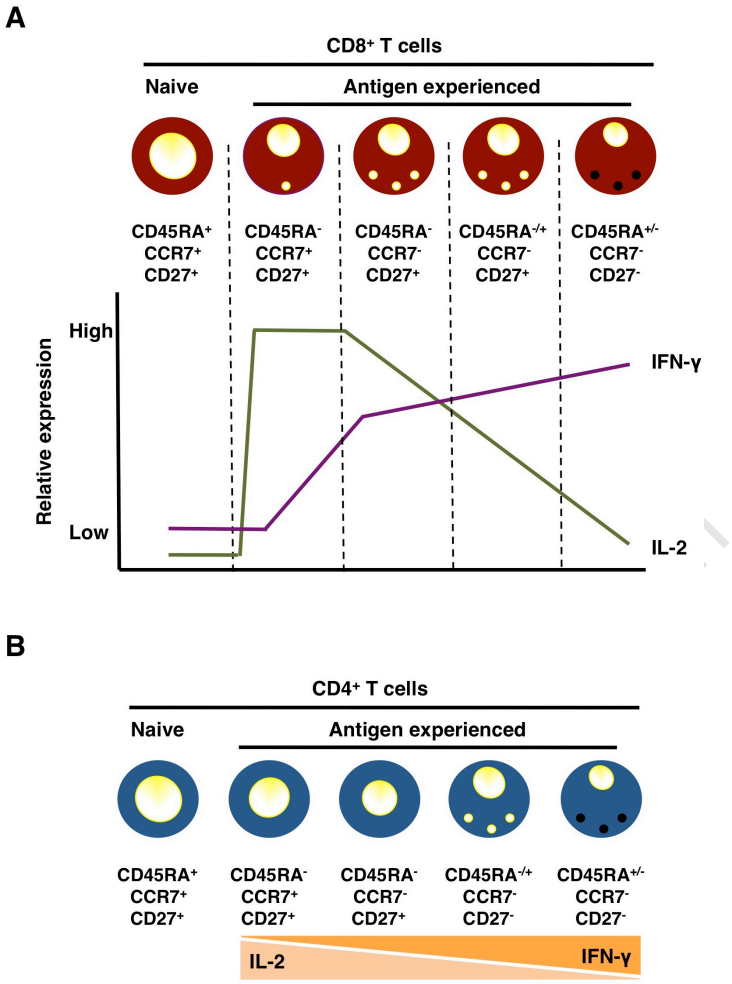

Figure 1.7: CD8 and CD4 T cell maturation in response to antigen
(A) CD8 and (B) CD4 T cell phenotypic associations to functional attributes. Five distinct subsets of circulating T cells are defined according to CD45RA, CCR7, and CD27 expression. Adapted from (Appay *et al.*, 2008).

The developmental pathway of memory T cell subsets from naive T cells remains a subject of debate in humans. The discussion centres, around whether the cell differentiation pathway is linear or branched, one-way or reversible. A linear differentiation pathway has been described in CD8 T cells from murine viral and bacterial infection models and is suggested to be progressive, from naïve → effector → T_{EM} → T_{CM} (Seder and Ahmed, 2003; Wherry and Ahmed, 2004). In contrast, studies of T cell differentiation in human viral infection suggest that T_{EM}-like cells with shorter telomere length

compared to T_{CM} are the end point of memory development (Champagne *et al.*, 2001; Appay *et al.*, 2002; Gamadia *et al.*, 2003; Papagno *et al.*, 2004). Appay *et al.* propose a progressive model of differentiation of CD4 and CD8 T cells from early → intermediate → late differentiated subsets (Appay *et al.*, 2002; Appay and Rowland-Jones, 2004), thus supporting a linear pathway of differentiation.

These models of memory development have mostly been studied in viral infections of animal models. More human studies of T cell memory phenotypes are needed in chronic bacterial infections such as TB. To date, there is no clear consensus on the development of memory/effector T cell differentiation (Appay *et al.*, 2008; Ahmed *et al.*, 2009), and the terminology of effector and memory responses has often been used inconsistently. Additional studies would need to better define the lineage factors and interplay of the different subsets in a more comprehensive fashion. However, the general agreement is that successful vaccination requires long-lived immunological memory that confers protection against infection or disease (Lambert *et al.*, 2005). In the context of TB vaccine development, the generation of memory T cells and thus the characterisation of mycobacteria-specific T cells is of particular importance, and has not been studied in great detail in HIV-infected children.

1.10 Effects of human immunodeficiency virus infection on the immune system and impact of antiretroviral therapy (ART)

Infection with HIV has a detrimental effect on several parts of the immune system, but its primary impact is on the population of CD4 T cells (Mogensen *et al.*, 2010). Progression of HIV infection to AIDS is characterised by a steady decline of this particular T cell subset, due to (although not limited to) preferential infection via certain cell surface receptors, such as CCR5 and CXCR4 (Mehandru *et al.*, 2007). The result is a profound defect in T cell function and depletion of cells, which places the host at risk of disease caused by opportunistic pathogens (Lloyd, 1996; Edgar, 2008; Geldmacher *et al.*, 2010). *M. tuberculosis* control requires CD4 T cells, thus HIV-infection is one of the most frequently encountered co-infections in areas with a high prevalence of TB. Through clinical experience it is now well established that the dramatic decline of CD4 T cells increases the risk to contract potentially lethal bacterial and viral co-infections (Lloyd, 1996; Wilcox and Saag, 2008).

The aim of anti-retroviral therapy (ART) is to prevent or slow down the profound decline of the CD4 T cells as well as delay or prevent onset of infections by interfering with the replication of HIV at several life cycle steps. The different drugs used in the combination therapy, which is the most effective treatment starts often at different stages of the HIV-life cycle in order to prevent successful replication (Nielsen *et al.*, 2005). Despite the success of protease and reverse transcriptase inhibitors in suppressing HIV-1 replication, new drugs are being evaluated. Among these new targets, inhibitors of virus attachment to target cells and integration are the most promising candidates (Moore and Stevenson, 2000). The aim of ART is to maintain an undetectable level of virus in the blood. It is not yet possible to completely eradicate HIV once a person is infected, and combination therapy needs to be taken continuously to avoid replication and development of viral resistance (Wilcox and Saag, 2008).

When to start ART in children was debated until recently, as the advantage of rapid viral suppression is potentially offset by the risk associated with very

long term use of powerful medication. However, it has now become clear that any infant found to be HIV infected should start ART as soon as possible after diagnosis, independent of the CD4 count (South African Department of Health, 2009). A reliable CD4 count result is not necessarily available in all resource poor settings and many children are only monitored clinically (Lloyd, 1996). As in adults, TB is a leading co-morbidity and ART decreases susceptibility. A large number of older children in HIV clinics in South Africa have never received ART and are in need of urgent treatment to prevent infections and death. The detrimental impact of abnormal function of CD4 T cells is easily appreciated from the extensive discussion of the T cell populations and their mediators in the above sections. The impaired cellular responses to mycobacterial antigens in both adults and children have been well characterised in this context, in particular reduced levels of IFN-γ (Tena *et al.*, 2003; Mansoor *et al.*, 2009), which is of central importance for containing mycobacterial infection, as discussed above. IFN-γ release assays (IFGRAs), used in the diagnosis of TB are promising, however, further investigation is needed for their use in high-risk groups such as HIV infected adults and children (Mori, 2009; Fujita *et al.*, 2010).

1.11 Prevention of tuberculosis through vaccination

1.11.1 BCG vaccine and basis for new vaccine candidates

Bacillus Calmette-Guérin (BCG), developed by two French researches, Albert Calmette and Camille Guérin, is still the only licensed vaccine against TB (WHO, 2004). It is a live attenuated strain of *M. bovis,* which is the causative agent of TB in cattle. The less virulent BCG vaccine strain was developed through serial subculture (231 passages) on slices of potato soaked in a glycerol-bile mixture, over a 13-year period (Benevolo-de-Andrade *et al.*, 2005). It was first given as a vaccine to humans in June 1921 (McKone, 1948). The vaccinee was a newborn, whose likely caregiver, the infant's grandmother, had TB. The infant's mother had succumbed to TB shortly after childbirth (Benevolo-de-Andrade *et al.*, 2005). Today, only a few countries do not use BCG for routine vaccination mostly because TB is relatively rare, for example, the USA and the Netherlands (Fine *et al.*, 1999). The BCG vaccine is used throughout TB-endemic countries, and is estimated to be administered to more than 100 million newborns annually as part of the WHO Expanded Programme for Immunization (EPI) (Hussey *et al.*, 2007).

BCG is safe and affords variable and mostly poor protection against pulmonary TB. Despite this, it does protect against severe childhood forms of TB, such as miliary TB and TB meningitis (Trunz *et al.*, 2006), which are associated with high mortality rates. Its efficacy in preventing pulmonary TB in adults has varied dramatically in various studies in different parts of the world from 80% in the United Kingdom to 0% in Chingleput, India (Fine, 1995). Environmental factors may contribute to this difference in efficacy. For example, it has been suggested that exposure to environmental mycobacteria may interfere with or mask the BCG induced immune response (Brandt *et al.*, 2002; Black *et al.*, 2002). Many other factors may also contribute to this discrepancy including BCG vaccine strain, age, genetic predisposition and immunosuppression amongst others.

Because of case reports of disseminated BCG infection (BCGosis) (Hesseling

et al., 2007; Hesseling *et al.*, 2009), this vaccine is contraindicated in HIV infection according to the revised WHO Vaccine Safety Committee guidelines (WHO, 2007). However, in reality, the HIV status of most infants is unknown at birth, when the BCG vaccine is routinely administered and many HIV-infected infants continue to receive it. In addition to BCGosis as a result of severe immune deficiency, BCG may also cause immune reconstitution syndrome (BCG-IRIS) once combination anti-retroviral therapy (ART) is commenced, particularly in children with very low CD4 T cell counts (Smith *et al.*, 2009). A more efficacious vaccine that is safe and protective in HIV-infected children is urgently needed. An increasing effort is being made to design better anti-tuberculosis vaccines including viral vectors, recombinant BCGs, and the use of *M. tuberculosis* proteins with an adjuvant (subunit vaccines) to induce more optimal immune responses.

1.11.2 Novel anti-TB vaccination strategies in the immunodeficient host

Despite its poor protection against pulmonary TB, BCG has good safety records and will continue to be used due to its beneficial effects against severe forms of TB. Thus novel TB vaccine development has focused on boosting BCG induced immunity using prime-boost vaccination strategies (Kaufmann *et al.*, 2006; Kaufmann, 2006; Kaufmann, 2010; Parida and Kaufmann, 2010) rather than eliminating the BCG vaccine altogether. Several promising vaccine candidates are currently in human clinical trials (McShane *et al.*, 2005; Brennan *et al.*, 2007; Hussey *et al.*, 2007; Hoft, 2008; Kaufmann *et al.*, 2010). The current development of new anti-TB vaccines centres around strategies for the generation of an improved BCG, such as recombinant BCG (rBCG) (Horwitz and Harth, 2003; Grode *et al.*, 2005; Horwitz *et al.*, 2006), viral vectored vaccines or the use of immunodominant antigens in subunit vaccines to boost the BCG prime (Weinrich Olsen *et al.*, 2001; McShane *et al.*, 2005; Reed and Lobet, 2005; Hoft, 2008). Since application of a live replicating vaccine to immunocompromised individuals carries the potential risk of dissemination (Hesseling *et al.*, 2003; Hesseling *et al.*, 2007; Hesseling *et al.*, 2009), the TB epidemic in HIV-infected populations

has urged the development of efficacious, non-replicating TB vaccines that are suitable for CD4 T cell deficient hosts such as subunit vaccines.

Novel subunit vaccines currently under development typically carry antigens, that are highly immunogenic and recognized by a high proportion of individuals infected with *M. tuberculosis*, or vaccinated with BCG, such as ESAT-6, TB10.4 and Ag85B (Brock *et al.*, 2004; Dietrich *et al.*, 2006). The coding gene for ESAT-6 is not present in any of the *M.bovis* BCG vaccine strains or in most environmental mycobacteria and consequently, reactivity to this antigen is an indication of *M. tuberculosis* infection. TB10.4 is also a member of the ESAT-6 gene family. In contrast to ESAT-6, the gene coding for TB10.4 is also present in BCG. Cells isolated from immune competent, BCG vaccinated individuals as well as TB patients produced high levels of IFN-γ after stimulation with TB10.4 (88% and 71% responders, respectively) (Skjot *et al.*, 2000). Ag85B is present in both *M. bovis* BCG, and *M. tuberculosis* and is well described in the literature as another potent IFN-γ inducer in both *M. tuberculosis* infected and BCG vaccinated individuals (Wiker and Harboe, 1992).

All three antigens have been shown to induce control of *M. tuberculosis* infection in animal models when given as subunit vaccines. Weinrich Olsen *et al.* have shown efficacy of an Ag85B-ESAT-6 fusion protein in both mice (Weinrich Olsen *et al.*, 2001) and guinea pig models (Olsen *et al.*, 2004). Langermans et al. also showed protection against M. tuberculosis in a non-human primate model (Langermans *et al.*, 2005). Further, Dietrich *et al.* have found even better control of infection with a fusion protein containing Ag85B and TB10.4 in a murine model (Dietrich *et al.*, 2005). The subunit vaccine has been formulated with the adjuvant IC31, which promotes a strong Th1 response (Aagaard *et al.*, 2009). This novel construct represents another new promising vaccine candidate against TB.

A successful vaccine needs to induce protective immune responses by generating immunological memory. However, in HIV-infected individuals,

profound T cell defects might prevent successful vaccination, as antigen-experienced (memory) T cell populations are depleted and or dysfunctional. For example, immune responses to Influenza A and measles vaccination are impaired in HIV-infected children (Obaro *et al.*, 2004; Lyall *et al.*, 1997). ART results in strong and sustained inhibition of viral replication and is associated with a rapid rise in CD4 T cells in patients with advanced disease. In contrast to adults, the rise in CD4 T cells in children in response to ART is predominantly within $CD45RA^+$ cells. Thymic output of T cells is recognised to be greater in children, and thymic rearrangement excision circle (TREC) assays confirm that a high proportion of naïve cells are recent thymic emigrants (De Rossi *et al.*, 2002). It remains to be determined whether this higher proportion of naïve cells results in broader functional immune reconstitution in children as opposed to adults. In adults receiving ART, reconstitution of antigen-specific cellular immune responses has been demonstrated *in vitro*, but more importantly, *in vivo* studies show that susceptibility to TB is significantly reduced (Badri *et al.*, 2002). This reduced susceptibility to TB was highest in symptomatic patients and those with advanced immune suppression. Despite this the overall susceptibility still remains well above that of HIV-negative individuals. The question whether it could be further reduced by vaccination remains unanswered in both adults and children.

1.12 Whole blood assays used to study mycobacteria-specific responses

1.12.1 Whole blood intracellular cytokine staining (ICS) assay

Whole blood assays are attractive as a tool to study immune responses to *M. tuberculosis* as all relevant cell types as well as humoral factors are present and can contribute to the interaction with mycobacteria (van Crevel *et al.*, 1999). In contrast to conventional assays using peripheral blood mononuclear cells (PBMCs), the whole blood intracellular cytokine-staining (ICS) assay uses smaller blood volumes without the need for sophisticated processing of blood (Hanekom *et al.*, 2004). This is particularly relevant when studying paediatric populations and for the laboratory set up of many developing countries. The ICS assay allows the study of antigen-specific T cell responses, giving a snapshot of the immune response at the time of blood sampling. Whole blood is stimulated with mitogen, and pathogen-specific antigens. Surface and intracellular molecules such as cytokines are then detected using flow cytometry in a stimulated sample and compared with an unstimulated control sample. In this thesis we used an ICS assay to measure intracellular production of IFN-γ and IL-2 and characterised surface phenotype of CD4 and CD8 T cells after stimulation of whole blood with mycobacterial antigens, as detailed in chapter 2.

1.12.2 Basic principles of multiparameter flow cytometry

Flow cytometry allows detailed characterisation of multiple parameters on a single cell level as cells flowing in a fluid stream are interrogated by a series of lasers (Shapiro, 2003). Light scattered in the forward direction (forward scatter, FSC), gives information about the cell size, and light scattered at a 90° angle (side scatter, SSC) gives information about the internal complexity or granularity of the cell, Surface and intracellular markers of interest, stained with fluorescent molecules (e.g. fluorochrome-conjugated monoclonal antibodies), allow detailed characterisation of specific cell populations. These

fluorescent molecules are excited by the lasers and emit light at longer wavelengths. The intensity of the emitted fluorescence is proportional to the number of binding sites on the cell of interest. The scattered light and emitted fluorescence are collected by detectors and converted to digital signals, which are displayed for analysis in one and two dimensional plots, such as histograms and dot plots, respectively.

1.12.3 Tools to study mycobacteria-specific responses

The purpose of any assay in the context of vaccine evaluation is to provide an *in vitro* measure of the vaccine-specific immune response (immunogenicity or vaccine take). In TB vaccine research most of the assays used evaluate the magnitude and quality of the vaccine-specific memory T cell response. These assays measure a recall response after stimulation of whole blood or PBMCs with vaccine antigens in vitro. These assays measure antigen-induced host responses through levels of secreted or expressed key cytokines using enzyme-linked immunosorbent assay (ELISA) (Hasan *et al.*, 2009), enzyme-linked immunosorbent spot (Elispot) assay (Hughes *et al.*, 2005), ribonucleic acid (RNA) assay (Hasan *et al.*, 2009), or frequencies of T cell responses using flow cytometry (Hanekom *et al.*, 2004; McShane *et al.*, 2005; Horton *et al.*, 2007; Scriba *et al.*, 2010; Abel *et al.*, 2010). Bactericidal assays, which enumerate mycobacteria in cell culture systems before and after host-pathogen interactions are also used in vaccine evaluation.

1.12.3.1 Cytokine assays

Although studies of single or multiple cytokines have provided important insights into the pathophysiology of tuberculosis infection and disease, correlation of cytokine levels with outcome have been poor. This is mainly a consequence of the wide inter- and intra-individual variability and reflects the complexity of the host-pathogen interactions. As a consequence, to date, no validated biomarkers exist that accurately predict progression from TB infection to TB disease. In TB vaccine assessment, several immunogenicity

markers, such as production of IFN-γ in response to vaccine antigens are currently being evaluated, but there is obvious heterogeneity. The assays used may vary in duration depending on the nature of the question being addressed as well as what is practical. The different types of assays include (1) the short term assay which is less than 24 hours, (2) the medium term assay is between 1 to three days and (3) the long term assay can be four to five days or more (Hanekom *et al.*, 2008). Each type of assay has distinct advantages and disadvantages and assay selection depends on what is practical within the study settings as well as what part of the immune response is investigated. Regardless, optimization of the chosen assay system is key in generating reliable immunological data. Resource permitting, performing a combination of short, medium and long term assays would be more informative. The data in this thesis was generated with a short term ICS whole blood assay as well as a 96-hour bactericidal assay, which are both practical in paediatric studies.

1.12.3.2 Bactericidal assays

Bactericidal assays in the context of tuberculosis vaccine research are well established in animal models, where they are applied to screen potentially promising vaccine candidates (Weinrich Olsen *et al.*, 2001; Langermans *et al.*, 2005; Dietrich *et al.*, 2005). Reduction of bacterial load in various organs functions as the main read-out of efficacy. The first generation of human bactericidal assays, were designed using Mø infected with *M. tuberculosis* (Silver *et al.*, 1998). Freshly harvested peripheral blood lymphocytes (PBL) were added to infected Mø (primary lymphocyte-dependent inhibition) and lymphocytes added subsequently. Co-cultures were lysed and viable mycobacteria counted by CFU plating. Worku *et al.* used T cells expanded from peripheral blood and stimulated with specific mycobacterial antigens prior to adding them to antigen-presenting cells *in vitro* (secondary lymphocyte-dependent inhibition) (Worku and Hoft, 2000).

One of the main impediments of work with *M. tuberculosis* in bactericidal assays is the need for containment facilities and the fact that bacterial

quantification is only available after weeks of incubation. To facilitate more rapid read-outs, new assays were designed which measure metabolic activity as a correlate of mycobacterial numbers. Two different systems have been designed: reporter–gene tagged mycobacteria (Kampmann *et al.*, 2000) or a radiometric detection system using BacTec bottles (Cheon *et al.*, 2002). These assays were adapted for whole blood rather than PBMC (whole blood inhibition assays). Whole blood assays require considerably smaller volumes of blood compared with PBMC based assays, making them more suitable under field conditions, especially when children are studied. Some investigators have used BCG rather than virulent *M. tuberculosis* to avoid the requirement for BSL3 conditions. However validation with virulent strains is important and has been carried out (Al-Attiyah *et al.*, 2006; Martineau *et al.*, 2007).

The mycobactericidal assay used in this thesis utilises diluted whole blood and reporter-gene tagged mycobacteria (*M. tuberculosis* H37Rv). The reporter-gene (lux AB) is carried on a plasmid (pSMT1) under the control of the inactivated heat-shock promoter (hsp60) (Snewin *et al.*, 1999). Metabolic activity, which is the viability marker of the bacteria, is measured by recording the emitting bioluminescence signal as relative light units (RLU). The bioluminescence signal is induced by adding, an exogenous substrate, n-decyl-aldehyde. Bioluminescence, the biological production of light, is ATP-dependent and only viable bacteria emit light. It is a marker of mycobacterial viability, which was shown to correlate directly and consistently with mycobacterial quantification by counting CFU (Snewin *et al.*, 1999). **Figure 1.8** shows a schematic representation of the whole blood luminescence assay.

In this thesis, we used the reporter-gene tagged mycobacterial assay developed by Kampmann *et al.* to complement and correlate the data gathered by ICS whole blood cytokine assays with a functional read-out. (Kampmann *et al.*, 2000).

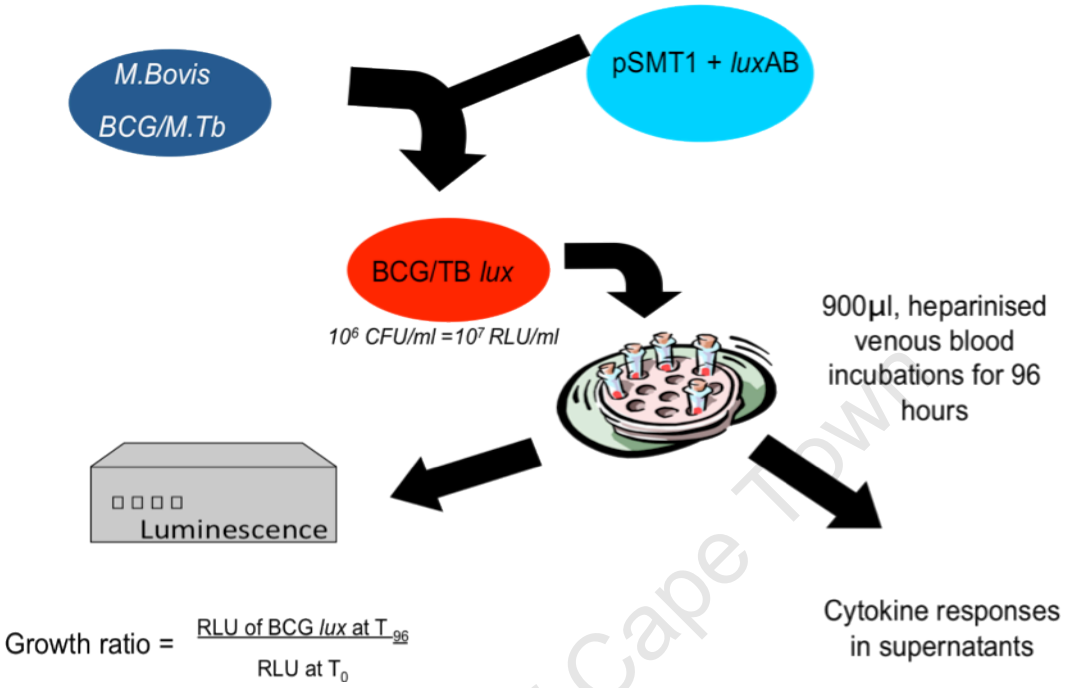

Figure 1.8: Schematic of the whole blood growth inhibition assay

Reporter-gene tagged mycobacteria (*M. tuberculosis* H37Rv or BCG) were transformed with the plasmid (pSMT1). This plasmid carries the reporter-gene luciferase A and B (*lux* AB) genes, under the control of an inactivated heat-shock promoter (hsp60). Heparinised whole blood diluted 1:2 with RPMI and inoculated with luminescent bacteria is incubated at 37°C for 96 hours on a rocking platform. Supernatants are removed and frozen for future cytokine analysis. The growth ratio is determined by dividing the RLU reading at 96 hours (T_{96}hrs)/ by the reading at baseline (T_0hrs). Schematic obtained from B Kampmann.

1.13 Aims of the study

ART has completely transformed both life expectancy and quality of life for HIV-infected individuals in developed countries and the introduction of ART into sub-Saharan Africa is now a major world health priority. In the coming years, increasing numbers of children with HIV will be treated in sub-Saharan Africa, but despite extensive therapy with ART, a large number of these children will subsequently succumb to TB, as they reside in an environment highly endemic for TB. Protection from TB must therefore rank as a fundamental component of health care delivery in these countries. Apart from providing long-term preventative anti-TB therapy with its potential complications, vaccination of this vulnerable group must remain an alternative option. Most of the novel TB vaccines aim to boost BCG immunity through a heterologous prime-boost vaccination strategy. Whether vaccination could successfully boost anti-mycobacterial immunity in HIV-infected children receiving ART remains unanswered. There are presently no data on baseline mycobacteria-specific T cell memory responses in paediatric HIV infection that could be boosted with an anti-TB vaccine. This study will address the fundamental question whether ART will enable the reconstitution of mycobacteria-specific immune responses to antigens contained in novel anti-TB vaccines, in HIV-infected children receiving ART.

Hypothesis:

Our hypothesis is that T cells from children with previous BCG vaccination, with or without HIV infection, can recognize mycobacterial antigens that are under investigation for inclusion into novel anti-TB vaccines.

We hypothesize that mycobacteria-specific memory CD4 and CD8 T cell responses are lower in HIV-infected children before ART, compared with non-HIV-infected children, but are reconstituted during the course of ART.

Objective:

Our objective is to describe effector and memory responses to selected mycobacterial antigens in African children with and without HIV infection and to study the impact of ART on such responses in the HIV-infected group.

Specific Aims:

1. To develop and optimise an 8-colour flow cytometry panel to functionally and phenotypically characterise memory sub-populations of mycobacteria-specific CD4 and CD8 T cells.
2. To quantify the frequency and memory phenotype of mycobacteria-specific T cells in children with and without HIV-infection.
3. To quantify the frequency and memory phenotype of mycobacteria-specific T cells in HIV-infected children during the course of ART.
4. To determine mycobacterial growth inhibition in whole blood of HIV-infected children before and during the course of ART.
5. To determine the association between data obtained by ICS and whole blood growth inhibition assays.

CHAPTER 2

Materials and methods

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2.2 Materials and methods

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2.1 Introduction

In this chapter the general methods and reagents used in the project are described in detail.

2.2 Materials and methods

2.2.1 Study design

This prospective study addressed three aims: 1) to develop and optimize an 8-colour flow cytometry antibody panel to study T cell responses, 2) cross-sectional analysis of T cell responses in HIV^+ children and control groups, and 3) longitudinal analysis of T cells in HIV-infected cohort during 12 months of therapy. The aim of this study was to compare the function and phenotype of T cell responses in children, in order to characterise the type of responses that might be observed in children of different immunocompetence in response to antigens currently included in novel candidate TB vaccines. To accomplish this, memory T cell responses in small volumes of whole blood were evaluated.

2.2.2 Study participants

Ethical approval for the study was obtained from the Research Ethics Committees of the University of Cape Town, South Africa (REC 081/2006), and of Imperial College London, UK (02/GB/23E). Healthy adult volunteers and three groups of children, who had all received BCG vaccination at birth, were targeted for enrolment. Adults were enrolled following informed consent. Legal guardians of children gave informed consent. All children were recruited and followed up by the same dedicated research nurse. Demographic characteristics of all children are tabulated and described in chapter 4.

2.2.2.1 Healthy adult volunteers

Development and optimisation of multiparameter flow cytometry panels were completed using whole blood from healthy adults. The volunteers were recruited at the Institute of Infectious Disease and Molecular Medicine (IIDMM) at the University of Cape Town (UCT). Only individuals over the age of 18, of ideal weight and with good general health were recruited. None of the volunteers were taking medication for chronic illness.

2.2.2.2 Healthy children

Healthy children (HC), under 15 years of age, of normal weight for age and born to HIV-negative mothers, were recruited from community clinics, routine immunisation clinics and pre-booking clinics for planned surgery at Red Cross Children's Hospital, Cape Town. Any children, who had reported prior exposure to an active TB case, children with a past history of TB, children treated for LTBI or any evidence of acute or chronic health problems were excluded. This group served to determine the nature of T cell responses to mycobacterial antigens in BCG-vaccinated, but HIV and TB-negative population of healthy children.

2.2.2.3 HIV-seronegative children diagnosed with TB

HIV-seronegative children, under 15 years of age, diagnosed with TB, and had been receiving standard anti-TB therapy (rifampicin, isoniazid and pyrazinamide) for a minimum of 2 months, were recruited from TB clinics. TB diagnosis was either by culture confirmation (definite TB) or by a combination of suggestive signs and symptoms, a history of TB exposure, abnormal chest radiology, positive tuberculin skin test (TST) response (>10 mm) and response to TB therapy (probable TB). This group served to determine T cell responses to mycobacterial antigens in immunocompetent children who were expected to have *M. tuberculosis*-specific immunological memory. Any children who were acutely unwell, malnourished or on immunosuppressive medication were excluded.

2.2.2.4 HIV-infected children

Children above the age of 3 months, with perinatally acquired HIV-1 infection, about to start triple combination antiretroviral therapy (ART) were identified by clinicians and/or the research nurse at HIV-community clinics, at pre-treatment meetings or at the Red Cross Children's Hospital. All children were receiving regular follow-up and fulfilled either clinical or immunological criteria for starting ART according to WHO guidelines. However, children under 12 months were started on ART regardless of their clinical or immunological status in line with the new guidelines (WHO, 2008). None of the children had previously received ART or treatment for TB. Any children, who had prior reported exposure to an active TB case, children who were acutely unwell or had signs/symptoms of TB or other opportunistic infections, were excluded. Children with a history of rapidly progressing HIV disease, such as opportunistic infections, recurrent severe bacterial infections, specific encephalopathy and wasting syndrome were excluded. Children with a CD4 count of less than 50 cells/μl, who required multiple hospital admissions for opportunistic infections or other complications of HIV disease, were excluded.

2.2.3 Tissue culture

Throughout all tissue culture procedures, sterile conditions were maintained.

2.2.3.1 Blood sample collection

A total of 10 ml heparinised venous blood was collected into vacuum collection tubes (BD Biosciences), from all children. All bloods were processed within two hours of blood draw. In the healthy and TB groups blood samples were used to prepare duplicate antigen stimulations of whole blood for intracellular cytokine staining (ICS) assay. In HIV-infected children 5 ml of blood was used for the ICS assay and 5 ml was used for the mycobacterial growth inhibition assay (see section 2.2.4 and 2.2.5, respectively). A further 2 ml of ethylenediaminetetraacetic acid (EDTA) blood was collected from HIV-

infected children: 500 μl blood was sent for determination of CD4 count, 500 μl for full blood count and differential count as well as 1 ml for HIV-viral load determination.

2.2.3.2 Stimulations

A whole blood ICS assay, which allows characterisation of T cell responses to specific antigens in relatively small volumes of blood, was used to identify the memory phenotype and cytokine expression of mycobacteria-specific T cells (Hanekom *et al.*, 2004). Whole blood was stimulated with the recombinant proteins, Ag85B (Rv1886c) and TB10.4 (Rv0288) (added together due to limited blood volumes, at 10 μg/ml each), *M. tuberculosis* purified protein derivative (PPD, at 20 μg/ml,) or early secretary antigenic target-6 (ESAT-6) peptides, (Rv3875) at 10 μg/ml. All antigens were kindly provided by P. Andersen, Staten Serum Institute, Denmark. Unstimulated blood served as negative control and blood incubated with staphylococcal enterotoxin B (SEB, at 10 μg/ml, Sigma) as positive control. 1 ml of whole blood was incubated with antigens in 2 ml polypropylene tubes (Sarstedt) for a total of 7 hours in a programmable water bath at 37°C. Brefeldin A (Sigma, 10 μg/ml) was added after the first 2 hours of incubation, to trap cytokines intracellularly. The water bath was programmed to cool down to room temperature after a further 5 hours at 37°C.

Cells were harvested within 20 hours of start of incubation, and incubated with 2 mM EDTA (Sigma) for 15 minutes at room temperature to reduce cell clumping. To lyse red blood cells and fix white blood cells, the sample was transferred into 15 ml conical Falcon tubes containing 9 ml FACS lysing solution (BD Biosciences), and incubated at room temperature for 10 minutes. Cells were centrifuged at 300 x g for 7 minutes at room temperature. Supernatants were decanted and the remaining cells resuspended in a cryopreservation solution consisting of 500 µl RPMI 1640 medium with L glutamine (Sigma) and 500 µl 20% dimethyl sulfoxide (DMSO, Sigma) in heat inactivated fetal calf serum (FCS, Gibco, Invitrogen). Resuspended cells were

transferred into cryovials (Corning) and placed in Mr. Frosty's (Merck) containing isopropanol (Sigma), which allows gradual freezing at a rate of -1°C/min. Mr Frosty's were placed at -80°C. Cells were transferred to liquid nitrogen for longer term storage the following morning.

2.2.4 Intracellular cytokine staining (ICS) assay

Optimisation of this assay is described in detail in chapter 3.

2.2.4.1 Antibodies

The following combination of surface antibodies was used: anti-CD3 Pacific Blue (UCHT1), anti-CD8 PerCP-Cy5.5 (SK1), anti-CD27 PE (MT271), anti-CD45RA PE-Cy7 (L48), all from BD Biosciences, anti-CD4 QD605 (S3.5, Invitrogen) and anti-CCR7 APC (150503, R&D Systems). Anti-IFN-γ AlexaFluor700 (B27) and anti-IL-2 FITC (5344.111, both from BD Biosciences) were used for intracellular cytokine staining.

2.2.4.2 Intracellular cytokine staining assay

T cell functional and phenotypic profiles were measured using a two-step staining method: cells were permeabilised, stained first for surface markers followed by staining for intracellular cytokines. The cryopreserved cells were rapidly thawed in a water bath at 37°C. Thawed cells were transferred into 5 ml 2054 Falcon tubes (BD Biosciences) containing 2 ml 1% bovine serum albumin (BSA, Sigma) in 1 X Dulbecco's phosphate buffered saline (PBS, BioWhittaker), washed twice at 300 x g for 7 minutes. The cells were transferred to a 96 well plate (Nunc) and permeabilised using 200 μl 1XPerm/Wash Solution (BD Biosciences) for 10min at 4°C. Cells were then pelleted at 1800 x g for 3 minutes at 4°C in a refrigerated centrifuge. The cells were resuspended in 50 μl 1XPerm/Wash and then stained at 4°C, first with surface antibodies for 30 minutes - after a wash step using 180 μl 1XPerm/Wash, the cells were stained with intracellular antibodies for 1 hour at

4°C in the dark in the remaining 50 μl Perm/Wash. After intracellular staining, the cells were washed with 180 μl 1XPerm/Wash and again pelleted at 4°C for 3 minutes at 1800 x g, resuspended in 100 μl 1XPerm/Wash and transferred to 5ml 2054 Falcon tubes.

2.2.4.3 Multiparameter flow cytometry

Stained samples were acquired on the same day on a LSRII flow cytometer. The details of the LSRII instrument configuration, flow cytometry panel optimisation, data acquisition and analysis are described in chapter 3.

2.2.4.4 Data analysis

All flow cytometry data were analyzed using FlowJo v8.8.2 (Tree Star), Pestle (v1.6.1) and Spice (v4.9) (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) (Roederer *et al*., 2011). Results were expressed as the frequency of positive events, of the stimulated sample, above those of the negative control, the unstimulated sample. Memory phenotypes of cytokine producing, mycobacteria-specific T cells were only analysed if at least 40 cytokine+ (specific) cells were detected, and if this response was at least double that from the unstimulated control.

2.2.5 Mycobacterial growth inhibition assay

The mycobacterial species used in this study was the virulent *M. tuberculosis* H37Rv lux strain, provided by Dr Kampmann, Imperial College London. All work with the virulent strain was done in a BSL3, containment facility.

2.2.5.1 Mycobacterial media

7H9 broth: 4.7 g of powdered 7H9 broth (Middlebrook, BD, Difco) was dissolved in 900 ml dH_20 (containing 2 ml glycerol, BDH, Merck) and 500 μl

Tween 80 (Sigma). The broth was autoclaved for 15 min at 121°C. The prepared media was supplemented with 10% (20 ml) ADC enrichment (Middlebrook, BD, Difco) and 10 mg hygromycin B (200 μl of 50 mg/ml stock, Boehringer Mannheim, Roche Diagnostics) before use as liquid growth medium for MTB lux and stored at 4°C.

7H11 agar: 10.5 g of powdered 7H11 agar (Middlebrook, BD, Difco) in 450 ml dH_20 (containing 2.5 ml glycerol) was boiled to dissolve completely. The agar was then sterilized by autoclaving for 15 min at 121°C. The prepared media was supplemented with 10% (50 ml) OADC enrichment (Middlebrook, BD, Difco), 10 mg of amphotericin B (50 mg/ml stock) and 25 mg hygromycin B (500 μl of 50 mg/ml stock) before use as solid growth medium for MTB lux. The medium was then poured into plates under sterile conditions and left to set. Plates were stored at 4°C until use.

2.2.5.2 Preparation and storage of mycobacteria

Recombinant mycobacteria (MTB lux) were grown into the logarithmic phase over 72 hours in a shaking incubator at 37°C in 7H9/ADC/Tween 80/hygromycin medium. Logarithmic growth of MTB lux was initially detected by plating 100 μl aliquots of serial dilutions of MTB lux culture on solid media and counting colony-forming units (CFU) after 4 weeks of incubation at 37°C. Subsequently, mycobacterial growth was determined by measuring relative light units (1 CFU = 10 RLU). The culture was dispensed into 1 ml vials; glycerol (Sigma) was added to a final concentration of 25% and the vials frozen at -80°C. Each vial was shown to contain 1.8 x 10^7 RLU/ml viable mycobacteria.

2.2.5.3 Preparation of mycobacterial culture for inoculation into whole blood

Prior to each experiment, a vial of stored MTB lux was thawed, added to 15 ml of 7H9/ADC/ Tween 80/hygromycin medium in a sterile disposable 250 ml Erlenmeyer flask (Corning) and incubated with shaking at 120 rev/min in a

37°C incubator for 72 hours. The mycobacteria were then resuspended in sterile PBS to a concentration of 4-5 x 10^6 RLU/ml prior to inoculation.

2.2.5.4 Mycobacterial growth inhibition assay protocol

A whole blood luminescence assay, as previously described (Kampmann *et al.*, 2000), was performed. 5 ml of heparinised whole blood was diluted 1 in 2 with RPMI 1640 medium (Sigma). 900 μl volumes of blood were then aliquoted into 7 ml screw top, endotoxin-free tubes (Sterilin, Merck), and inoculated with 100 μl luciferase-labelled *M. tuberculosis* H37Rv (MTB lux) at 4-5 x 10^6 RLU/ml to give a final volume of 1 ml per tube. Cells were inoculated in triplicate with MTB lux at two time points: 0 and 96 hours to measure mycobacterial growth. A 24-hour lux sample was also set up in duplicate for supernatant analysis. An unstimulated control sample was also set up at each time point with blood and RPMI only. The samples were then incubated at 37°C (no CO_2) on a platform shaker (Merck, Stuart Scientific, STR6) set at 20 rev/min. The 0 hour time point samples were analysed immediately after inoculation, on the day of blood collection.

To harvest, samples were centrifuged for 10 minutes at 2000 x g and 400 μl supernatant was collected from each tube and filtered before storage at -80°C for future cytokine measurement. To readjust the volume, 400 μl of sterile PBS was added to the samples, before transferring to 25 ml Sterilin universal tubes and adding 10 ml dH_2O to lyse the red blood cells. The samples were incubated for 10 minutes at room temperature and centrifuged for 10 minutes 2000 x g to recover the MTB lux. The supernatant was discarded and the pellet was vortexed to disperse the MTB lux, and resuspended in 1 ml of sterile PBS. Samples were diluted 10-fold in duplicate (to minimise any reduction of luminescence in opaque samples [quenching]) prior to luminescence measurement.

2.2.5.5 Bioluminescence detection

Luminescence was measured using a single tube luminometer (Turner Design 20/20, Germany). This design uses an injectable port to inject 100 μl of the substrate, 1% n-decyl aldehyde, (Sigma) in ethanol into each luminometer tube (Sarstedt). Measurements were collected over 20 seconds from each tube and reported as a mean. Duplicate measurements were reported for each sample prepared in triplicate. An unstimulated control sample incubated with whole blood and RPMI only was included at each time point. Growth of MTB lux in mycobacterial culture medium only was also measured after 96h. Luminescence was measured in units of relative light units and results were expressed as a calculated growth ratio (GR), which relates the RLU reading at 96 hours, to the RLU reading of the inoculum (0 hours), using the following formula:

$$\text{Growth ratio} = \frac{\text{RLU of MTB lux at } T_{96}}{\text{RLU of MTB lux at } T_0}$$

2.2.6 CD4 and differential count and viral load determination

The full blood count (cells/μl and percentages), differential count (cells/μl and percentages) and viral load (log_{10} RNA copies/ml) determinations were routinely done at the National Health Laboratory Services (NHLS) and results were accessible to treating clinicians in the HIV clinic. CD4 T cell enumeration and viral load measurements were performed at enrolment and then at 3 monthly intervals up to 12 months by the clinical laboratory services. The lower limit of detection for the viral load assay is <1.7 log_{10} RNA copies/ml.

2.2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 for Mac OS X. Differences between groups were calculated first with a Kruskal Wallis

test, then Mann-Whitney or Wilcoxon matched pairs test. Correlations were calculated by non-parametric Spearman test. All tests were two-tailed, and a value of $p < 0.05$ was considered significant.

CHAPTER 3

Optimisation of an 8-colour flow cytometry panel to study mycobacteria-specific T cell populations

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3.4 Discussion

3.1 Introduction

Flow cytometry is a sensitive technique that allows detailed characterisation of immune responses at the single cell level (Appay *et al.*, 2000; Betts *et al.*, 2003; Betts *et al.*, 2006). Polychromatic flow cytometry (PFC), also called multiparameter flow cytometry, has evolved to allow measurement of more than 4 colours plus two physical characteristics; forward scatter (FSC) and side scatter (SSC) (Roederer *et al.*, 1996; Roederer *et al.*, 1997; De Rosa *et al.*, 2003; Perfetto *et al.*, 2004; De Rosa *et al.*, 2004). These recent advances have greatly increased our understanding of the complexity of the immune system. Recent advances in flow cytometry instrumentation, software and reagents has shown increased application of this technique, especially in the field of T cell immunology (Harari *et al.*, 2004; Betts *et al.*, 2006; Darrah *et al.*, 2007) and vaccine development (Seder *et al.*, 2008).

This chapter describes the methods and systematic approach that were employed in this study, to develop an optimal antibody panel for measuring intracellular cytokine expression by multiparameter flow cytometry. To this end, an 8-colour antibody panel was developed and optimised to characterise function and memory phenotype of mycobacteria-specific CD4 and CD8 T cells. Optimisation of flow cytometry based polychromatic assays becomes more complex and time consuming as more parameters are included (Mahnke and Roederer, 2007). However, the wealth of information gained usually justifies the technical challenges. It is important for each researcher to titrate flow cytometry reagents and optimise the combinations of antibody-fluorochromes in flow cytometry panels. Before the final flow cytometry assay was adopted in this study a large number of optimisation experiments were performed. The optimised panel was used to address the aims of this project, as stated in chapters 4 and 5.

3.2 Methods

3.2.1 Immunofluorescence staining

Optimisation experiments were performed using a single staining step when staining for surface markers only or the two-step staining method described in chapter 2 for ICS (section 2.2.4.2) on samples obtained from healthy adults.

3.2.2 Antibody titration

Each antibody was individually titrated by serial dilution to determine optimal antibody titre and staining performance. Titrations were performed on samples that had under gone the same treatment as the experimental staining, since temperature, light exposure and staining buffer might influence staining and antibody performance. To ensure antigen saturation twice the concentration recommended by the manufacture was selected as the starting titre. At least 10 two-fold serial dilutions were completed per antibody in 100 μl volumes.

3.2.3 Effects of fixation on surface marker resolution

Since all whole blood samples used in this study were fixed prior to staining with antibodies, the effects of fixation on the resolution of surface markers was assessed. A 1 ml fresh blood sample was ether stained immediately prior to fixing or 1 ml blood was add to 9 ml FACS lysing solution, vortexed and incubated at room temperature for 15 min, to lyse red blood cells. Cells were pelleted and stained with surface antibodies.

3.2.4 Fluorescence minus one

For fluorescence minus one, (FMO) experiments, one antibody was omitted at a time to assess potential false positives or spectral overlap issues, which could arise from interactions between fluorochromes in large antibody panels. A sample was stained with all the antibodies in the complete panel and

compared with a series of samples stained with the same panel while omitting one antibody in consecutive samples. FMO experiments were performed under the same conditions as the final experimental set-up. Data was analysed using the same gating strategy applied to the experimental samples.

3.3 Antibody panel design and optimisation results

3.3.1 Selecting markers of Interest

Markers of interest were included in the final panel by considering (1) the research question and (2) the ability to detect the markers of interest with the proposed assay system. The markers of interest characterised in this thesis include markers of T lymphocyte lineage (CD3, CD4, CD8), phenotype (e.g. CD45RA, CCR7, CD27) and function (IFN-γ, IL2). The aim was to characterise antigen experienced CD4 and CD8 T lymphocytes ($CD3^+$ T cells) in peripheral blood from children and healthy adult controls using multiparameter flow cytometry. Multiparameter flow cytometry allows integrated measurement of functional and phenotypic parameters relevant to TB immunity in limited volumes of paediatric blood samples.

The rationale for inclusion of the markers selected in this study is reviewed in detail in chapter 1. CD4 and CD8 T, cell type 1 cytokine responses are known to be important in the immune response against *M. tuberculosis* (Schluger and Rom, 1998). This kind of immunity is therefore the most commonly measured when evaluating mycobacterial immunity. The memory T cell compartment is important in the context of successful vaccination and consists of both CD4 and CD8 T cells that can rapidly acquire effector functions to kill infected cells and/or secrete inflammatory cytokines that inhibit replication of the pathogen. Sallusto *et al.*, have characterised memory T-cells into effector memory ($CD27^-$, $CD62L^-$, $CCR7^-$) and central memory T cells ($CD27^+$, $CD62L^+$, $CCR7^+$), with different functions and migratory properties (Sallusto *et al.*, 1999).

CD4 and CD8 T cell populations in this thesis were analysed for memory markers (e.g. CD45RA and CCR7), differentiation markers (e.g. CD27), as well as markers of cellular function (e.g. IFN-γ).

3.3.2 Instrumentation

3.3.2.1 Instrument Configuration: Laser and Detector considerations

In order to design and optimise ICS panels the type of flow cytometry instrumentation that is available for sample acquisition must first be identified. The antibody-fluorochrome combinations discussed in the following sections was guided by the choice of instrument configuration (laser and detector: mirror/filter combinations) of the South African Tuberculosis Vaccine Initiative (SATVI) laboratory BD LSR II. **Table 3.1** illustrates the configuration of the SATVI laboratory BD LSR II instrument, which is equipped with three different excitation light sources (lasers).

The three lasers excite particles at distinct wavelengths: 488nm (blue), 405nm (violet) and 633nm (red). Detectors/photomultiplier tubes (PMTs) arranged in an octagon-shaped array collect light from the blue laser (**Figure 3.1 A**) and those in two trigon-shaped arrays collect light from the violet and red lasers (**Figure 3.1 B and C**, respectively) The blue laser PMT arrays measure up to seven parameters and those for the violet and red lasers up to three parameters each. Fluorescent emission wavelengths detected by the PMTs are based on filter combinations placed in front of each PMT. The LSR II is equipped with two types of filters: dichroic long pass (DCLP) and band pass (BP) filters (Perfetto *et al.*, 2004). LP filters allow light above a specified wavelength to pass through the filter to be detected by the PMT. All light below this wavelength is reflected to the following PMT. The BP filter, in turn, only transmits light within a specified wavelength range to be detected by the following PMT. Any wavelengths, which fall outside this range, are not measured.

For example, in **Table 3.2**, for the blue laser, wavelengths of light longer than 735 nm are transmitted through a 735 LP filter and shorter wavelengths are reflected. The wavelengths are then passed through a 780/60 band pass (BP) filter that allows transmission of wavelengths longer than 750 and shorter than 810 nm. This cascade of light culminates in measurement of the PE-Cy7

maximal emission (E_m) wavelength (785nm) in PMT A. The wavelengths shorter than 735 nm are reflected to the next PMT (PerCP-Cy5.5). The instrument configuration thus dictates which types of fluorochrome reagents can be used with the available configuration (Baumgarth and Roederer, 2000; Perfetto *et al.*, 2004; Mahnke and Roederer, 2007).

Laser (λ)	Mirror LP (nm)	Filter BP (nm)	PMT	Possible Fluorochromes
Blue (488nm)	735	780/60	A	PE-Cy7
	685	695/40	B	PerCP-Cy5.5
	635	670/14	C	PE-Cy5, PerCP
	600	610/20	D	PE-Texas Red
	550	576/26	E	PE
	505	530/30	F	FITC, AlexaFluor 488
	Blank	488/10	G	SSC
	Blank	Blank	H	
Violet (405nm)	630	655/8	A	Quantum Dot 655
	505	605/12	B	Quantum Dot 605
	Blank	440/40	C	Pacific Blue
Red (633nm)	735	780/60	A	APC-Cy7
	690	720/40	B	AlexaFluor 700
	Blank	660/20	C	APC

Table 3.1: Optical configuration of the SATVI Laboratory BD LSR II instrument
λ: wavelength, LP: long pass, BP: band pass, FSC: forward scatter, SSC: side scatter

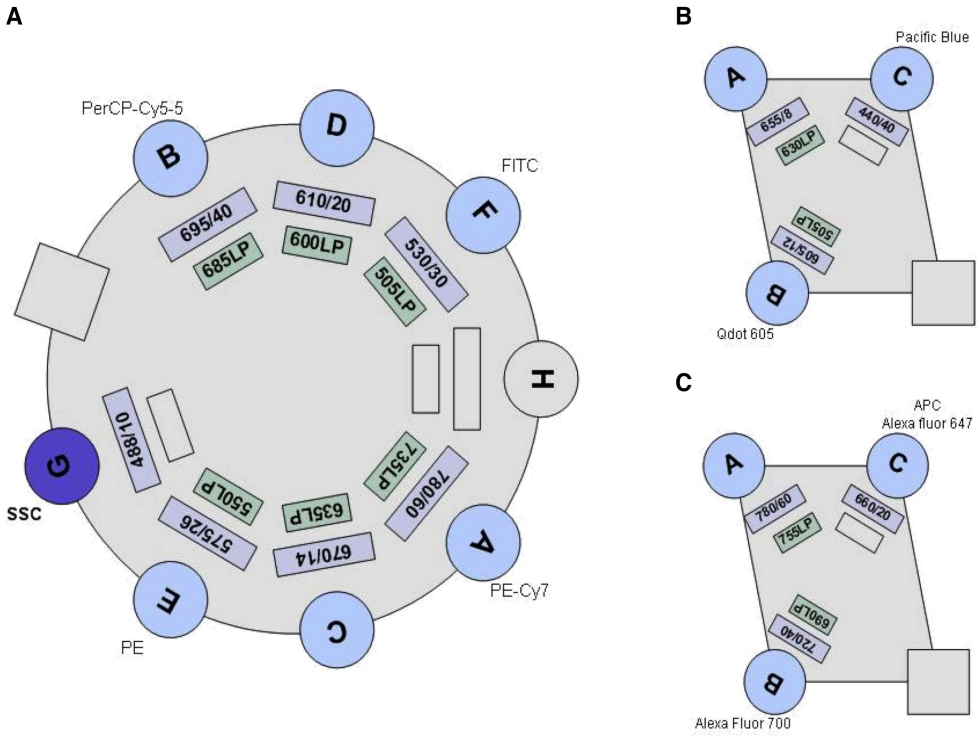

Figure 3.1: Representation of photomultiplier tubes arranged in octagon and trigon arrays with fluorochromes selected for an for an 8-colour panel

(A) Blue laser octagon array of PMTs and filters that can detect side scatter (SSC) and up to six fluorescence signals. (B) Violet laser trigon and (C) Red laser trigon array of PMTs and filters that can detect up to three fluorescent signals. From the BD Biosciences flow cytometry manual 2003.

3.3.2.2 Instrument quality control and performance tracking

At the start of the study, instrument settings were optimised to ensure reproducible and reliable longitudinal experiments. Instrument baseline settings were established using Cytometer Setting and Tracking (CST) beads, (BD Biosciences). The LSR II used for this analysis was calibrated each day prior to sample acquisition using the CST beads. This procedure ensured that PMT voltages were adjusted to set fluorescence target values for each experiment and that flow cytometer instrument variation would be independent of this parameter.

Validation of PMT voltages incorporates the CV, signal to noise ratio and PMT linearity curves. A tolerance target range was established for each channel and daily quality control (QC) standardization (performance checking) of the LSR II involved adjustment of PMT voltages within the target range in addition to laser alignment check. A daily QC ensures that any variation noted between different samples, within or between experiments is independent of the performance of the cytometer.

Following this procedure, baseline PMT voltages for the flow cytometry antibody panel used in this study were established and target values were recorded for the baseline settings. These were saved as application settings and were applied to all subsequent acquisition sessions.

3.3.3 Assignment of antibody-fluorochrome combinations

In order to describe the desired aspects of T cell function and memory phenotype it was necessary to combine 8 different markers. Fluorescently labelled monoclonal antibodies specific for these markers of interest were selected for an 8-colour flow cytometry panel. Assignment of antibody-fluorochrome combinations was an empirical and integrated process.

The following considerations were taken into account when designing the multiparameter panel: antibody brightness and antigen density, spectral overlap and compensation requirements. This process was further constrained by the available instrument configuration (lasers and PMTs) as well as the commercial availability of antibody conjugates. Because many of the antibody-fluorochrome combinations were already established in the SATVI laboratory, we established the study panel based on the following combinations: anti-CD3 Pacific Blue, anti-CD4 PerCP-Cy5.5, anti-CD4 QD605, anti-CD8 Pacific Orange, anti-CD8 PerCP-Cy5.5, anti-CD45RA PE-Cy7, anti-CCR7 APC, anti-CD27 PE, anti-IFN-γ AlexaFluor700 and anti-IL-2 FITC.

3.3.3.1 Antigen density and antibody brightness

Table 3.2 illustrates the fluorochromes, which were investigated for inclusion in the 8-colour staining panel. CD3/4/8 are abundantly expressed proteins, which identify major lineages of cells. Antibodies to these markers stain cells very brightly. This allows good resolution between negatively and positively staining cell populations resulting in a bimodal distribution. The expression pattern of CD45RA and CCR7 is often a continuum, resulting in less distinct resolution between negatively and positively staining cell populations.

It is important to assign the brightest fluorochromes to dim markers, and vice versa (Baumgarth and Roederer, 2000; Maecker and Trotter, 2009). However, care must be taken to avoid spillover from bright cell populations into channels requiring high sensitivity for those populations (Baumgarth and Roederer, 2000; Maecker and Trotter, 2009). Following convention, dim markers were assigned to bright colours with little compensation spread and markers that are present at high density were assigned to dimmer fluorochromes (**Table 3.2**).

The staining index (SI) of a fluorochrome gives a measure of the brightness of the fluorochrome relative to its level of noise in unstained cells (Baumgarth

and Roederer, 2000; Maecker and Trotter, 2009). Fluorochromes with high SI allow clear discrimination between positive and negative populations and were, therefore, assigned to dim markers. Markers with high expression were assigned to fluorochromes with lower SI. For example, a bright fluorochrome, such as PE-Cy7, was chosen for CD45RA, while a dimmer fluorochrome, like Pacific Blue, was used for CD3.

	Marker	Fluorochromes tested
Phenotype	CD3	Pacific Blue
	CD4	PerCP-Cy5.5, Quantum Dot 605
	CD8	Pacific Orange, PerCP-Cy5.5
	CD45RA	PE-Cy7
	CCR7	APC
	CD27	PE
Function	IFN-γ	AlexaFluor700
	IL-2	FITC

Table 3.2: Fluorochromes investigated for inclusion into the 8-colour staining panel

3.3.3.2 Spectral overlap considerations and compensation

Fluorochromes emit light over a wide wavelength range. This can lead to spectral overlap between dyes that emit light at similar wavelengths and are detected in more than one detector (**Figure 3.2**) (Baumgarth and Roederer, 2000; BDBiosciences, 2010). For example in (**Figure 3.2**), FITC emits in the FITC channel (primary fluorochrome) as well as in the PE channel (secondary

fluorochrome). This spectral overlap is accounted for by compensation (Bagwell and Adams, 1993; Roederer *et al.*, 1997; Roederer, 2001). This subtracts light of the primary fluorochrome from the signal in the secondary fluorochrome. It is best to choose fluorochromes that do not require too much compensation (i.e. have little fluorescence overlap), but with multicolour experiments this becomes unavoidable (Baumgarth and Roederer, 2000).

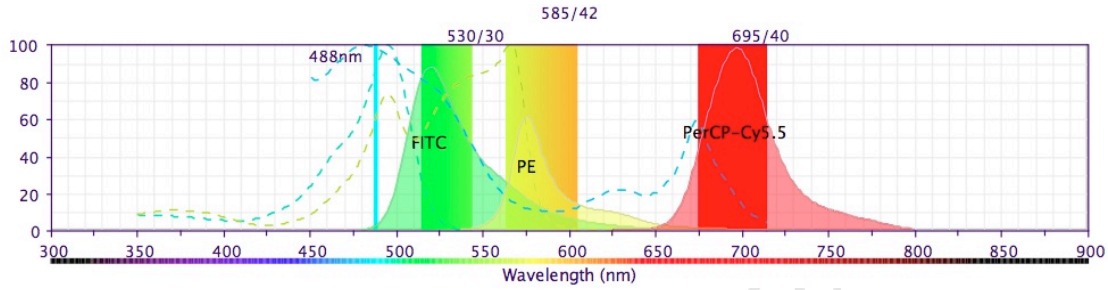

Figure 3.2: Fluorescence spectra showing overlap between closely emitting fluorochromes

The spectral emission wavelength of FITC overlaps into the PE detector channel. Adapted from (BDBiosciences, 2010).

We used anti-mouse immunoglobulin (Ig) kappa (κ) CompBeads (BD Biosciences), to calculate compensation for each flow cytometry experiment, (**Figure 3.3 A-D**). The CompBeads provide distinct negative (background fluorescence) and positive stained bead populations and sufficient numbers of positive events to allow calculation of accurate compensation (BDBiosciences, 2009a). They consist of two populations of microparticles: the positive control anti-mouse Ig κ CompBeads bind any mouse Ig, while the negative control CompBeads do not bind Ig (BDBiosciences, 2009b). The CompBeads were stained with a single antibody-fluorochrome conjugate (single stain compensation control) for all fluorochromes included in an experiment.

Compensation aligns the median of the negative and positive cell populations (**Figure 3.3 B and D**) (Bagwell and Adams, 1993; Herzenberg *et al.*, 2006). **Figure 3.3 B and D** illustrates that individual cells fall above or below the

median (e.g. when plotting FITC against PE or PerCP-Cy5.5 against Alexafluor700), resulting in data spread. This apparent broadening of the data is a result of applying compensation, when the correction or subtraction of fluorescence is the result of two relatively large numbers (i.e. the statistical variance of the fluorescence measurements increases with the correction applied) (Herzenberg *et al.*, 2006). Data spread can give false positives with brightly stained cells. Therefore, care must be applied when choosing fluorochrome combinations and gating strategies should be optimised.

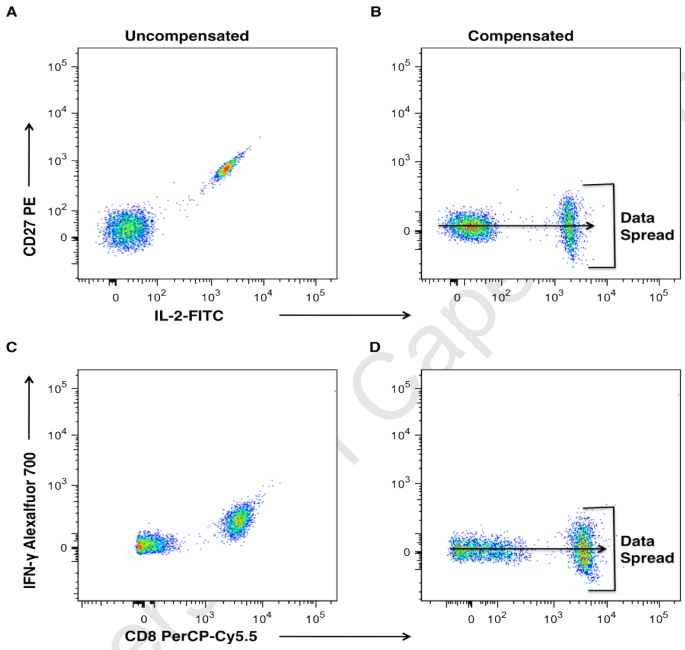

Figure 3.3: Compensating for spillover and data spread

(A) Uncompensated cells stained with FITC fluoresce in the PE channel. (B) After compensation the median of the negative population is aligned with the positive population. (C) Prior to compensation CD8 cells stained with PerCP-Cy5-5 fluoresce in the AlexaFluor700 channel. (D) Compensation correctly aligns the negative and positive populations.

3.3.4 Empirical process of final antibody selection

We initially investigated the use of a Pacific Orange conjugated antibody (CD8 Pacific Orange) to detect CD8 T cells. However, because of the poor resolution between the CD8 negative and positive populations with this antibody as shown in **Figure 3.4 A**, this antibody was excluded from the panel. CD8 PerCP-Cy5.5 showed a better resolution between the positive and negative CD8 populations, and was used instead for the final panel (**Figure 3.4 B**). We then investigated inclusion of a relatively new fluorochrome, Quantum Dot 605 (QD605), which has relatively narrow emission spectra (Chattopadhyay *et al.*, 2006) and therefore, less compensation requirements. Initially, QDot605 was only available as a biotinylated antibody, requiring a two-step staining method. The cells were first stained with CD4 biotin for 30 minutes, followed by a 30 minute staining step with streptavidin QDot605. We elected to change to the directly conjugated antibody when it became commercially available, to avoid the two-step labeling.

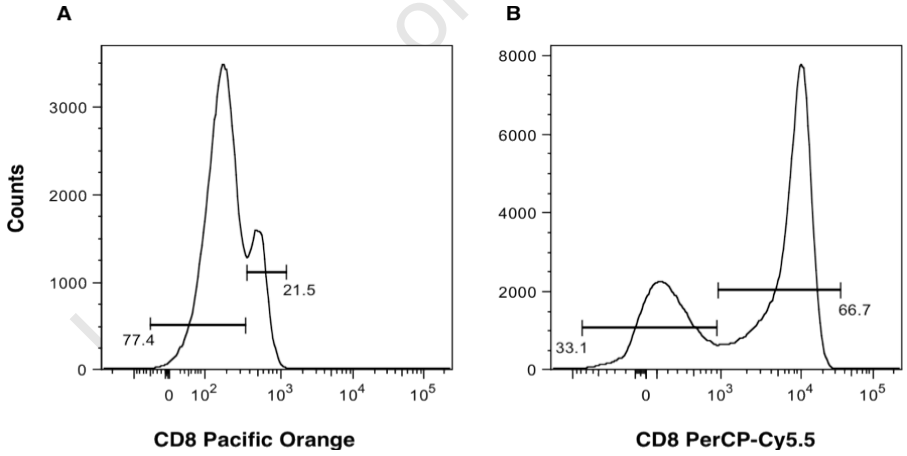

Figure 3.4: Histograms showing whole blood staining of $C3^+CD8$ T cells with two different fluorochrome-conjugated antibodies

(A) CD8 Pacific Orange-conjugated antibody shows decreased resolution between negative and positive population in the 8-colour panel. (B) CD8 PerCP-Cy5.5-conjugated antibody shows optimal resolution of negative and positive populations in the 8-colour panel.

3.3.4.1 Antibody titration

It was important to determine the optimal concentration for each antibody as this allows the identification of the lowest titer that could be used while allowing near maximal staining and achieving good resolution between the positive and negative cell populations. This is referred to as the signal-to-noise (S/N) ratio. The CCR7 APC titration illustrates this point: the optimal titer for CCR7 APC was chosen by identifying the positive and negative cell populations for CCR7 and, for each of these, the median fluorescence intensity (MFI) was calculated. The ratio between the MFI of the positive and negative cells was then calculated and plotted along with the frequency of the positively staining cell population for each antibody volume. **Figure 3.5 A and B** illustrates the optimal titer chosen for CCR7 APC. The optimal titre lies on the plateau of a titration curve indicating maximal staining of the positive staining cells (saturation point). The saturation point was reached at 2.5 μl of CCR7 APC antibody (**Figure 3.5 B**). The best resolution of positive and negative staining cells (highest S/N ratio) was reached at 5 μl. To achieve better resolution between the positive and negative cell populations of CCR7 APC the 5 μl volume was chosen, especially, since the expression pattern of CCR7 is often a continuum. More coluors mean more spillover issues, with loss of resolution sensitivity in affected detectors. The optimal staining volume/concentration was titrated for each fluorochrome-conjugated antibody.

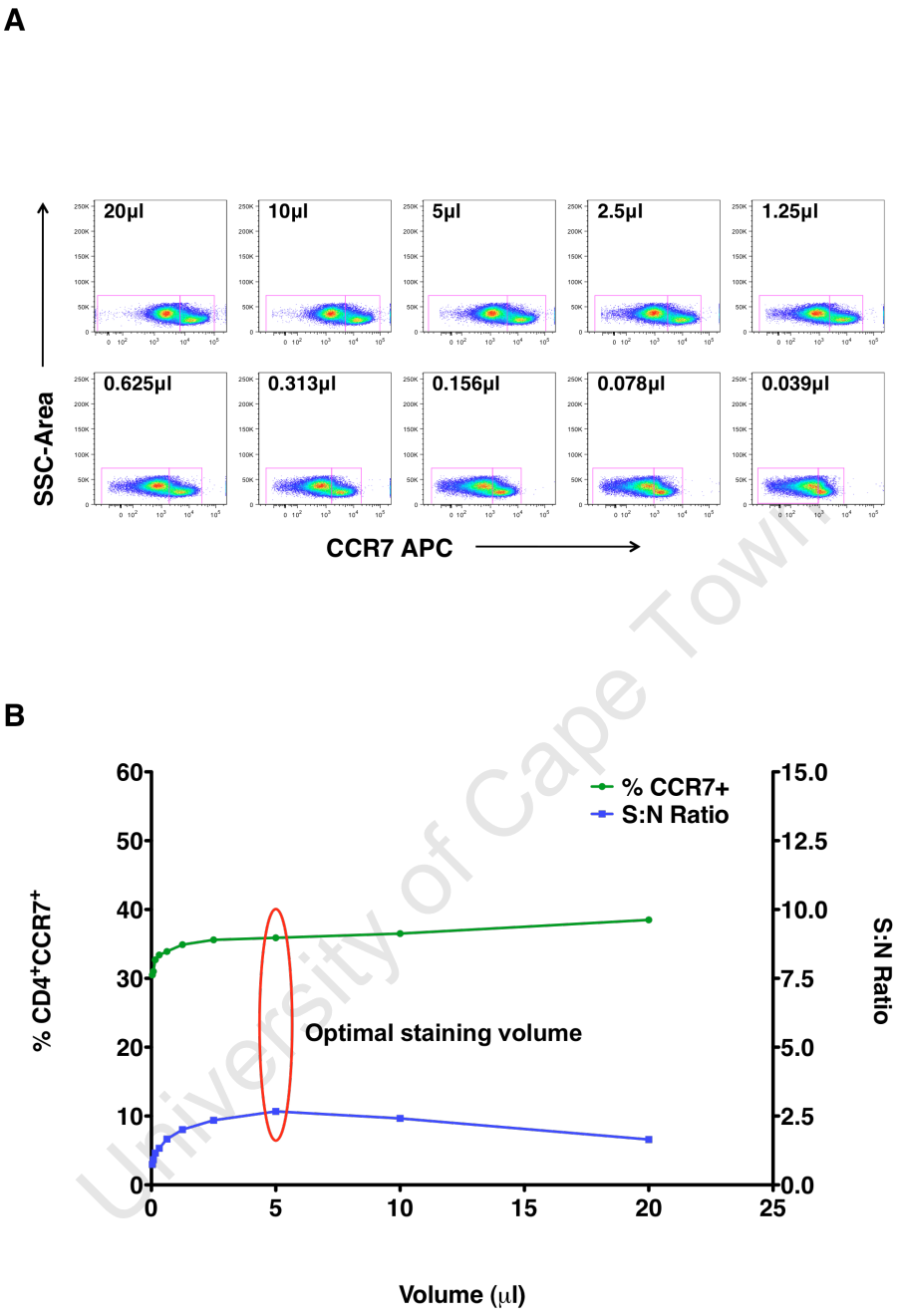

Figure 3.5: Titration of APC conjugated CCR7 antibody

(A) The gates represent the $CD3^+$ $CCR7^+$ and $CD3^+$ $CCR7^-$ populations displayed as contour plots after stimulation of whole blood with Ag85B/TB10.4. The antibody was titrated by serial dilution from a volume of 20µl to 0.039µl in 100µl. (B) The frequencies of $CD3^+$ $CCR7^+$ cells and signal-to-noise ratios in relation to antibody volume.

3.3.4.2 Effects of fixation on surface marker resolution

To determine the effects of fixation on surface marker staining and antibody performance, fresh whole blood was stained with antibodies before fixing the cells and compared with whole blood stained after fixation with FACS lysing solution. **Figure 3.6** illustrates that resolution between positive and negative staining cells was maintained for CD45RA PE-Cy7 after fixation and results were comparable to freshly stained samples. Similarly, CCR7 APC and CD27 PE maintained reliable resolution after fixation between positive and negative staining cells (data not shown).

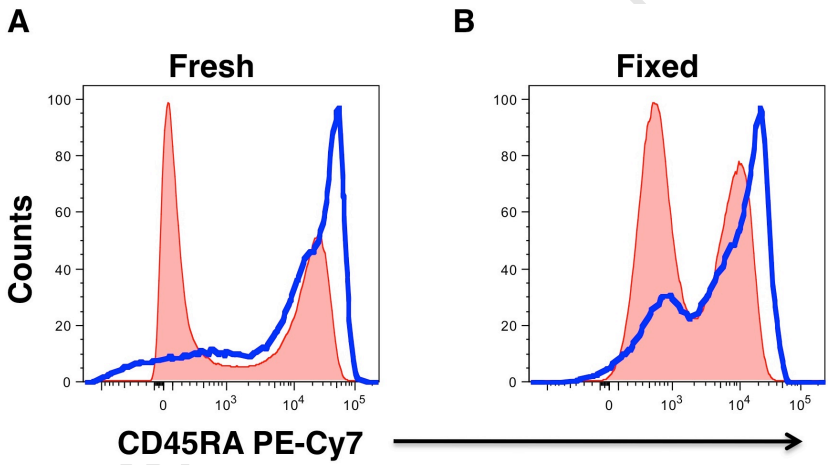

Figure 3.6: Effect of fixation with FACS lysing solution on surface marker staining CD4 (red shading) and CD8 (blue line) T cell histogram overlays illustrating resolution of positive and negative CD45RA populations in (A) fresh and (B) fixed whole blood.

3.3.4.3 Staining protocol optimization for the analysis of surface markers

The effect of the staining technique on the resolution of surface markers and on antibody performance was determined by staining with staining buffer and compared with Perm/Wash buffer. **Figure 3.7** illustrates that better resolution of positive and negative populations of CCR7 was achieved for both CD4 and CD8 T cells when staining with Perm/Wash buffer compared with 1% BSA (staining buffer). Good resolution was achieved for all other surface markers when staining with Perm/Wash buffer.

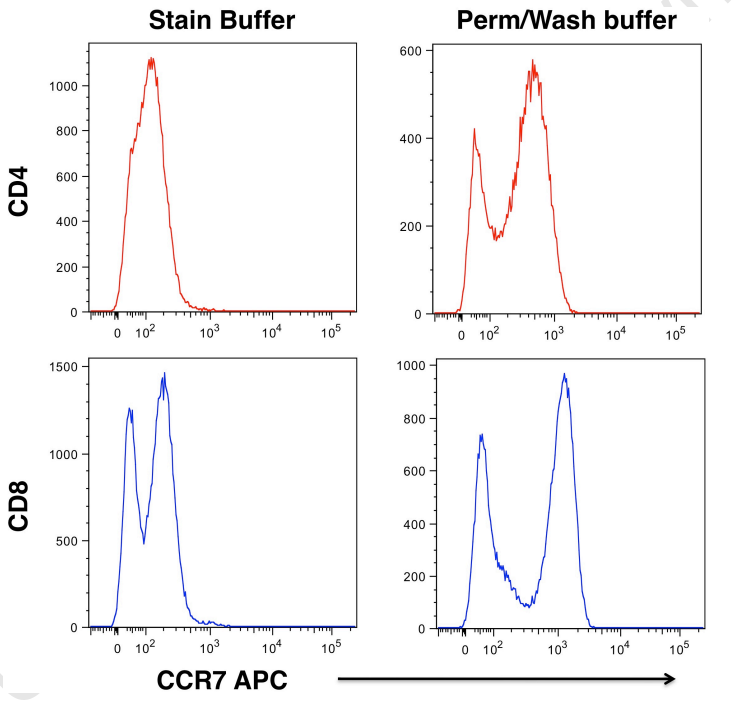

Figure 3. 7: Staining protocol for the analysis of surface markers
CCR7 APC staining in (A) Staining buffer and (B) Perm/Wash buffer.

3.3.5 Final antibody panel performance

To test for fluorescence spillover effects in the 8-colour panel as well as overall performance of the 8-colour panel the fluorescence minus one (FMO) experiment was performed, where cells are stained with all antibodies except one. **Figure 3.8** shows an example of a FMO experiment where all antibodies were included except CD4 QDot 605. This method allows detection of fluorescence signal caused by any of the other fluorochromes in the QDot 605 detector (false positive). The spectral overlap was assessed by recording the frequencies of cells expressing each marker on cells with or without each antibody on cells within the lymphocyte gate. **Table 3.3** shows the frequencies obtained when each antibody fluorochrome was excluded. No significant false fluorescence signal was detected in any of the channels (**Table 3.3**). Table 3.4 illustrates the final optimal staining panel and optimal staining volumes.

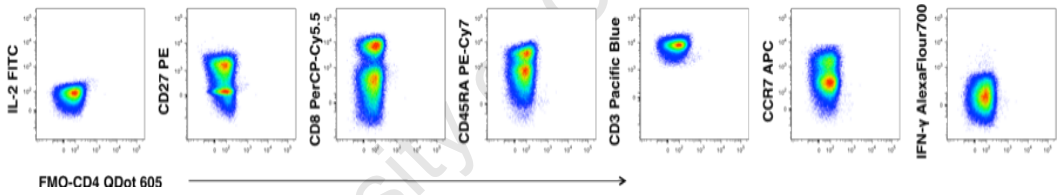

Figure 3.8: Example of fluorescence minus one experiment missing CD4 Qdot605 Density plots weree gated on $CD3^{+}$ T cells except for CD3 Pacific Blue, which is gated on lymphocytes.

	Immunological Marker (% positive expression)							
FMO	CD3	CD4	CD8	CD45RA	CCR7	CD27	IFN-γ	IL-2
Pacific Blue	0.091	58.124	34.399	36.734	29.409	67.172	0.202	0.059
QD605	65.698	0.033	34.176	34.499	27.754	65.133	0.145	0.067
PerCP-Cy5.5	65.698	58.123	0.038	36.735	28.614	67.576	0.166	0.063
PE-Cy7	65.698	58.513	33.930	0.002	29.410	67.172	0.160	0.065
APC	65.966	58.382	35.332	37.880	0.088	66.459	0.160	0.068
PE	66.348	57.691	33.674	36.734	30.252	0.043	0.176	0.066
Alexa700	65.870	57.836	33.674	34.500	31.096	65.805	0.048	0.069
FITC	65.699	57.985	34.176	34.500	29.409	65.805	0.154	0.005
Full Antibody Panel	65.854	58.093	34.194	35.940	29.425	66.446	0.166	0.075

Table 3.3: Summary chart of frequencies (%) of immunological markers detected in the 8-colour panel when incubated with all antibodies minus one (FMO)

Whole blood from an adult volunteer was stimulated with Ag85B/TB10.4 and stained with or without each single antibody. The amount of background fluorescence within a specific channel when the antibody in that particular channel is omitted is indicated in the pink shaded boxes. Samples were gated on $CD3^+$ T cells except for CD3 Pacific Blue, which is gated on lymphocytes. The full antibody panel indicates the frequencies obtained when all antibodies are included.

Antibody	Fluorochrome	Clone	Optimal volume (µl)
CD3	Pacific Blue	UCHT1	1
CD4	Quantum Dot 605	S3.5	0.5
CD8	PerCP-Cy5.5	SK1	5
CD45RA	PE-Cy7	L48	5
CCR7	APC	150503	5
CD27	PE	MT271	10
IFN-γ	AlexaFluor700	B27	1
IL-2	FITC	5344.111	5 µl

Table 3.4: Final 8-colour panel and antibody titers

3.3.6 Experimental set up

3.3.6.1 Sample size calculation for rare event analysis

When assessing antigen specific responses relatively small frequencies are expected (<5%). The power calculator developed by Holden Maecker was used to determine the number of events required to obtain a power of 90%, p<0.05, (downloaded from http://maeckerlab.typepad.com). **Table 3.5** shows the number of events of CD4 and CD8 T cells required for acquisition for reliable rare event analysis. The median levels for backgrounds and Ag85B-TB10.4-specific responses for IFN-γ and IL-2 were calculated from a whole-blood experiment performed on six adult volunteers. The analysis revealed that approximately 152000 CD4 T cells and about 687000 CD8 T cells were required to allow reliable detection of IFN-γ IL-2 expressing cells over background. The power calculator was used as a tool/guide to predict the necessary sample size/number of cells to be acquired to be confident to have enough positive events above background in any given marker/cell sub-population. However, due to small numbers of possible events, especially in HIV infection the entire sample was acquired.

	CD4		CD8	
Cytokine	IFN-γ	IL-2	IFN-γ	IL-2
Frequency of cells in unstimulated control (% Background)	0.02	0.02	0.01	0.02
Frequency of cells in stimulated sample (% Positive events)	0.08	0.04	0.02	0.03
Number of events required	23877	128961	257961	429893

Table 3.5: Sample size required for rare event analysis
The power calculator developed by Holden Maecker was used to determine the number of events to acquire when assessing cytokine production. Downlaoded from (http://maeckerlab.typepad.com).

3.3.6.2 Intra-assay variability

To assess intra-assay variability of the ICS assay, six identical samples from the same adult volunteer were simultaneously stained with the 8-colour panel shown in **Table 3.4**. **Table 3.6** shows the coefficient of variation (CV) calculated for the six experiments. The CVs were calculated using the mean percentage of cytokine positive cells (IFN-γ^+ or IL-2^+) for both CD4 and CD8 T cells and standard deviation. The CVs for cytokine positive CD4 (IFN-γ^+ or IL-2^+) and for IFN-γ^+CD8 T cells were <10%. However, the CV increased to >20% at very low frequencies of positive cells as seen with IL-2^+CD8 T cells. The reliability of significant differences becomes questionable at these very low values.

	CD4		CD8	
	IFN-γ	IL-2	IFN-γ	IL-2
Minimum (%)	0.301	0.154	0.097	0.028
Median (%)	0.366	0.174	0.106	0.048
Maximum (%)	0.383	0.199	0.119	0.055
Mean (%)	0.355	0.174	0.106	0.046
Std. Deviation	0.03	0.02	0.01	0.01
Coefficient of variation (%)	8.47	9.65	8.28	22.23

Table 3.6: Intra assay variability: coefficient of variation of cytokine positive T cells

3.3.6.3 Gating strategy & Data Analysis of multiparameter data sets

We started by excluding doublets from the analysis by gating on forward-scatter (FSC)-height vs. FSC-area. Doublet events may either be the consequence of two cells sticking together, or two cells have passed through the laser beam so close to each other that they have been interpreted as a single event. When this involves two different cell types the flow cytometer will record that a cell displaying the characteristics of both cell-types was present in the sample (double-positive). This can be highly misleading, especially when evaluating a rare marker.

Small lymphocytes were selected by gating on SSC-Area and FSC-Area. T lymphocytes were selected by gating on the CD3+ cells, which were subsequently differentiated into CD4 and CD8 T cells. Backgating confirmed that the cytokine expressing cells were found at the centre of the lymphocyte gate (i.e. within the mid fluorescent range). Intracellular cytokine expression was analysed in the predefined CD4 and CD8 T cell populations by using Boolean gating to determine the combination of cytokines produced.

Figure 3.9 illustrates the performance of the final panel and the gating strategy that was used to characterize cytokine producing CD4 and CD8 T cell populations. This gating strategy was used to analyse both the cross-sectional and the longitudinal data, in chapter 4 and 5, respectively.

To account for background or spontaneous cytokine production frequencies of cytokine expressing cells in the negative control (unstimulated sample) were subtracted from frequencies measured in the stimulated samples. Participants were excluded from the analysis for any of the following reasons: (1) a positive control (SEB) response less than the median plus 3 median absolute deviations (MAD) above median background of the negative control, (2) less than 40 positive events in the stimulated sample for phenotypic analysis. All values of zero were adjusted to 0.001 for plotting on the logarithmic scale.

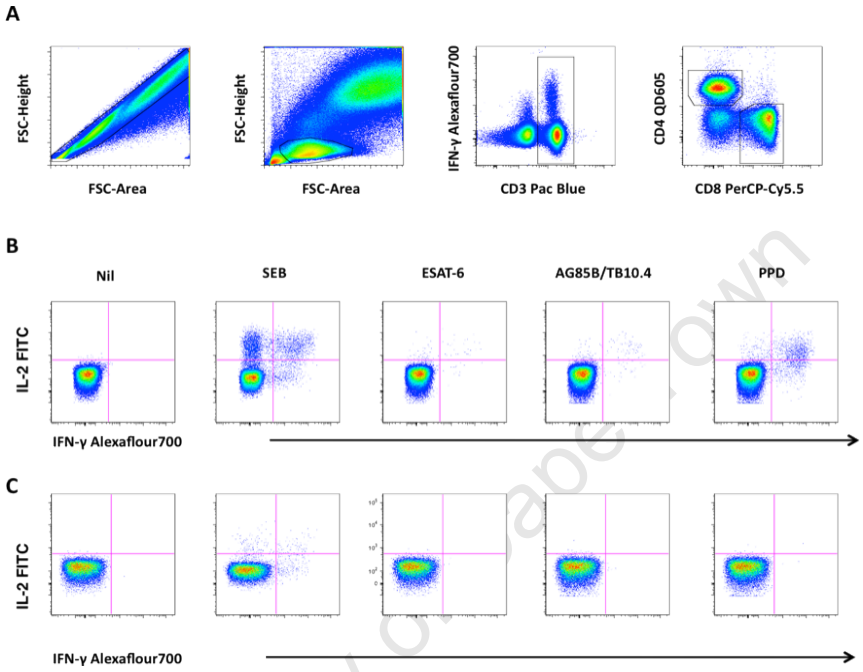

Figure 3.9: Final performance of the panel used to identify mycobacteria-specific CD4 and CD8 T cells

(A) Representative density plots showing the gating strategy used to identify CD4 and CD8 T cells from whole blood. From left to right, cell doublets were excluded using forward scatter-area and forward scatter-height parameters. Small lymphocytes were then selected. T cells were selected by gating on $CD3^{+}$ cells and this was then followed by selection of CD4 and CD8 T cells. (B) Representative plots showing IFN-γ and IL-2 expression by unstimulated (Nil), SEB, ESAT-6, Ag85B/TB10.4, or PPD, stimulated CD4 or (C) CD8 T cells.

3.4 Discussion

In the present chapter the key points in the optimisation process were titration of individual fluorochrome conjugated antibodies, spectral overlap considerations and control of compensation. Establishing a multi-parameter 8-colour panel to simultaneously assess phenotypic and functional T cell markers has presented technical challenges, but these were overcome by a meticulous, step-wise approach to assess all variables that may influence outcome. We have confirmed that the following steps or considerations should be adhered to when starting up a flow cytometry panel: 1) select fluorochromes for brightness and least spillover, 2) optimize antibody panels by taking into account reagent brightness and data spread, 3) Standardise longitudinal experiments with proper QC, 4) put systems into place to allow streamlined and reproducible analysis. Optimisation experiments give valuable knowledge on the strengths and weaknesses of an assay, thereby aiding the extraction of meaningful information from complex data sets.

We were successful in the development and optimization of an 8-colour flow cytometry panel to comprehensively and reliably assess mycobacteria-specific T cell cytokine expression combined with the ability to phenotype the memory T cells in small whole blood samples collected from paediatric populations. Relevant markers were selected to address the research questions in this study allowing the characterisation of functional profiles and memory subsets of CD4 and CD8 T cells. Various antibody-fluorochrome combinations were assessed and suboptimal or poorly performing reagents were excluded from the final antibody panel.

CHAPTER 4

CD4 and CD8 T cell responses to mycobacterial antigens

4.1 Introduction

4.2 Methods

4.3 Results

4.3.1 Participant recruitment and demographics

4.3.2 Mycobacteria-specific CD4 and CD8 T cell frequencies were detectable in all groups of children

4.3.3 Frequencies and proportions of polyfunctional CD4 and CD8 T cells

4.3.4 Co-variants of the Ag85B/TB10.4-specific CD4 and CD8 T cell response

4.3.5 Antigen-specific CD4 and CD8 T cells express an effector memory phenotype

4.3.6 T cell memory phenotypes are comparable in healthy and diseased children

4.4. Discussion

4.1 Introduction

Children are known to have a much higher risk of severe disease from tuberculosis (TB) than adults and better protection from TB has been identified as an international health and research priority in both adults and children (Nelson and Wells, 2004; Lienhardt *et al.*, 2003). As part of the World Health Organisation (WHO) expanded program of immunisation (EPI), the only licensed vaccine against TB, bacille Calmette-Guérin (BCG), is given to more infants worldwide than any other childhood vaccine. However, it is well established that BCG has limited value in curbing the global TB epidemic, as it affords variable and mostly poor protection against pulmonary TB (Trunz *et al.*, 2006). HIV infection is a major contributor to the TB epidemic, as associated immune compromise dramatically increases the risk of TB disease (Lawn and Churchyard, 2009; Abdool Karim *et al.*, 2009). HIV-infected children are more susceptible to TB than HIV-uninfected children. Mortality in children with concomitant HIV infection has been reported to be as high as 13% in HIV-infected children compared with 1.5% in HIV-uninfected children in a South African study in children (Madhi *et al.*, 2000).

Currently, there is no evidence to suggest that BCG confers protection against TB in HIV-infected infants and children (WHO, 2004), but might in fact be potentially harmful by causing dissemination, resulting in BCGosis (Hesseling *et al.*, 2007; Hesseling *et al.*, 2009). The HIV status of most infants is unknown at birth, when the BCG vaccine is routinely administered, especially in resource poor settings where there is lack of public health infrastructure to assess HIV status of the mother. Many infants with HIV infection therefore continue to receive the vaccine. BCG-related disease may also manifest as an immune reconstitution syndrome (BCG-IRIS) once antiretroviral therapy (ART) is commenced, particularly in children with very low CD4 T cell counts (Smith *et al.*, 2009). A more efficacious vaccine that is safe in immunocompromised persons and confers protection to HIV-infected individuals is therefore urgently needed (Newton *et al.*, 2008).

Any successful vaccine is required to generate long-lived immunological memory and induce protective immune responses. However, despite longstanding research efforts, the “correlates of protection”, i.e. markers that can be measured in peripheral blood to reflect protective immune responses against TB, continue to be ill defined. Animal and human studies have shown that antigen-specific CD4 T cells are critical, (Mogues *et al.*, 2001; Saunders *et al.*, 2002; Havlir and Barnes, 1999), since impairment of CD4 T cell responses, such as in HIV, lead to dramatic increase in TB susceptibility, partially reversible by treatment with ART. CD8 T cells also appear important for protection (Behar *et al.*, 1999; Derrick *et al.*, 2004; Billeskov *et al.*, 2007; Bruns *et al.*, 2009; Chen *et al.*, 2009). The generation of antigen-specific memory/effector T cell populations by a given TB vaccine candidate has therefore been the focus of immunological read-outs for novel TB vaccines. These immune responses have not been studied in great detail in HIV infected patients (Wilkinson *et al.*, 2009), particularly not in children.

Since human studies have demonstrated disease susceptibility to intracellular pathogens in persons with genetic defects in the IFN-γ and IL-12 pathways (Flynn *et al.*, 1993; Orme *et al.*, 1993; Newport *et al.*, 1996; Ottenhoff *et al.*, 1998; Ottenhoff *et al.*, 2005), it is widely accepted that “protective” T cells should express T helper type 1 (Th1) cytokines, such as IFN-γ, IL-2 and TNF-α. IL-2 is required for secondary expansion of memory T cells (Williams *et al.*, 2006; Dooms *et al.*, 2007). Further, polyfunctional T cells, which co-express two or more cytokines, have been associated with more effective control of murine intracellular infections (Darrah *et al.*, 2007), including *M. tuberculosis* (Forbes *et al.*, 2008), but convincing human data are currently lacking.

T cell cytokine expression has been linked with cell surface expression of markers of memory phenotype in viral infections in mice (Sallusto *et al.*, 1999) and humans (Harari *et al.*, 2005). CD45RA, which is lost by T cells upon priming, differentiates naïve and memory (antigen-experienced) T cells. However, this marker can be re-expressed, in some more extensively differentiated populations. Within the antigen-experienced population,

expression of CCR7 defines central memory T cells (T_{CM}) which classically express IL-2 and are able to home to lymphoid organs. Although these cells are thought to lack immediate effector function, they are long lived and proliferate rapidly upon antigen reencounter. By contrast, effector memory T cells (T_{EM}), which lack CCR7 expression, predominantly express effector cytokines, such as IFN-γ (Sallusto *et al.*, 1999; Harari *et al.*, 2005). These populations can be further dissected according to their degree of differentiation, based on the expression level of CD27. This co-stimulatory marker is sequentially lost during T cell differentiation (Fritsch *et al.*, 2005).

To date, very few studies have analyzed mycobacteria-specific T cell immunity in detail in children beyond infancy (Tena *et al.*, 2003). It is unknown whether HIV-infected children can mount similar T cell responses to healthy children upon vaccination with novel TB vaccines. In this cross-sectional study we aimed to delineate the type of T cell responses that may be boosted by new TB vaccines in three groups of children. To achieve this aim the function and memory phenotype of mycobacteria-specific T cells were characterised in African children with and without HIV-infection.

4.2 Methods

Whole blood was collected from 3 groups of BCG-vaccinated children: HIV unexposed healthy children (n=30), HIV-seronegative children receiving TB treatment (n=30) and HIV-infected children without TB (n=30). Blood was stimulated with specific mycobacterial antigens for a total of 7 hours as described in chapter 2. T cell cytokine production by CD4 and CD8 T cells as well as the memory phenotype of these antigen specific T cells was determined using an 8-colour flow cytometry panel as detailed in chapter 3.

4.3 Results

4.3.1 Participant recruitment and demographics

Children were recruited at clinics in African communities from TB endemic areas in the Western Cape region, as described in chapter 2, in section 2.2.2. The demographic characteristics of all children are summarised in **Table 4.1**.

We enrolled 30 children of similar age into each of the three study groups (overall median age: 3 years, interquartile (IQR) range: 1-5 years). All children had routinely received BCG vaccination at birth, as part of the Expanded Programme on Immunisation (EPI) in South Africa. All children (n=30) enrolled into the healthy control group (HC) were clinically well, had normal full blood count and C-reactive protein (CRP) levels and no other signs of any ongoing infection or inflammation.

All children enrolled into the TB group (TB) were receiving standard treatment for TB with isoniazid, rifampicin and pyrazinamide for a median of 4 months (range: 2-6 months). Twenty-nine children had pulmonary TB, 1 child had TB of the spine. In 7 cases, TB had been microbiologically confirmed by positive culture. In the other 23 cases TB was diagnosed based on epidemiological history, signs and symptoms consistent with TB, abnormal chest X-ray, a positive tuberculin skin test (induration > 10mm) and clinical improvement after start of TB treatment.

All HIV-infected children had perinatally acquired HIV infection and had advanced HIV disease, fulfilling clinical and/or immunological criteria for starting antiretroviral therapy (ART), according to WHO guidelines (WHO, 2008). The CD4 percentages of these HIV-infected children are well below percentages reported for age-matched healthy African children from Uganda (IQR: 18.8%–45.8%) (Lugada *et al.*, 2004). Any children with reported exposure to an active TB case in their household were excluded from the HC and HIV groups.

	Healthy Controls (n=30)	TB Diseased (n=30)	HIV-Infected (n=30)
Male/female	17/13	17/13	15/15
Ethnicity			
Black	27	27	26
Mixed race	1	3	4
White	2	0	0
Age in years, median (IQR)	3.5 (1-5)	3 (1-4)	3.5 (1-6)
<1	2	4	6
1 - 5	23	22	16
6 - 12	5	3	8
>12	0	1	0
CD4 absolute count (cells/µl), median (IQR)	N/A	N/A	531 (284-1259)
CD4 percentage, median (IQR)	N/A	N/A	17.85 (12.93-21.45)
Viral load (log_{10} RNA copies/ml), median (IQR)	N/A	N/A	4.8 (4.30-5.76)

Table 4.1: Study participant demographic characteristics
IQR; interquartile range, N/A; not available

4.3.2 Mycobacteria-specific CD4 and CD8 T cell frequencies were detectable in all groups of children

We used flow cytometry to evaluate the frequency of CD4 and CD8 T cells expressing IFN-γ and IL-2 in whole blood from the three groups of children after antigen stimulation (**Figure 4.1**). Cytokine expression in unstimulated samples was very low and mostly undetected. All groups of children responded to the positive control (SEB). There were no significant differences in response to the positive control between any of the different study groups (data not shown). When blood was stimulated with Ag85B/TB10.4, CD4 T cells typically expressed IFN-γ and/or IL2 (Figure 4.1 B), whereas CD8 T cells expressed mostly IFN-γ.

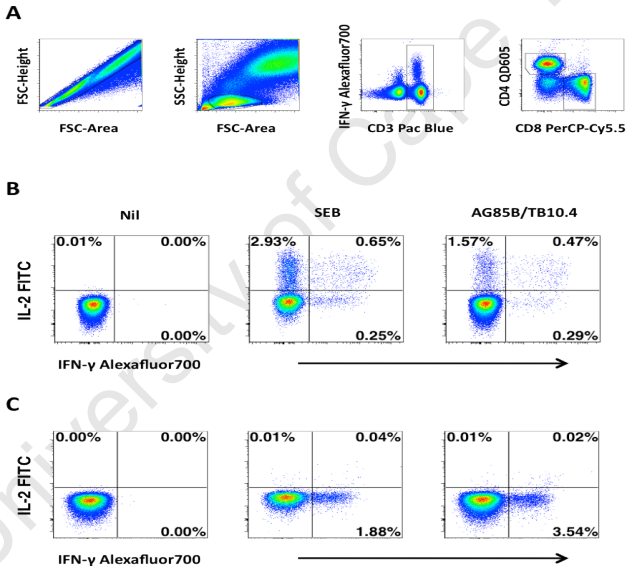

Figure 4.1: Functional characterisation of Ag-specific T cell cytokine expression by intracellular cytokine staining and flow cytometry

(A) Representative density plots, from a single healthy child, showing the gating strategy used to identify cytokine expressing CD4 and CD8 T cells from whole blood. From left to right, cell doublets were excluded using forward scatter-area and forward scatter-height parameters. Small lymphocytes were then selected. T cells were selected by gating on CD3+ cells, followed by selection of CD4 and CD8 T cells. (B and C) Representative plots showing IFN-γ and IL-2 expression in unstimulated (Nil), SEB and Ag85B/TB10.4 stimulated CD4 (B) or CD8 (C) T cells.

Although fewer CD3+ cells were available for analysis from HIV-infected children (median (IQR): 154,670 (104,156–294,199)), compared with the number of CD3+ T cells from HC children (median (IQR): 254,468 (165,733–345,048); p=0.026) and non-HIV-infected children with TB (median (IQR): 247,026 (165,000–393,612); p=0.036), mycobacteria-specific CD4 and CD8 T cells were readily detectable, even in HIV-infected children. Frequencies of these specific cells were observed in a remarkably broad range, between 0 and 10.36% in all three groups (**Figure 4.2**).

Total cytokine-expressing (IFN-γ^+ and/or IL-2^+), total IFN-γ or total IL-2 Ag85B/TB10.4-specific CD4 T cell frequencies were significantly lower in HIV-infected children, compared with the TB group (**Figure 4.2 A-C**). There were no differences in Ag85B/TB10.4-specific CD4 T cell frequencies between HIV-infected children and children in the HC group. Frequencies of PPD-specific CD4 T cells, expressing total cytokine, or IFN-γ or IL-2 were also significantly higher in the TB group, compared with both the HC and HIV-infected groups (**Figure 4.3 A-C**). Similarly, ESAT-6-specific CD4 T cells were detectable in all three groups of children, although mostly at very low frequencies. Again, total cytokine-expressing or total IFN-γ ESAT-6-specific CD4 T cell frequencies were significantly lower in the HIV-infected children, compared with the TB group (**Figure 4.4 A-C**).

There was no difference in frequencies of total cytokine or total IFN-γ-expressing Ag85B/TB10.4-specific CD8 T cells between the 3 groups (Figure 4.5 A-C). Total IL-2-expressing Ag85B/TB10.4-specific CD8 T cells were significantly lower in HIV-infected children, compared with both the TB and HC groups (Figure 4.5 C). However, the validity of this difference was questionable since the Ag85B/TB10.4-specific IL-2-expressing CD8 T cell frequencies were very low. Peptide antigens are perceived to be more optimal for stimulating CD8 T cell responses as these antigens would require less processing and are better recognized by CD8 T cells. However, as our own data confirm, strong CD8 responses can also be elicited using protein antigens. Concentrations and type of antigen and time-course are likely to

play a role in the quality and quantity of such responses. However, control experiments using peptides of the same antigens revealed highly correlated results (data not shown). Frequencies of PPD-specific CD8 T cells were low in all groups of children and no differences between groups were observed (data not shown). Similarly, no difference in total cytokine, total IFN-γ or total IL-2 expression by ESAT-6-specific CD8 T cells was observed (data not shown).

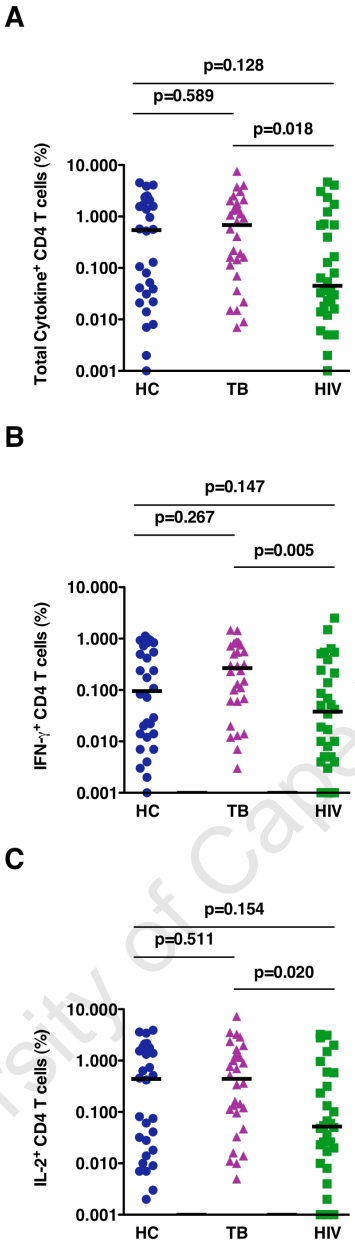

Figure 4.2: Ag85B/TB10.4-specific CD4 T cell responses measured by intracellular cytokine staining and flow cytometry in whole blood from the three groups of children

Each symbol represents an individual and for each plot, the median is represented by the horizontal line. (A) Frequencies of Ag85B/TB10.4-specific, total cytokine$^+$ (IFN-γ$^+$ and/or IL-2$^+$) CD4 T cells (B) total IFN-γ$^+$ or (C) total IL-2$^+$ CD4 T cells after incubation with recombinant proteins. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

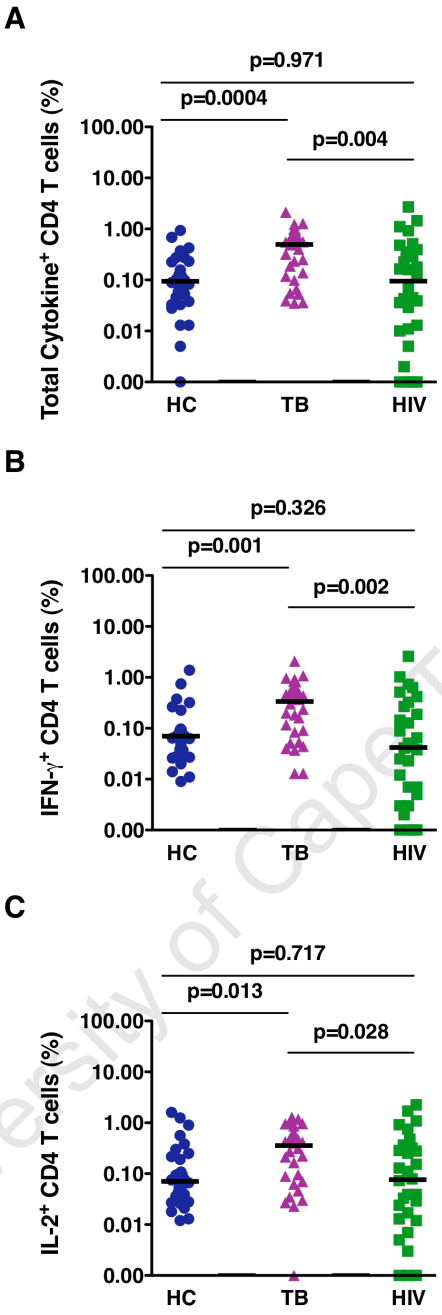

Figure 4.3: PPD-specific CD4 T cell responses in the three groups of children

Each symbol represents an individual and for each plot, the median is represented by the horizontal line. (A) Frequencies of PPD-specific, total cytokine$^+$ (IFN-γ$^+$ and/or IL-2$^+$) CD4 T cells (B) total IFN-γ$^+$ or (C) total IL-2$^+$ CD4 T cells. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

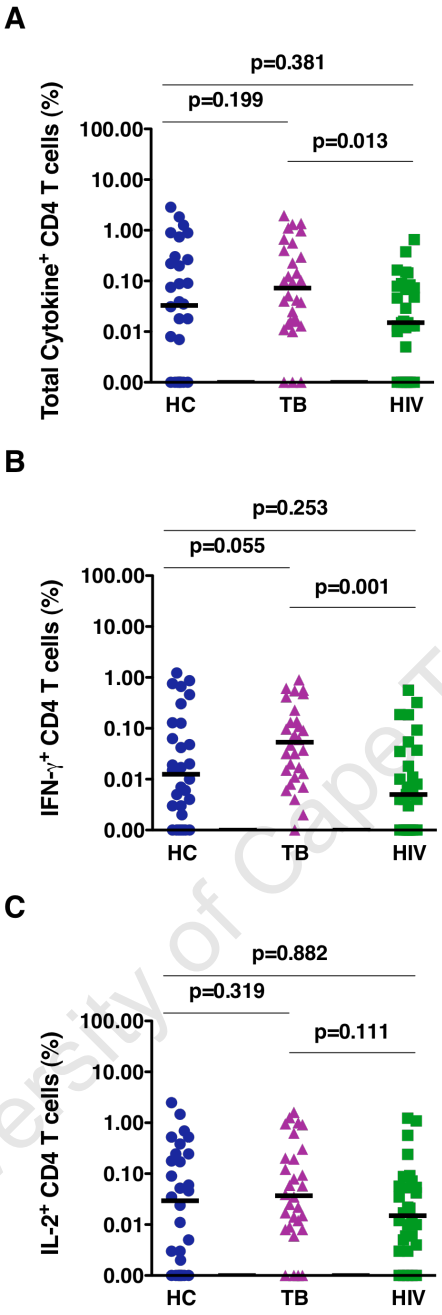

Figure 4.4: ESAT-6-specific CD4 T cell responses

Each symbol represents an individual and for each plot, the median is represented by the horizontal line. (A) Frequencies of ESAT-6-specific, total cytokine$^+$ (IFN-γ$^+$ and/or IL-2$^+$) CD4 T cells (B) total IFN-γ$^+$ or (C) total IL-2$^+$ CD4 T cells. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

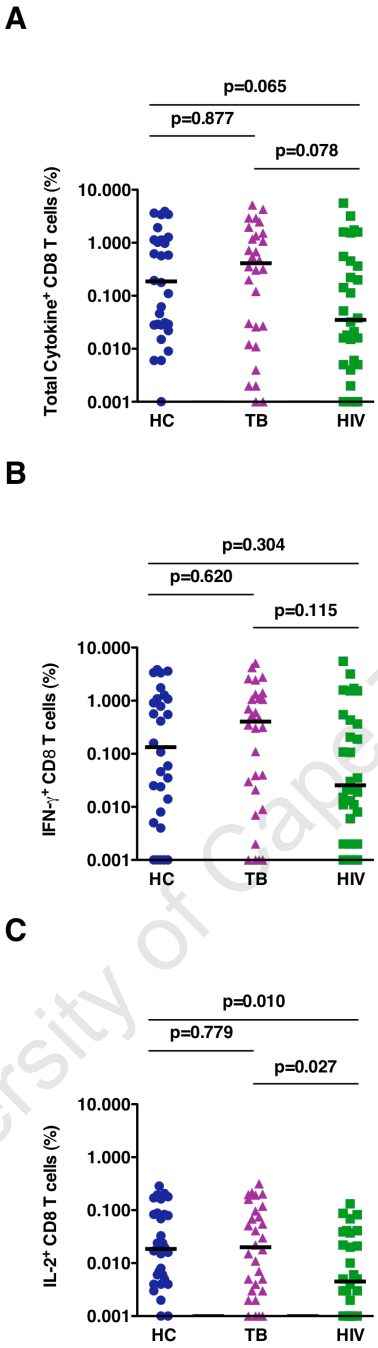

Figure 4.5: Ag85B/TB10.4-specific CD8 T cell responses in the three groups of children

(A) Frequencies of Ag85B/TB10.4-specific, total cytokine$^+$ (IFN-γ$^+$ and/or IL-2$^+$) CD8 T cells. Each symbol represents an individual and for each plot, the median is represented by the horizontal line. (B) total IFN-γ$^+$ or (C) total IL-2$^+$ CD8 T cells. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

4.3.3 Frequencies and proportions of polyfunctional CD4 and CD8 T cells

Cells that co-express cytokines have been suggested to provide better protection against intracellular infections than cells that express only a single cytokine (Darrah *et al.*, 2007). We compared the frequencies of cytokine co-expressing cells between the 3 groups of children. HIV-infected children had significantly lower frequencies of Ag85B/TB10.4-specific CD4 T cells co-expressing IFN-γ and IL-2 than children with TB (p=0.002, **Figure 4.6 A**). Ag85B/TB10.4-specific CD4 T cells expressing IFN-γ alone were also significantly less frequent in HIV-infected children, compared with children with TB (p=0.029).

Frequencies of bi-functional expressing PPD-specific CD4 T cells were significantly lower in HIV-infected and HC groups compared to children with TB (p=0.002 and p=0.004, respectively; **Figure 4.7 A**). This was also true for mono-functional IFN-γ-expressing CD4 T cells in HIV-infected and HC groups compared to the TB group (p=0.028 and p=0.014, respectively).

Similarly, ESAT-6-specific bi-functional CD4 T cells were significantly lower in HIV-infected compared with children with TB (p=0.0003, **Figure 4.8 A**). Healthy children appeared to have lower ESAT-6-specific bi-functional CD4 T cells, but this was not significant (p=0.05).

Despite the low frequencies of Ag85B/TB10.4-specific CD8 T cells producing IL-2 alone or co-expressing IFN-γ and IL-2 in all three groups of children, these subsets were significantly less frequent in HIV-infected children, compared with the TB group (p=0.024 and p=0.040, respectively; **Figure 4.9 A**). IL-2 expressing mono-functional CD8 T cell frequencies were also lower in the HIV-infected group compared with healthy controls (p=0.01, **Figure 4.9 A**).

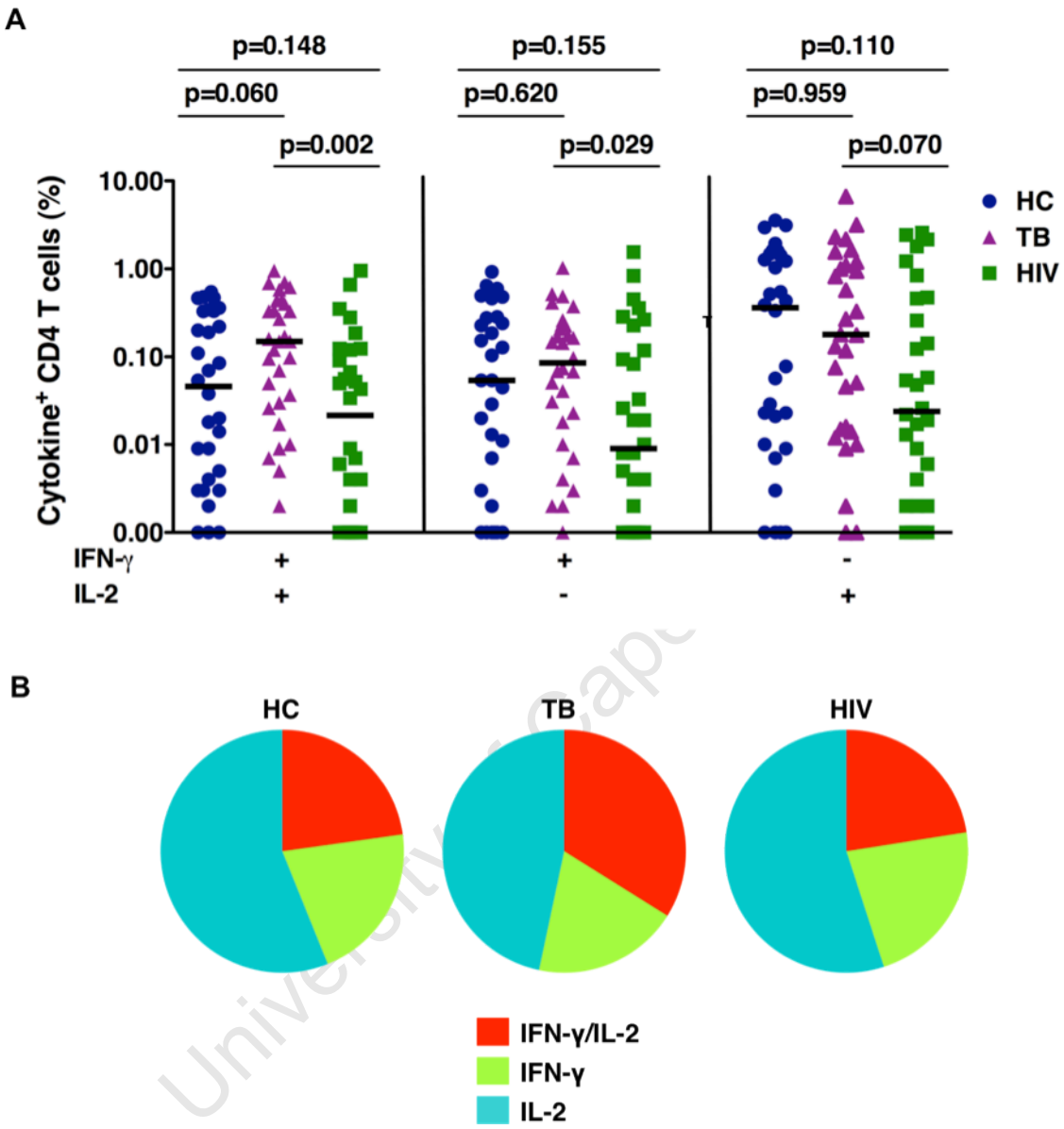

Figure 4.6: Analysis of the quality of Ag85B/TB10.4-specific CD4 T cells in the three groups of children

(A) Frequencies of bifunctional or monofunctional specific CD4 T cells. Each symbol represents an individual and for each plot the median is represented by the horizontal line. (B) Pie charts represent the mean proportions of cells co-expressing IFN-γ and/or IL-2, among the total CD4 T cell response, after stimulation with Ag85B/TB10.4. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

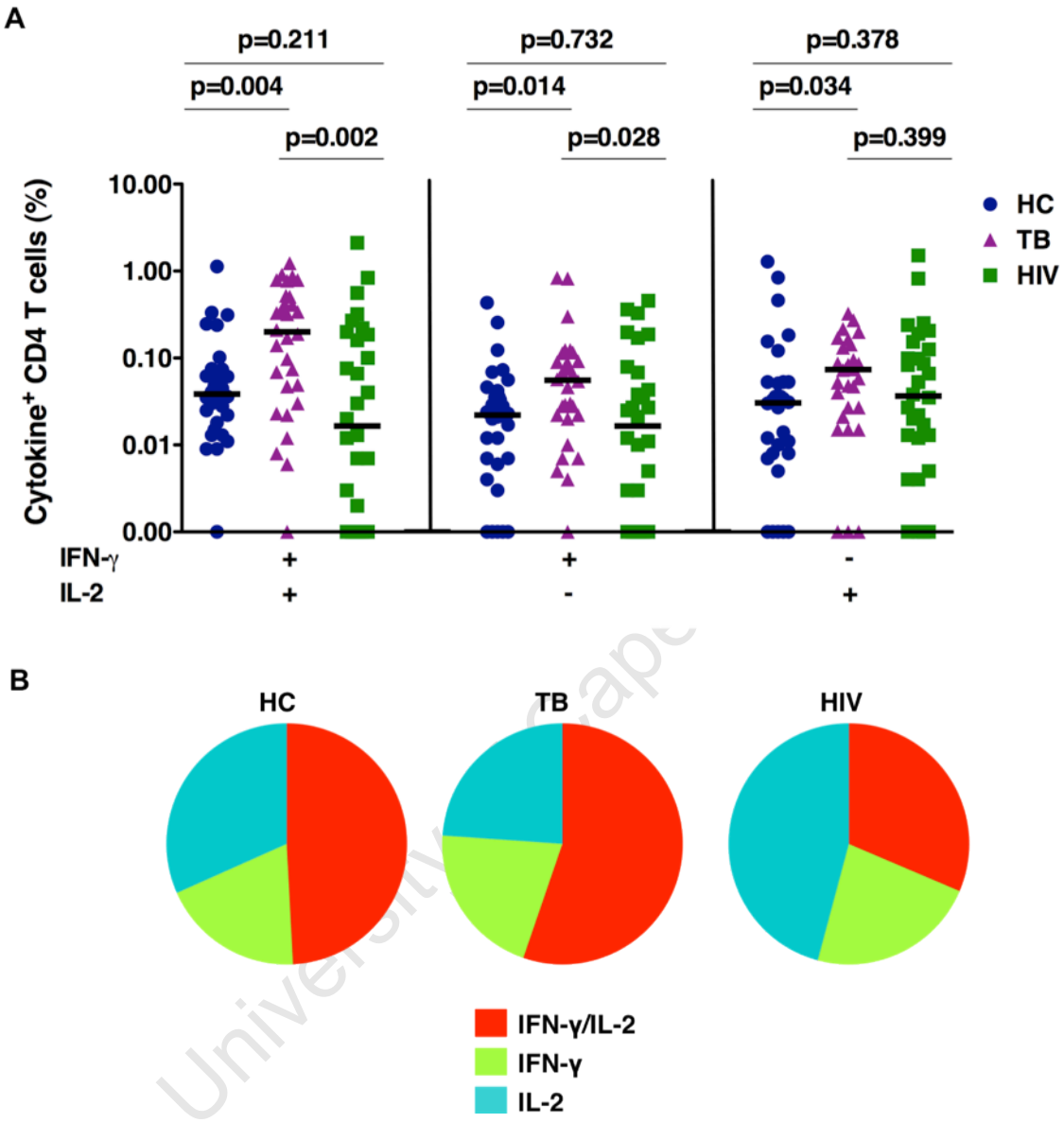

Figure 4.7: Analysis of the quality of PPD-specific CD4 T cells

(A) Frequencies of bifunctional or monofunctional specific CD4 T cells. Each symbol represents an individual and for each plot, the median is represented by the horizontal line. (B) Pie charts represent the mean proportions of cells co-expressing IFN-γ and/or IL-2, among the total CD4 T cell response, after stimulation with PPD. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

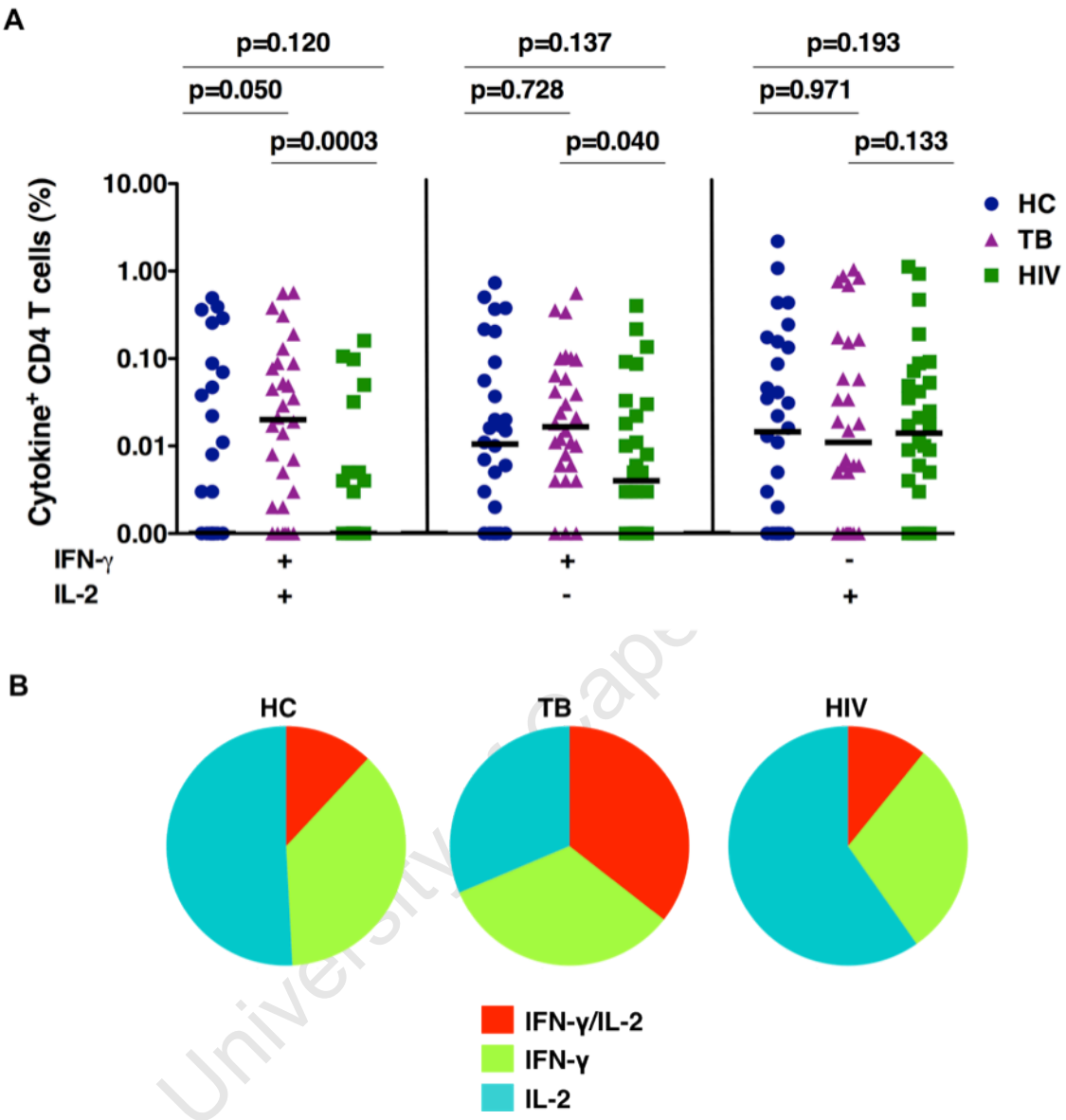

Figure 4.8: Analysis of the quality of ESAT-6-specific CD4 T cells in the three groups of children

(A) Frequencies of bifunctional or monofunctional specific CD4 T cells. Each symbol represents an individual and for each plot, the median, is represented by the horizontal line. (B) Pie charts represent the mean proportions of cells co-expressing IFN-γ and/or IL-2, among the total CD4 T cell response, after stimulation with ESAT-6. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

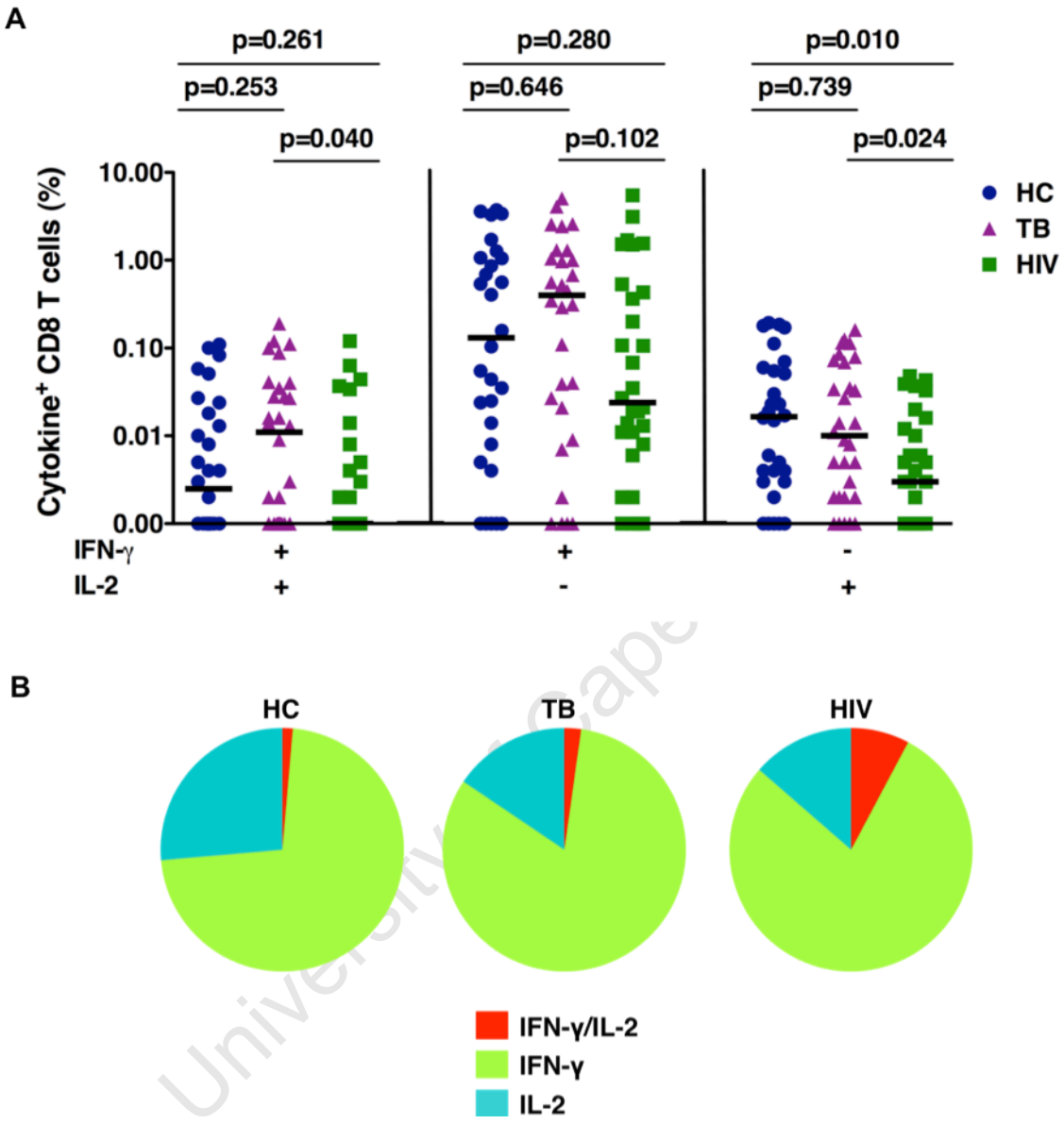

Figure 4.9: Analysis of the quality of Ag85B/TB10.4-specific CD8 T cells in whole blood from three groups of children

(A) Frequencies of bifunctional or monofunctional specific CD8 T cells. Each symbol represents an individual and for each plot, the median, is represented by the horizontal line. (B) Pie charts represent the mean proportions of cells co-expressing IFN-γ and/or IL-2, among the total CD8 T cell response, after stimulation with Ag85B/TB10.4. Differences between any two groups were calculated using the Mann Whitney U test. HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection.

To further characterise the quality of the T cell response, we assessed combinations of cytokine expression among antigen-specific T cells. Overall, Ag85B/TB10.4-specific cells expressing IL-2 alone comprised the predominant subset of specific CD4 T cells in all groups of children (**Figure 4.6 B**), whereas cells expressing IFN-γ alone or in combination with IL-2 comprised similarly sized subsets.

PPD-specific CD4 T cells were more polyfunctional, with IFN-γ/IL-2 co-expressing cells comprising approximately 50% of the total response in the TB and healthy control groups (**Figure 4.7 B**).

Both the HC and the HIV-infected groups had lower proportions of polyfunctional CD4 T cells after stimulation with ESAT-6 compared to the TB group. IFN-γ/IL-2 co-expressing cells comprised 30-40% in the TB group compared to approximately10% in the HC and HIV-infected groups (**Figure 4.8 B**).

By contrast, CD8 T cells expressed almost exclusively IFN-γ after stimulation with Ag85B/TB10.4 (**Figure 4.9 B**). PPD- and ESAT-6-specfic CD8 T cells also predomiminantly expressed IFN-γ (data not shown). The PPD- and ESAT-6-specific CD8 T cell response was too low to further reliably characterise polyfunctionality (data not shown).

4.3.4 Co-variants of the Ag85B/TB10.4-specific CD4 and CD8 T cell response

Frequencies of the antigen-specific CD4 and CD8 T cell response were highly correlated. Total cytokine-producing Ag85B/TB10.4-specific CD4 T cell frequencies in all groups of children correlated with cytokine-producing CD8 T cell frequencies (**Figure 4.10 A-C**). In HIV-positive children, the routinely conducted CD4 counts (measured as absolute number/μl or in percentage) did not correlate with viral load (r=0.15, p=0.453; **Figure 4.11**). Viral load and CD4 percentages also did not correlate with the total cytokine-producing Ag85B/TB10.4-specific CD4 or CD8 T cell frequencies (data not shown). We also investigated the relationships between age or time on TB treatment with the frequencies of specific T cells. We observed an inverse correlation between age and the magnitude of the total Ag85B/TB10.4-specific CD4 T

cell response in healthy children (**Figure 4.12 A**). This was not observed in the TB group (r=-0.18, p=0.353) or the HIV-infected children (r=-0.34, p=0.07, data not shown). Frequencies of specific CD8 T cells also did not correlate with age in the 3 groups. Time on TB treatment did not correlate with the magnitude of CD4 or CD8 T cells (**Figure 4.12 B and C**, respectively).

Similar trends were observed in co-variants after stimulation with PPD and ESAT-6 (data not shown).

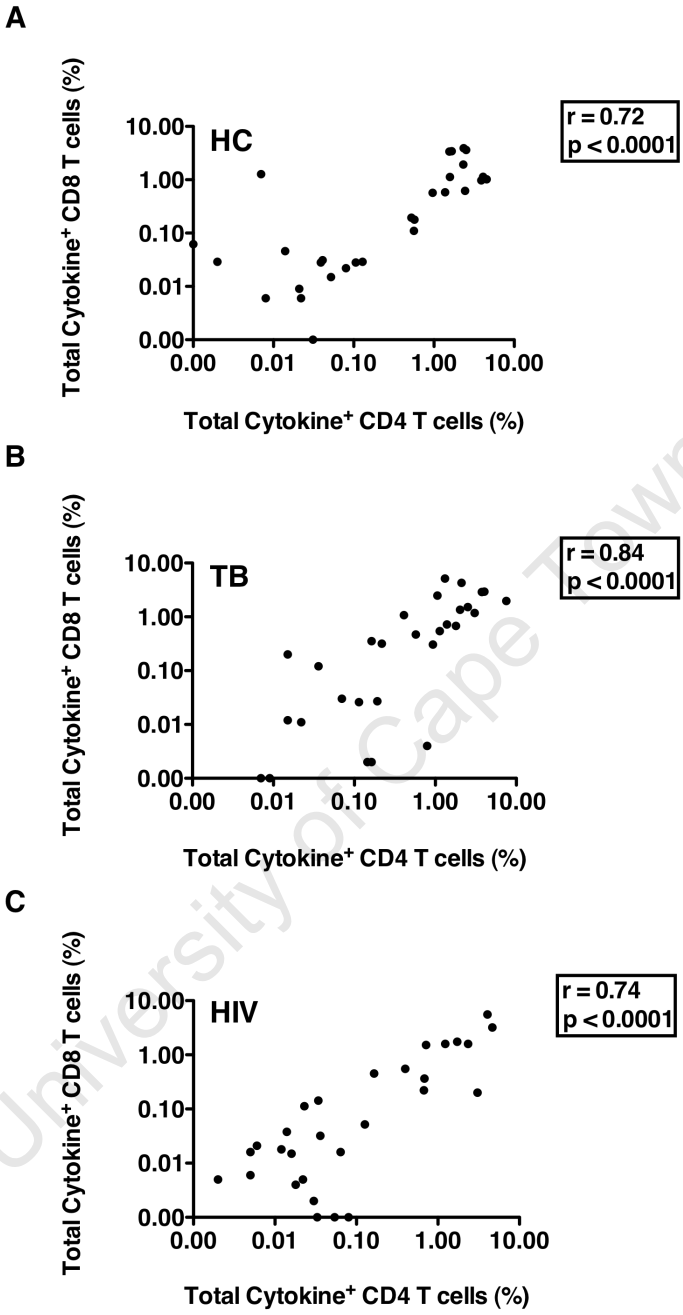

Figure 4.10: Relationship between the total Ag85B/TB10.4-specific CD4 and CD8 T cell response in (A) healthy children, (B) children with TB and (C) HIV-Infected children

Correlations were calculated by nonparametric Spearman test.

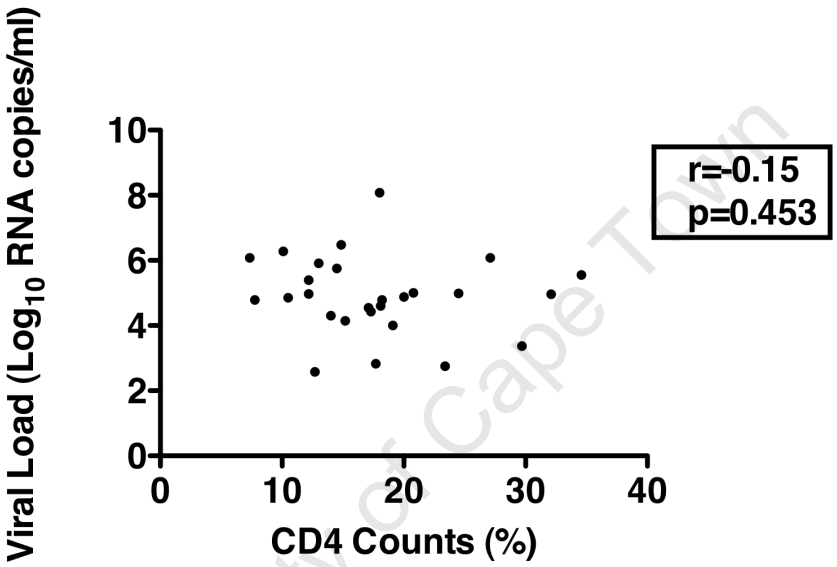

Figure 4.11: Percentage CD4 T cell counts compared to plasma viral load in HIV-infected children at enrollment

Correlations were calculated by nonparametric Spearman test.

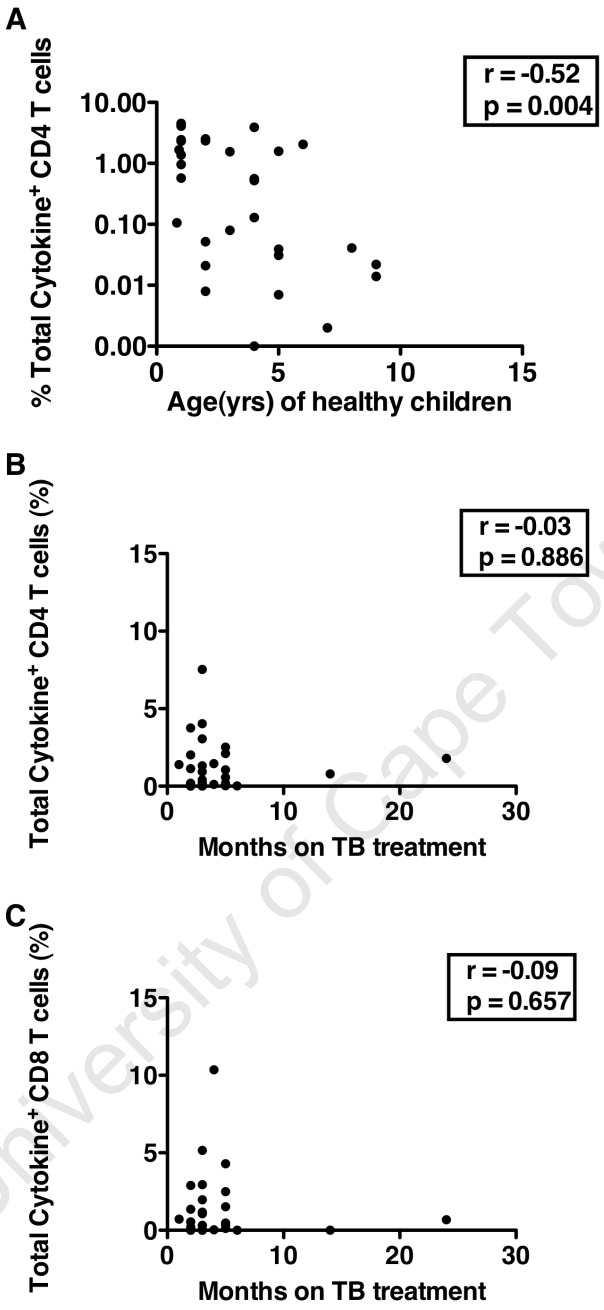

Figure 4.12: Relationship between Ag85B/TB10.4-specific CD4 or CD8 T cell response, age, and time on TB treatment

(A) Frequencies of total cytokine$^+$ CD4 T cells against age in healthy children. (B) Ag85B/TB10.4-specific CD4 T cell responses or (C) specific CD8 T cell responses were compared to months on TB treatment in the TB group. Correlations were calculated by nonparametric Spearman test.

4.3.5 Antigen-specific CD4 and CD8 T cells express an effector memory phenotype

To examine the link between T cell cytokine expression profile and memory phenotype, we analysed the expression of the phenotypic markers, CD45RA, CD27 and CCR7, on mycobacteria-specific T cells. **Figure 4.13** illustrates the memory phenotype marker co-expression pattern by Ag85B/TB10.4-specific IFN-γ^+ or IL-2^+ CD4 T cells, relative to the total CD4 T cell population, following stimulation of whole blood from a healthy child.

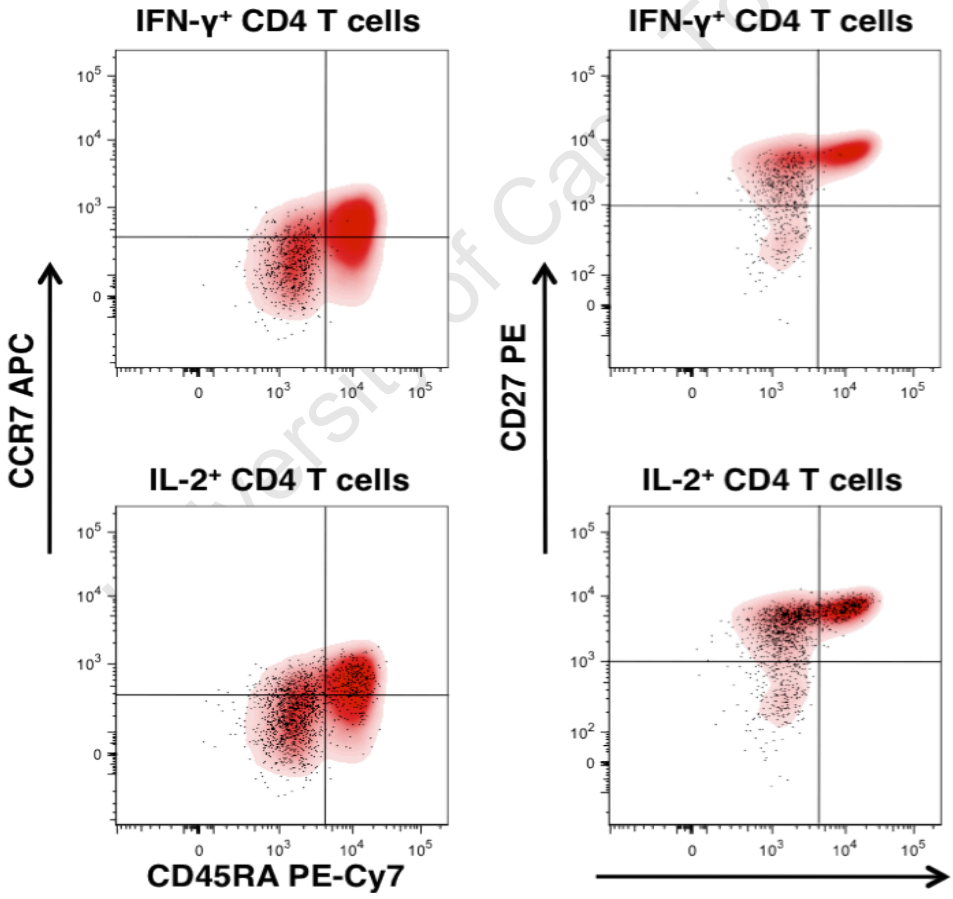

Figure 4.13: Flow cytometric analysis of CCR7, CD45RA and CD27 expression on total CD4 T cells (red background) or Ag85B/TB10.4-specific CD4 T cells, expressing IFN-γ (black dots, upper plots) or IL-2 (black dots, lower plots)

CD4 T cells producing IFN-γ, or IFN-γ and IL-2, were predominantly of a $CD45RA^{-}CCR7^{-}$ effector memory phenotype. Both presence and absence of the differentiation marker, CD27, was observed on these effector memory cells (**Figure 4.14**). A larger proportion of $IFN\text{-}\gamma^{+}IL\text{-}2^{+}$ (p=0.02) and $IFN\text{-}\gamma^{+}$ (p=0.01) CD4 T cells expressed the effector memory phenotype $CD45RA^{-}CCR7^{-}CD27^{-}$, compared with $IL\text{-}2^{+}$ CD4 T cells. IL-2-producing T cells were significantly more likely to be of a $CD45RA^{+}CCR7^{+}CD27^{+}$ central memory phenotype compared with $IFN\text{-}\gamma^{+}IL\text{-}2^{+}$ (p=0.0002) or $IFN\text{-}\gamma^{+}$ (p=0.0046) producing CD4 T cells.

Significantly higher proportions of $IFN\text{-}\gamma^{+}IL\text{-}2^{+}$ antigen-specific CD4 T cells belonged to an effector memory phenotype ($CD45RA^{-}CCR7^{-}CD27^{+/-}$), compared with the non-cytokine- producing total CD4 T cell subset (p<0.0001, **Figure 4.12**). A small subset of Ag85B/TB10.4-specific CD4 T cells also expressed a $CD45RA^{+}CCR7^{-}CD27^{-}$ phenotype, which in CD8 cells specific for chronic viruses has been termed terminally differentiated effector memory cells (Brenchley *et al.*, 2003). Similar trends were observed after stimulation with PPD or ESAT-6 in CD4 T cells (data not shown).

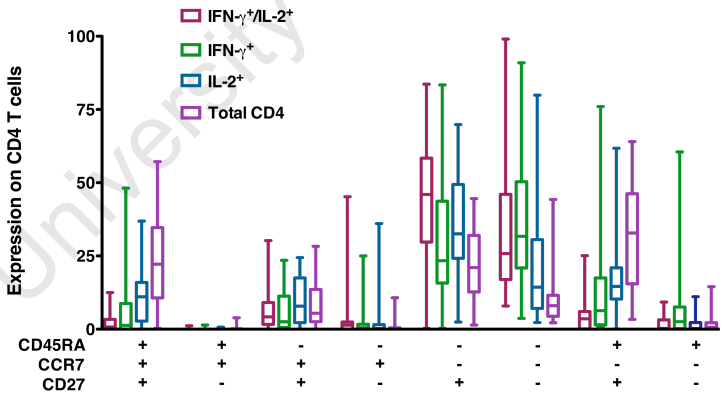

Figure 4.14: Memory phenotype of Ag85B/TB10.4-specific CD4 T cells in healthy children

Relative proportions of memory phenotype marker co-expression by the indicated cytokine-producing specific CD4 T cell populations, or the total CD4 T cell population. For each plot, the median is represented by the horizontal line, the interquartile range by the box and the range by the whiskers. Differences were calculated with the Kruskal–Wallis test, followed by the Mann–Whitney U test (p-values not shown).

In antigen-specific IFN-γ+ CD8 T cells this $CD45RA^{+}CCR7^{-}CD27^{-}$ terminally differentiated phenotype predominated (**Figure 4.15**). The non-cytokine producing, total CD8 T cell population were more likely to express a $CD45RA^{+}CCR7^{+}CD27^{+}$ phenotype compared with IFN-γ^{+} CD8 T cells. These cells comprised a significantly higher proportion of the total CD8 population, compared with antigen-specific IFN-γ+ CD8 T cells ($p<0.0001$, **Figure 4.15**). Specific CD8 T cells also expressed a $CD45RA^{-}CCR7^{-}$ phenotype, characteristic of effector memory T cells. However, more specific CD8 T cells expressed the $CD45RA^{+}CCR7^{-}$ phenotype than non-cytokine producing CD8 T cells (**Figure 4.15**). Approximately half of these IFN-γ- producing effector memory cells expressed CD27. Similar trends were observed after stimulation with PPD or ESAT-6 in CD4 T cells (data not shown).

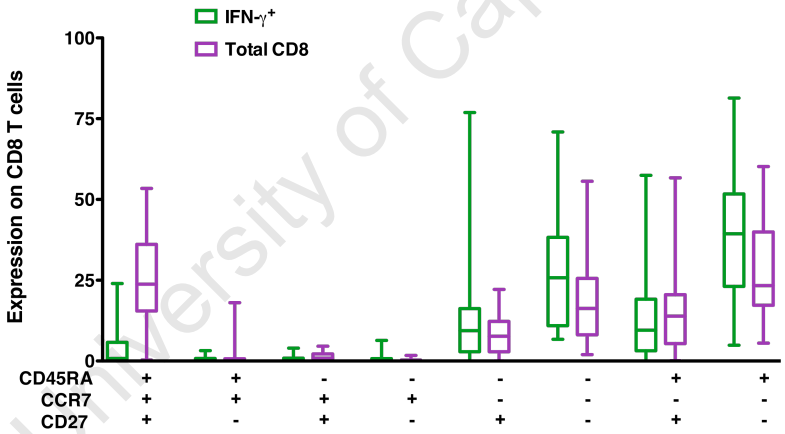

Figure 4.15: Memory phenotype of Ag85B/TB10.4-specific CD8 T cells in healthy children

Relative proportions of memory phenotype marker co-expression by IFN-γ+ CD8 T cells, or the total CD8 T cell population. Differences were calculated with the Kruskal–Wallis test, followed by the Mann–Whitney U test (p-values not shown).

4.3.6 T cell memory phenotypes are comparable in healthy and diseased children

In order to analyse whether HIV infection or TB disease might affect the memory phenotypes of mycobacteria-specific cells, we compared the phenotypes of mycobacteria-specific CD4 and CD8 T cells between all three groups (**Figure 4.16**). No significant differences in memory phenotype were observed amongst Ag85B/TB10.4-specific CD4 cells expressing IFN-γ (Figure 4.16 A) or IL-2 (**Figure 4.16 B**) between the three groups. Similarly, the memory phenotypes of IFN-γ-expressing CD8 T cells were also not different between the three groups (**Figure 4.16 C**). There was also no difference in CD4 and CD8 T cell memory phenotypes between the groups of children after PPD and ESAT-6 stimulation (data not shown).

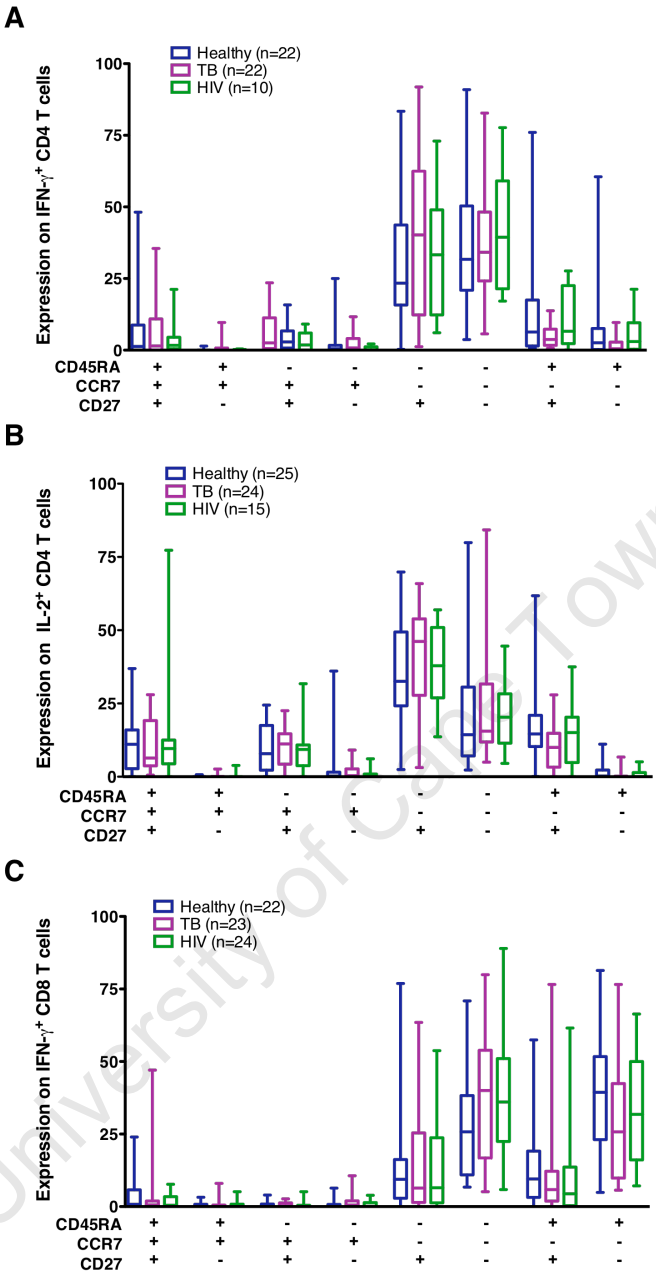

Figure 4.16: Comparison of Ag85B/TB10.4-specific T cell memory phenotype between the three groups of children

Relative proportions of memory phenotype marker co-expression by (A) IFN-γ+ CD4 T cells or (B) IL-2+ CD4 T cells (C) Relative proportions of memory phenotype marker co-expression by IFN-γ+ CD8 T cells. No significant differences were observed (p-values not shown). HC, healthy controls; TB, children with TB; HIV, children with HIV-1 infection. Differences between any two groups were calculated with the Mann-Whitney U test.

4.4. Discussion

Very little is known about the adaptive immune response to *Mycobacterium tuberculosis* in children from a setting of very high TB incidence. We characterised and compared mycobacteria-specific CD4 and CD8 T cell responses in healthy children, children with TB and anti-retroviral naïve, HIV-1-infected children. Five major points emerged: 1. mycobacterial antigens, which are included in novel TB vaccines were found to be highly recognised as recall antigens in our populations; 2. mycobacteria-specific CD4 and CD8 T cells were readily detected at surprisingly high magnitudes in children from all groups; 3. the frequencies of observed specific T cell responses covered a remarkably broad range; 4. no significant differences in magnitude, function or phenotype of specific T cells were observed in HIV-infected children, compared with healthy controls, but evidence of active TB significantly enhances the magnitude of the responses; 5. mycobacteria- specific CD4 and CD8 T cells were of an effector memory phenotype.

We detected mycobacteria-specific CD4 and CD8 T cell responses at high frequencies in children from all 3 groups, even in the HIV-infected group. These responses covered a remarkably broad range, extending from 0% up to 10%. Whether a high magnitude of T cell responses is associated with enhanced protection is not clear. Transfer of ESAT-6-specific memory Th1 cells to recipient mice before *M. tuberculosis* challenge showed that higher numbers of specific T cells confer better protection than lower numbers (Gallegos *et al.*, 2008). However, numerous other animal and human studies have shown that the CD4 T cell IFN-γ response does not necessarily correlate with protection, but may rather reflect bacterial load or degree of inflammation (Forbes *et al.*, 2008; Wolf *et al.*, 2008; Bennekov *et al.*, 2006; Mittrucker *et al.*, 2007; Tchilian *et al.*, 2009; Majlessi *et al.*, 2006; Kagina *et al.*, 2010). This is also consistent with our finding of the highest T cell responses in TB-affected children. Our data show that a substantial proportion of children have mycobacteria-specific T cell populations, expressing IFN-γ and/or IL-2, which may be enhanced by heterologous boosting vaccines. Whether such responses correlate with protection from TB disease remains to be further

validated in the human host.

We chose TB10.4 and Ag85B as mycobacterial recall antigens for the T-cell assays in our study. These antigens have recently been incorporated into a subunit vaccine in the form of a fusion protein, Ag85B-TB10.4, formulated with the adjuvant IC31 (Aagaard *et al.*, 2009). TB10.4 and Ag85B are expressed in a wide range of mycobacterial species and are commonly recognized in *M. tuberculosis*– infected and BCG-vaccinated individuals (Skjot *et al.*, 2000). Our data suggest these antigens may be immunodominant in children, including children with HIV infection, for the first time. We were surprised by the high frequencies of the responses in some children. To validate our findings, we conducted control experiments using peptides of the same antigens, which revealed highly correlated results (data not shown). We therefore concluded that our findings are not prone to experimental artifact, but reflect real antigen-specific T cell magnitudes.

Furthermore, although these antigens have previously been shown to induce high levels of CD8 responses in animal models the high magnitude of CD8 responses also surprised us (Elvang *et al.*, 2009). Our findings are encouraging news for the formulation of vaccines incorporating these antigens, because they appear to be able to induce both CD4 and CD8 responses. Detection of CD8 responses using recombinant proteins as recall antigens was most likely a result of cross-priming, also observed in the context of BCG vaccination of infants (Soares *et al.*, 2008).

Vaccine boosting may have different outcomes in BCG-primed, *M.tuberculosis* uninfected and latently infected children. To date, few data are available on the character of T cell responses in older children, who are more likely to have been exposed to TB. Most studies in children have been performed following BCG vaccination in infants (Soares *et al.*, 2008; Kagina *et al.*, 2009; Lalor *et al.*, 2010). These studies report lower frequencies of specific CD4 and also CD8 T cells in infants to those observed here in older children. This could reflect a less differentiated infant immune system. Naïve T cells constitute a larger proportion of the T cell compartment in infants,

compared with older children (Shearer *et al.*, 2003). Since the readout for intracellular cytokine assays is a frequency of T cells, the larger proportion of naïve cells may serve to lower the proportion of specific memory cells. Additionally, the differences between our results and the infant studies may also reflect the use of different antigens. Further, it is possible that the wide age range in our study may have confounded our results, although children were age-matched between the groups.

The finding that frequencies of mycobacteria-specific T-cell responses in HIV-infected and -uninfected children were not significantly different was unexpected. Lower mycobacteria- specific CD4 T-cell responses have been observed in HIV- infected adults when compared with HIV-uninfected control subjects (Elliott *et al.*, 1999; Sutherland *et al.*, 2006). We recently also showed that BCG-specific T-cell responses were markedly impaired in HIV-infected infants during the first year of life (Mansoor *et al.*, 2009). Further, blood of children with HIV was more permissive to mycobacterial growth in vitro and associated with lower levels of IFN-γ compared with HIV-negative children (Tena *et al.*, 2003). The reason for the similar frequencies between HIV-negative and HIV-positive children observed here is currently unclear. However, given the high rate of mortality in untreated, HIV-infected children in Africa—35% in children in the first year of life and 52.5% up to 2 years of age (Newell *et al.*, 2004)—our inclusion of HIV-infected children, with a mean age of 3.7 years, may have introduced a selection bias for children with slower HIV progression and better-preserved immune function. The wide range of responses observed in our study may also have led to less statistical power for detecting differences between the groups. Regardless, our data suggest that heterologous vaccine boosting may enhance T-cell responses in at least a proportion of HIV-infected children. Development of vaccines that augment T-cell immunity in this vulnerable group is particularly important.

We found that children with TB had significantly higher mycobacteria-specific CD4 and CD8 T-cell responses compared with HIV-infected children but not compared with the healthy control subjects. This is likely to reflect disease-associated inflammation and/or high levels of antigen exposure. This

contrasts with work showing that T-cell responses in patients with active pulmonary TB express lower levels of Th1 cytokines, such as IFN-γ and IL-2 (Hirsch *et al.*, 1999). However, these previously published studies did not examine cytokine production at the single-cell level.

The wide age range of participants in our study groups presents a study limitation. Because it is known that different ages are associated with differences in T-cell maturation and differentiation (Shearer *et al.*, 2003), this is likely to be an important contributor to the large range of mycobacteria-specific T-cell frequencies we observed. Ideally, a larger sample size would have allowed stratification of children by age, but this was beyond the scope of this study. We observed an inverse correlation between age and the magnitude of the CD4 T-cell response in healthy children. Multiple factors may affect the magnitude of response, including time since BCG vaccination (Weir *et al.*, 2008). Additional studies are required to understand this association.

Another caveat in our study was the variation in time on treatment and time since diagnosis of children in the TB group; however, there was no correlation with frequency of cytokine-producing T cells. Based on studies of culture conversion under treatment and longitudinal observations of immune responses using IFN-γ release assays (Herrmann *et al.*, 2009), we reasoned that a significant decline in antigenic load occurs during the early phase of TB treatment with more inherent variability than at later stages and that the longevity of effector responses is yet to be better defined. We therefore decided to enroll into the TB group those children who were already well established on their course of treatment. Another limitation was the unknown *M. tuberculosis* infection status in the HIV-infected and healthy control groups. Latent infection may have a marked effect on the magnitude and phenotype of T cell responses and analysis of infection status, as a covariate could have been additionally informative.

The aim of vaccination is the induction of immunological memory. It is

therefore essential to better understand memory profiles in children with and without co-morbidity who might receive such vaccines. We chose to extensively investigate the memory profiles of antigen-specific T cells, because such data in older children are lacking. Central memory T cells, along with IL-2 expression, are believed to be the optimal phenotype for long-lived protective immunity after infection or vaccination (Sallusto *et al.*, 1999). We report here that the Ag85B/TB10.4-specific T cell response was characterized by an effector memory phenotype. This is in agreement with memory phenotypes reported previously on mycobacteria-specific T cells in adults (Scriba *et al.*, 2008), children (Mueller *et al.*, 2008) and infants (Soares *et al.*, 2008; Kagina *et al.*, 2009).

Both presence and absence of the differentiation marker, CD27, was observed on these effector memory CD4 and CD8 T cells, suggesting an intermediate to an advanced stage of differentiation. This consistently observed effector memory phenotype may suggest high levels of exposure to mycobacterial antigens, as seen in chronic viral infections (Harari *et al.*, 2005), either through exposure to environmental mycobacteria, *M. tuberculosis* or, less likely, persistent antigen after BCG vaccination. Alternatively, mycobacteria-specific T cells may exhibit alternative differentiation or maturation pathways to those defined in chronic viral models (Harari *et al.*, 2005) Along these lines, a considerable proportion of mycobacteria-specific T cells express Th1 cytokines, but show a “naïve” CD45RA+CCR7+ phenotype. Such T cell phenotypes have previously been reported in other studies of anti-mycobacterial T cells by us and others (Soares *et al.*, 2008; Kagina *et al.*, 2009; Scriba *et al.*, 2008; Caccamo *et al.*, 2006). We previously suggested that this CD45RA+CCR7+ population reflects early differentiation into Ag-specific cells, before loosing CD45RA expression (Soares *et al.*, 2008). However, this phenotype is not observed in human viral infections (Harari *et al.*, 2005). Additional studies are required to investigate this further.

In summary, we report substantial frequencies of mycobacteria-specific effector CD4 and CD8 T cell responses, which may be augmented by boost

vaccines, even in HIV- infected children. These findings are encouraging in the context of ongoing trials of novel TB vaccines.

CHAPTER 5

Reconstitution of T cell immunity In HIV-Infected children receiving ART

5.1 Introduction

5.2 Methods

5.3 Results

5.3.1 HIV cohort follow-up and clinical outcome

5.3.2. Mycobacteria-specific CD4 and CD8 T cell responses during ART

5.3.3 Polyfunctional CD4 T cell response during ART

5.3.4 Memory T cell phenotype during ART

5.4.Discussion

5.1 Introduction

HIV infection is associated with increased risk of TB disease and death (Wood *et al.*, 2007; Corbett *et al.*, 2003; WHO, 2010a). Progressive loss of function and numbers of CD4 T cells are observed in infected individuals (Hazenberg *et al.*, 2000). In the absence of treatment, this eventually leads to the development of the Acquired Immunodeficiency Syndrome (AIDS), increasing the risk for developing opportunistic infections.

Antiretroviral therapy (ART) results in strong and sustained inhibition of viral replication and is associated with a rise in CD4 T cell numbers (Collier *et al.*, 1996; Autran *et al.*, 1997). This results in partial immune restoration of the immune deficiency (Autran *et al.*, 1997; Connick *et al.*, 2000; Li *et al.*, 1998). For the most part this improvement in the immune system protects people from opportunistic infections (Kaplan *et al.*, 2000). However, it is less clear whether functional immune responses can be fully restored, particularly in persons with advanced stages of HIV infection (Lange *et al.*, 2002; Lange *et al.*, 2003; Lederman *et al.*, 2003; Lawn *et al.*, 2005). In adults receiving ART, reconstitution of antigen-specific cellular immune responses has been demonstrated to opportunistic pathogens such as cytomegalovirus (CMV), *M. avium* complex (MAC), *Toxoplasma gondii* and *Candida albicans* (Li *et al.*, 1998; Komanduri *et al.*, 1998; Rinaldo *et al.*, 1999; Connick *et al.*, 2000; Havlir *et al.*, 2000; Fournier *et al.*, 2001). In contrast, Weinberg *et al.* observed inconsistent recovery of cytomegalovirus CMV-specific CMI in HIV-infected patients receiving ART (Weinberg *et al.*, 2001). Recurrences in CMV disease have been observed in HIV–Infected patients despite potent ART and apparent immune reconstitution (Johnson *et al.*, 2001).

Clinical susceptibility to TB is significantly reduced in HIV-infected individuals on ART (Ledergerber *et al.*, 1999; Girardi *et al.*, 2000; Kirk *et al.*, 2000; Jones *et al.*, 2000; Santoro-Lopes *et al.*, 2002; Badri *et al.*, 2002). Benefit of ART is significant across all WHO stages and CD4 cell counts. However, the overall susceptibility to develop TB in ART treated individuals does not return to that of HIV-negative individuals (Lawn *et al.*, 2005; Komati *et al.*, 2010). Thus

partial immune restoration of the immune deficiency is observed in HIV infection (Autran *et al.*, 1997; Connick *et al.*, 2000). Timing of ART initiation is suggested as a deciding factor of the capability of the immune system to reconstitute.

CD4 T cells are essential in the immune response to *M. tuberculosis* (Mogues *et al.*, 2001; Saunders *et al.*, 2002; Havlir and Barnes, 1999). HIV may disrupt the TB-specific responses by directly or indirectly causing the death of the CD4 T cells that most effectively respond to the mycobacterium. CD8 T cells are also important in the *M. tuberculosis*-specific response (Behar *et al.*, 1999; Derrick *et al.*, 2004; Billeskov *et al.*, 2007; Bruns *et al.*, 2009; Chen *et al.*, 2009). Early initiation of ART during acute infection might reduce the damage caused by HIV, allowing better development of early CD4 and CD8 T cell *M. tuberculosis*-specific responses.

It remains to be determined whether the effects of ART may influence broader functional immune reconstitution in children as opposed to adults. No studies have been conducted in children to analyse to what degree mycobacteria-specific T cell memory responses form part of this reconstitution. The objective of this part of the study was to characterise how mycobacteria-specific CD4 and CD8 T cell responses change in HIV-infected children once ART is commenced. To address this we performed a longitudinal follow-up of HIV-infected children prior to and during the first year of ART, and assessed changes to anti-mycobacterial specific T cell responses.

5.2 Methods

The cohort of HIV-infected children (described in chapter 4) was followed up at 3-monthly intervals for a total of one year, as described in chapter 2. Venous blood was collected at baseline (before commencing ART). Whole blood stimulations with mycobacterial antigens for a total of 7 hours were carried out as described in chapter 2. Cytokine expression profiles and memory phenotype of mycobacteria-specific CD4 and CD8 T cells were characterised by multi-parameter flow cytometry as described in chapter 3.

5.3 Results

5.3.1 HIV cohort follow-up and clinical outcome

A total of 30 HIV-infected children were enrolled into the study and followed-up at their regular visits to the HIV clinic at Red Cross Children's Hospital in Cape Town. None of the children had reported exposure to an adult with active TB in their household at baseline, or during the first 12 months of ART. All children who completed follow-up were compliant with their ART during their first 12 months of therapy. None of the children developed signs or symptoms of TB during the course of the study.

At 3 months, the cohort on ART comprised 28 children: 2 children were lost to follow-up, as one defaulted ART and the other relocated outside Cape Town. By 6 months, three further children were lost to follow-up; two children had relocated outside Cape Town and one child had died (of sepsis), a day prior to the 6 month visit. Twenty-five out of 30 children thus completed the 12 months study.

The median CD4 cell percentage at baseline was 17.85% (IQR: 12.93-21.45%) and the median absolute CD4 cell count was 531 cells/µl (IQR: 284-1249). The median $\log_{10}$ viral load RNA copies/ml at baseline was 4.88 (IQR range: 4.30-5.76). There were no significant differences in baseline CD4 cell counts between the children who completed 12 months follow-up compared with children who dropped out for the above reasons (median CD4 cell percentage: 18.10% (IQR: 13.35-23.95%) and 14.85% (IQR: 11.55-18.00%), respectively, p=0.221). The baseline $\log_{10}$ viral load RNA copies/ml were, however, significantly greater in the children who did not complete the study compared with the group that completed the study (median $\log_{10}$ viral load RNA copies/ml: 6.38 (IQR: 6.01-7.68 $\log_{10}$ RNA copies/ml) and 4.79 (IQR: 4.15-5.00 $\log_{10}$ RNA copies/ml), respectively, p=0.003).

By 3 months the CD4 cell percentage had already increased significantly (p=0.001) above baseline levels and continued to rise in the subsequent 6

months ($p<0.0001$, **Figure 5.1 A**). The median CD4 cell percentage remained significantly higher at 12 months of ART compared with percentages prior to ART. The median $\log_{10}$ viral load RNA copies /ml decreased significantly to below the detectable limit by 3months ($p<0.0001$, **Figure 5.1 B**) and the subsequent months up to 12 months. The overall decrease in viral load RNA copies /ml was significant with a p value of less than 0.0001. Twenty-two of the 25 children had fully suppressed viral loads by 12 months of therapy (**Figure 5.1 B**).

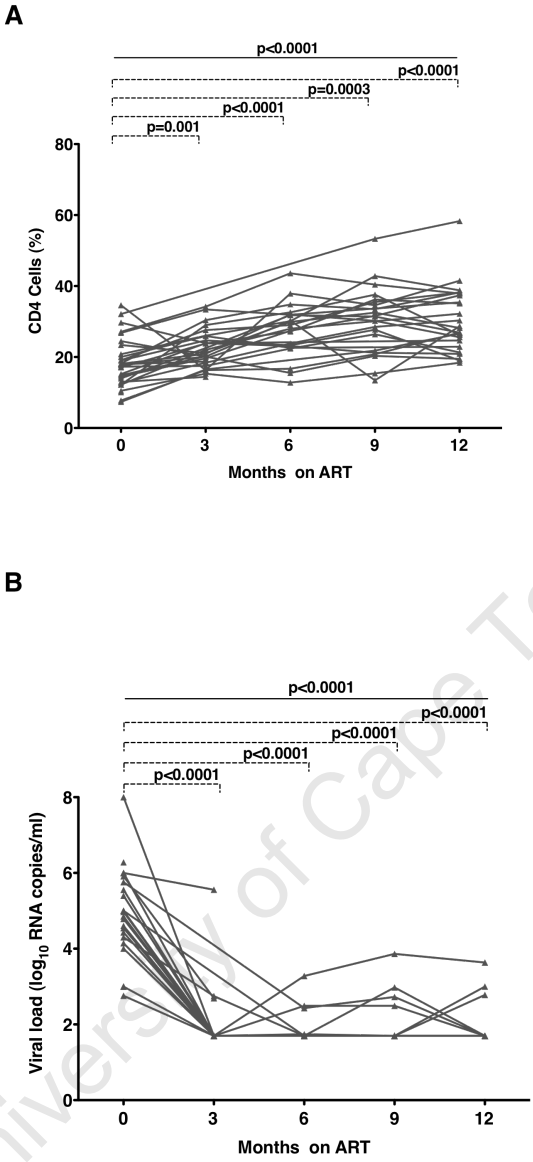

Figure 5.1: Changes in CD4 T cell percentages and viral load in the first 12 months of ART

(A) The CD4 T cell percentage and (B) log RNA copies/ml viral load were measured in HIV-infected children aged 0.33-12 years, at the time of enrolment into the study (T0), and at 3 monthly intervals in the first 12 months of ART. The changes over time are illustrated. A significant increase in percentage CD4 T cells was observed after the initiation of ART (p=0.001) and sustained throughout the study period. Data of all 30 patients are shown. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line).

5.3.2. Mycobacteria-specific CD4 and CD8 T cell responses during ART

Cytokine expression profiles of mycobacteria-specific T cells were measured as described in chapter 3. The frequency of mycobacteria-specific IFN-γ and/or IL-2 producing CD4 and CD8 T cells was assessed before and during 12 months of ART. SEB was used as a positive control at all time-points. Four participants, two at 6 months and the other two at 12 months, had SEB responses < 3 median absolute deviations above median background (unstimulated). Flow cytometry data obtained for these participants at these time-points were excluded from analysis for all antigens. The median background of unstimulated cells was above the detection limit of the SATVI flow cytometer (0.01%). For this reason backgrounds were subtracted for each individual at all time-points. Reported data have been corrected for background except for data on memory phenotypes. The frequency of cells in the unstimulated samples remained low at all time-points, thus allowing sensitive detection of low expressed cytokines. The median background for cytokine$^+$ CD4 T cells was 0.02% (IQR, 0.01-0.06) before ART and 0.01% (IQR, 0.003-0.02) at 12 months of ART (data not shown). The median background for cytokine$^+$ CD8 T cells was 0.02% (IQR, 0.01-0.03) before ART and 0.01% (IQR, 0.002-0.02) at 12 months of ART (data not shown). There were no differences in response to the positive control at all time-points for both CD4 and CD8 T cells (**Figure 5.2**, data shown for CD4 T cells only).

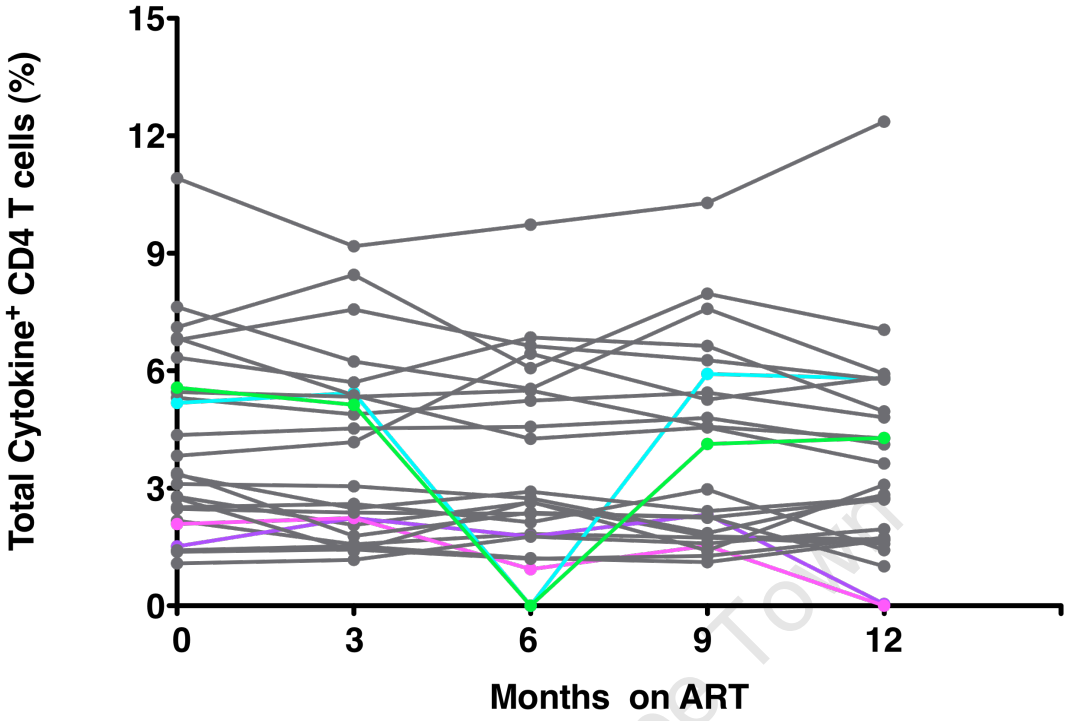

Figure 5.2: Cytokine producing CD4 T cells stimulated with SEB

Whole blood from HIV-infected children was stimulated with SEB and frequencies of cytokine$^+$ CD4 T cells were determined by flow cytometry at 0, 3, 6, 9 and 12 months of ART. Overall there were differences in frequencies of cytokine$^+$ CD4 T cells over the first year of therapy. Four individuals (coloured lines) were excluded from the analysis, since they had no response to SEB: two at 6 months and the other two at 12 months.

The overall frequency of total cytokine-expressing (IFN-γ$^+$ and/or IL-2$^+$) Ag85B/TB10.4-specific CD4 T cells decreased during ART (p=0.016, **Figure 5.3 A**). The median frequency of Ag85B/TB10.4-specific total cytokine producing CD4 T cells was low before of ART and decreased to below the sensitivity level of the assay at 12 months of ART (p=0.017, **Figure 5.3 A**). Ag85B/TB10.4-specific total IFN-γ$^+$ CD4 T cells also decreased during 12 months of ART (p=0.028, **Figure 5.3 B**). The frequencies of Ag85B/TB10.4-specific total IL-2$^+$ CD4 T cells did not change throughout the 12 months (**Figure 5.3 C**).

PPD-specific total cytokine producing, total IFN-γ^+ or total IL-2^+ CD4 T cells showed no change in the first 12 months of ART (Figure 5.4 A, data only shown for total cytokine$^+$ cells). The median frequencies of ESAT-6-specific total cytokine producing CD4 T cells were very low throughout the first 12 months of ART (**Figure 5.4 B**). Total IFN-γ^+ and total IL-2^+ ESAT-6-specific CD4 T cells were also low and did not change 12 months into ART (data not shown).

The median frequencies of Ag85B/TB10.4-specific total cytokine producing or total IFN-γ^+ CD8 T cells were low at the start of ART and declined during 12 months of ART (p=0,003 and p=0.001, respectively; **Figure 5.5 A and B**). Ag85B/TB10.4-specific total IL-2^+ CD8 T cells were very low throughout 12 months of ART and the validity of the significance difference in frequencies is questionable (p=0.009, **Figure 5.5 C**). The median frequencies of PPD-specific total cytokine producing CD8 T cells were low before ART and did not change by 12 months of ART (**Figure 5.6 A**). ESAT-6-specific total cytokine producing CD8 T cells also remained very low throughout the first 12 months of ART (**Figure 5.6 B**). Similar trends as with PPD or ESAT-6-specifc total cytokine$^+$ CD8 were observed for both total IFN-γ^+ and IL-2^+ CD8 T cells after stimulation with PPD or ESAT-6 (data not shown).

Overall, some children showed a dramatic decrease in frequencies of mycobacteria-specific responses over the first three months of ART. These children all had responses to the positive control and were all included in the analysis. The majority of the children showed a steady decline in the frequency of mycobacteria-specific responses during ART (**Figure 5.7 A and B**). Similar patterns of individual changes in cytokine responses were obtained for both PPD and ESAT-6-specific CD4 and CD8 T cells.

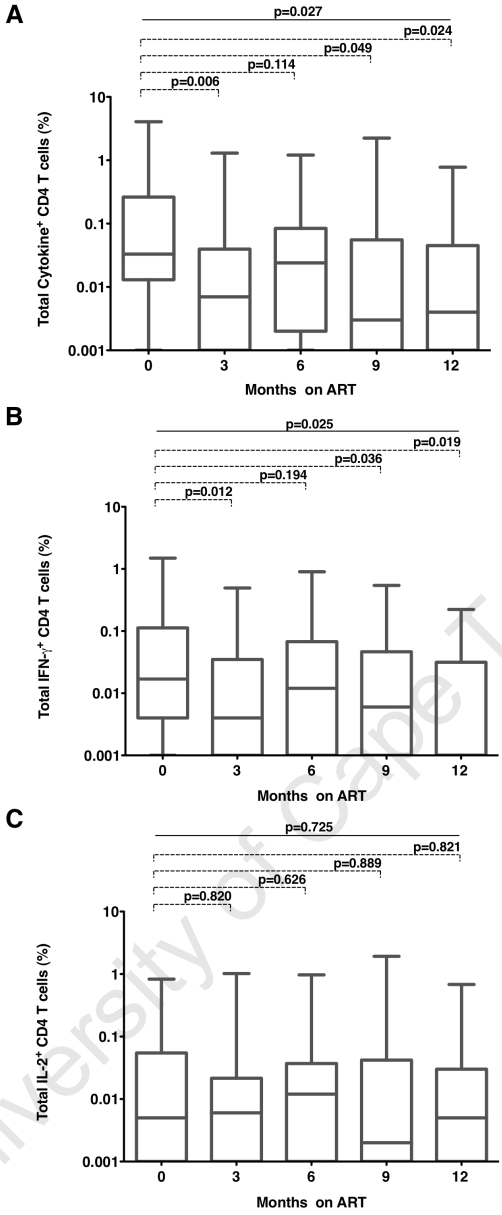

Figure 5.3: Ag85B/TB10.4-specific cytokine producing CD4 T cells during the first year of ART

(A) Total cytokine producing (B) total IFN-γ and (C) total IL-2 CD4 T cells, detected using an intracellular cytokine staining assay, following stimulation of whole blood with Ag85B/TB10.4. Box and whisker plots with horizontal lines indicating the median %, the whiskers the minimum and maximum values, and the boxes the inter-quartile range. Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

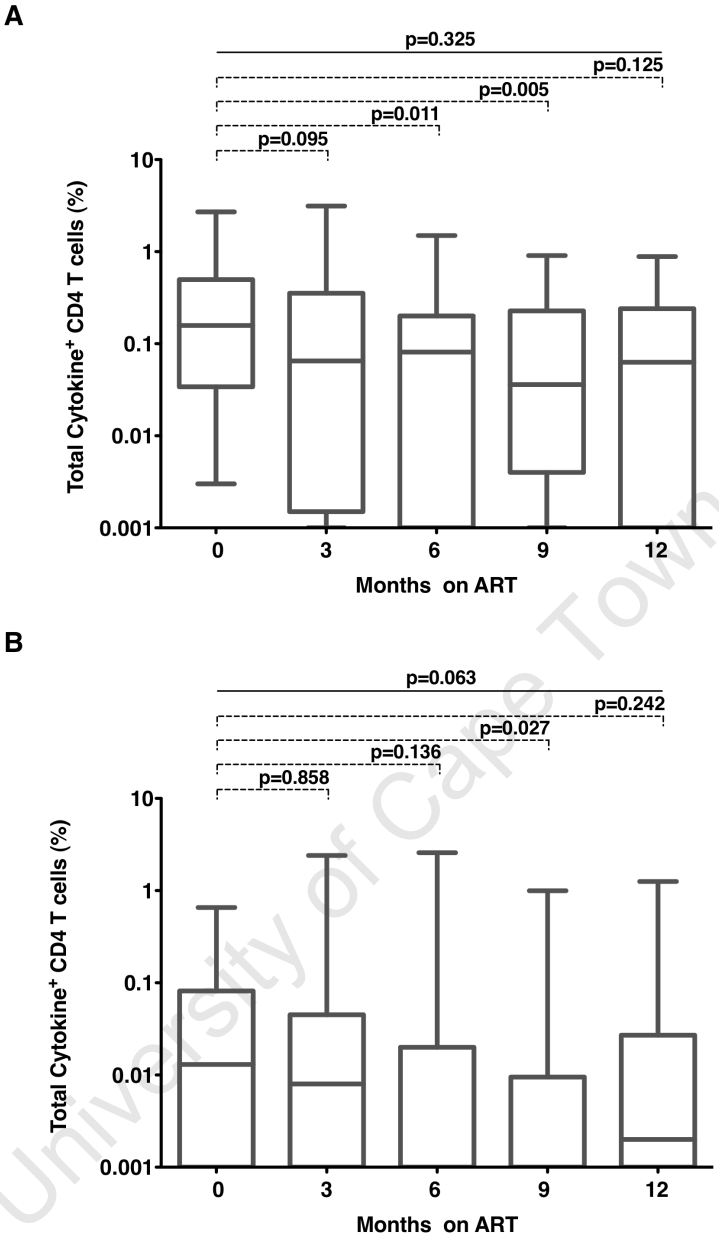

Figure 5.4: PPD and ESAT-6-specific CD4 T cells over the first year of ART

Frequencies of total cytokine producing CD4 T cells were assessed after stimulation of whole blood with either (A) PPD or (B) ESAT-6. Box and whisker plots with horizontal lines indicating the median %, the whiskers the minimum and maximum values, and the boxes the inter-quartile range. Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

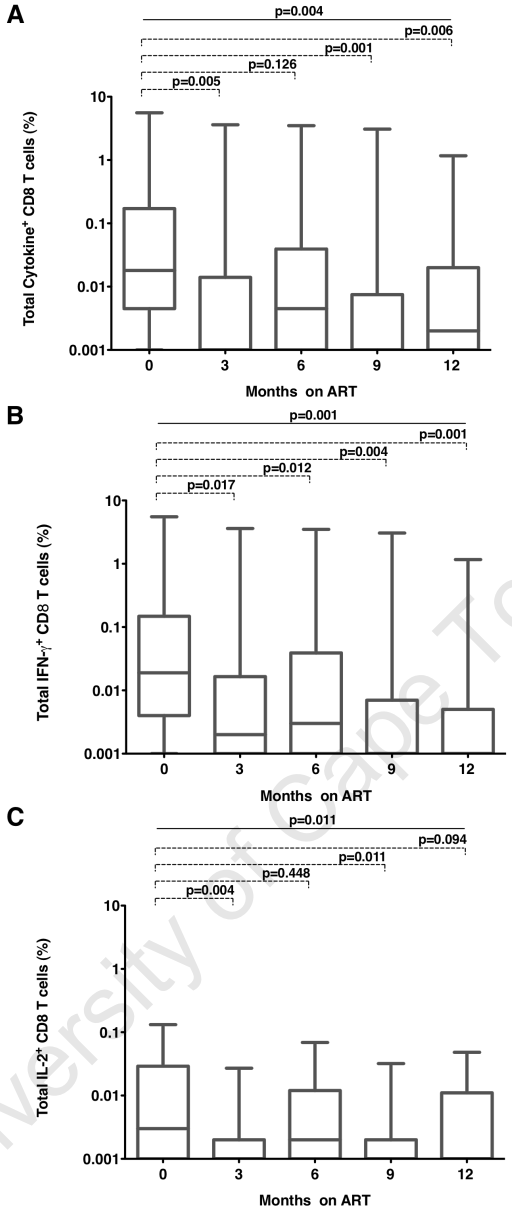

Figure 5.5: Ag85B/TB10.4-specific cytokine producing CD8 T cells during the first year of ART

(A) Total cytokine producing (B) total IFN-γ and (C) total IL-2 CD8 T cells, detected using an intracellular cytokine staining assay, following stimulation of whole blood with Ag85B/TB10.4. Box and whisker plots with horizontal lines indicating the median %, the whiskers the minimum and maximum values, and the boxes the inter-quartile range. Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

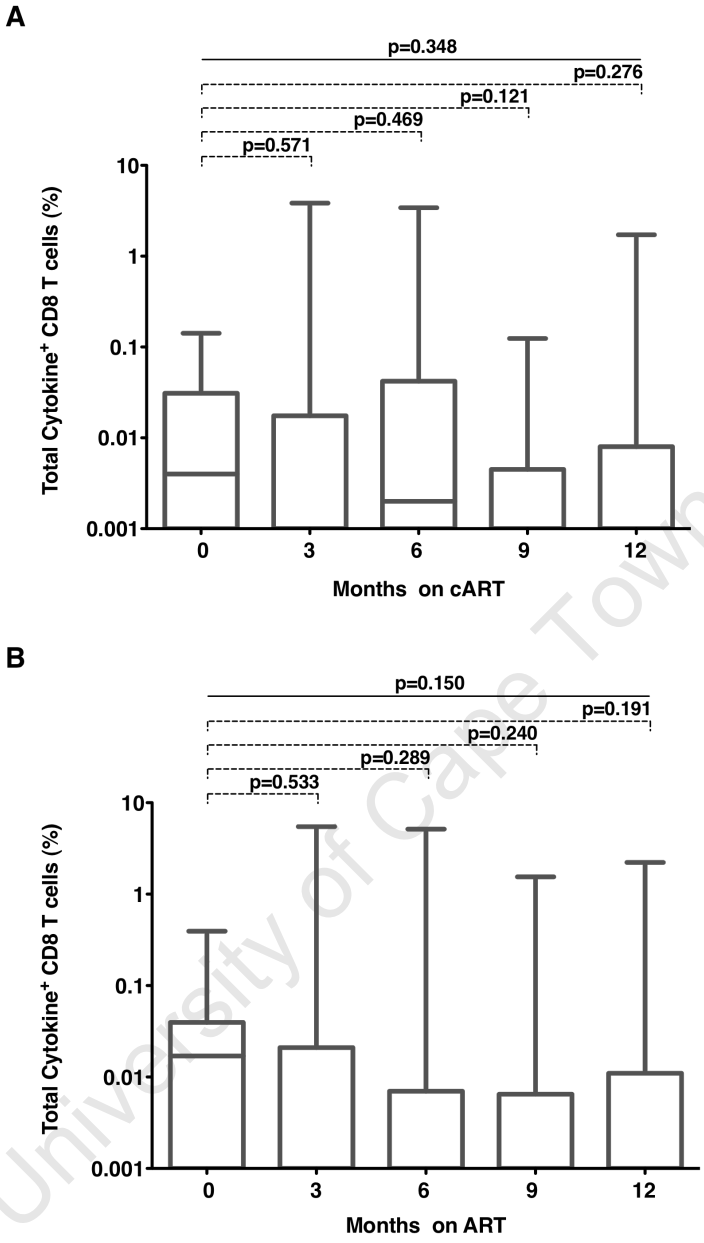

Figure 5.6: PPD and ESAT-6-specific CD8 T cells over the first year of ART

Frequencies of total cytokine producing CD8 T cells were assessed after stimulation of whole blood with either (A) PPD or (B) ESAT-6. Box and whisker plots with horizontal lines indicating the median %, the whiskers the minimum and maximum values, and the boxes the inter-quartile range. Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

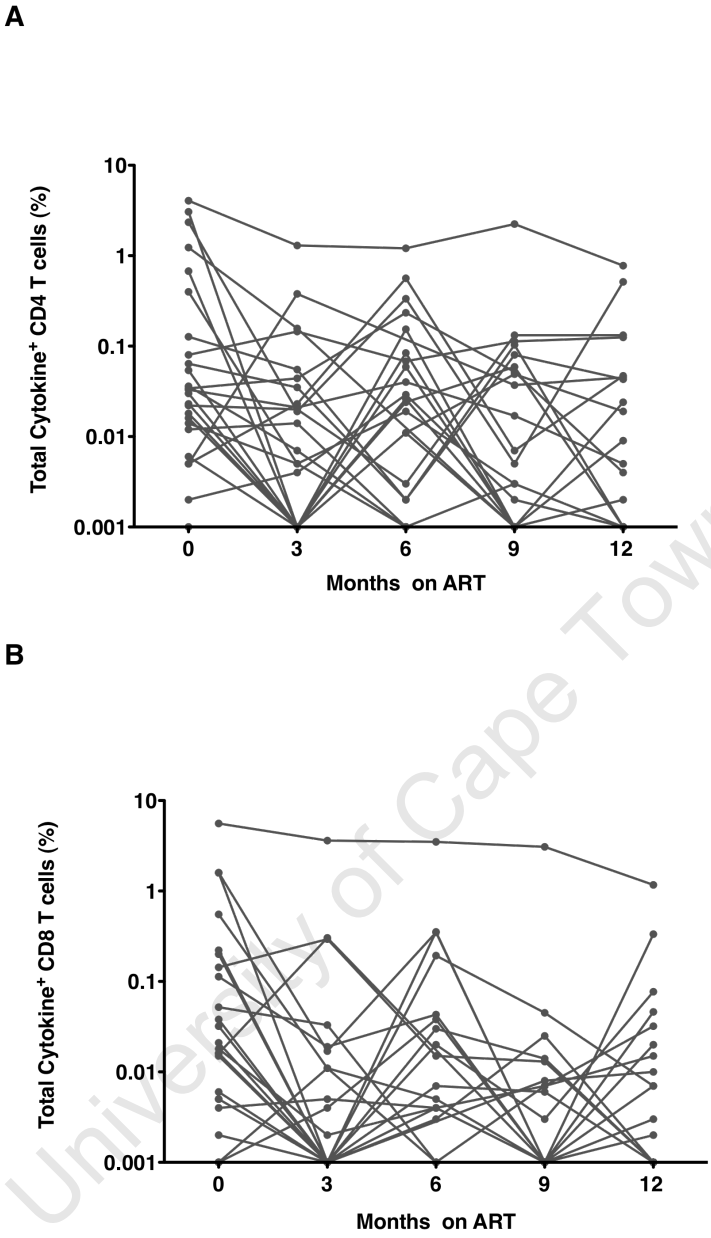

Figure 5.7: Representative graphs showing individual changes in CD4 and CD8 T cell cytokine frequencies during the first year of ART

Frequencies of Ag85B/TB10.4 total cytokine producing (A) CD4 T and (B) CD8 T cells at 0, 3, 6, 9 and 12 months of ART. Dots indicate individual frequencies for each time-point. All values of zero were adjusted to 0.001 for plotting on the logarithmic scale.

5.3.3 Polyfunctional CD4 T cell response during ART

Cells that co-express cytokines have been suggested to provide better protection against intracellular infections than cells that express only a single cytokine (Darrah *et al.*, 2007). We investigated the cytokine co-expression patterns in the children while on ART. The frequency of Ag85B/TB10.4-specific polyfunctional IFN-γ^+IL-2^+ CD4 T cells were low and did not change in the first 12 months of ART (**Figure 5.8 A**). CD4 T cells stimulated with PPD expressed high frequencies of IFN-γ^+IL-2^+ (Figure 5.8 B). However, these did not change during 12 months of ART. Frequencies of ESAT-6-specific IFN-γ^+IL-2^+ CD4 T cells were very low and no difference was observed during ART (**Figure 5.8 C**).

Frequencies of specific polyfunctional IFN-γ^+IL-2^+ CD8 T cells were too low to characterise further for all antigens (data not shown).

We next assessed the proportions of CD4 T cells expressing a polyfunctional profile at 3, 6, 9 and 12 months of ART using pie charts (**Figure 5.9**). The Ag85B/TB10.4-specific CD4 T cell response was dominated by a subset expressing only IL-2 before and during ART (Figure 5.9). The Ag85B/TB10.4-specific IL-2 producing subset appeared to decrease from approximately 60% at baseline to 50% at 3 months of ART, but returned to approximately 60-70% at 6 months and persisted at this level up to 12 months of ART. The IFN-γ and IL-2 co-expressing cells dominated the PPD-specific CD4 T cell response (**Figure 5.9**), before ART and persisted over 12 months of therapy. In ESAT-6-specific cells the IL-2-only producing cells remained at approximately 60-70% during 12 months of ART. Proportions of IFN-γ-only producing cells decreased from about 20% before ART to 10-15% in 12 months of therapy, after Ag85B/TB10.4 or ESAT-6 stimulation.

Proportions of CD8 T cells were too low for reliable longitudinal analysis of polyfunctionality for all antigens. IFN-γ continued to be the predominant cytokine expressed by specific CD8 T cells for all antigens (data not shown).

Median fluorescence intensity (MFI) of a functional parameter indicates the intensity of staining for the particular intracellular cytokine. This may reflect the amount of cytokine expressed by cells (Darrah *et al.*, 2007; Duvall *et al.*, 2008). Polyfunctional cells appear to produce considerably more cytokine from each cell (**Figure 5.10 A-F**). The MFI of Ag85B/TB10.4-specific IFN-γ^+ CD4 T cells was significantly higher in the dual positive cells compared with the single positive IFN-γ^+ CD4 T cells (**Figure 5.10 A**) before ART. This was also true for IL-2^+ CD4 T cells and IFN-γ^+ CD8 T cells (**Figure 5.10 B and C**, respectively). IL-2^+ CD8 T cells showed no difference in staining intensity whether dual positive or single positive (**Figure 5.10 D**).

This trend of higher MFIs of IFN-γ^+ and IL-2^+ CD4 T cells or IFN-γ^+ CD8 T cells was also observed for PPD or ESAT-6 stimulated cells (data not shown). Again there was no association between polyfunctionality and MFI for IL-2^+ CD8 T cells for both, PPD or ESAT-6 stimulations (data not shown).

To further characterize the quality of the polyfunctional response we calculated the MFI of the polyfunctional cells during ART. No major changes in MFIs were observed during ART for all antigens for CD4 T cells (**Figure 5.11 A-F**).

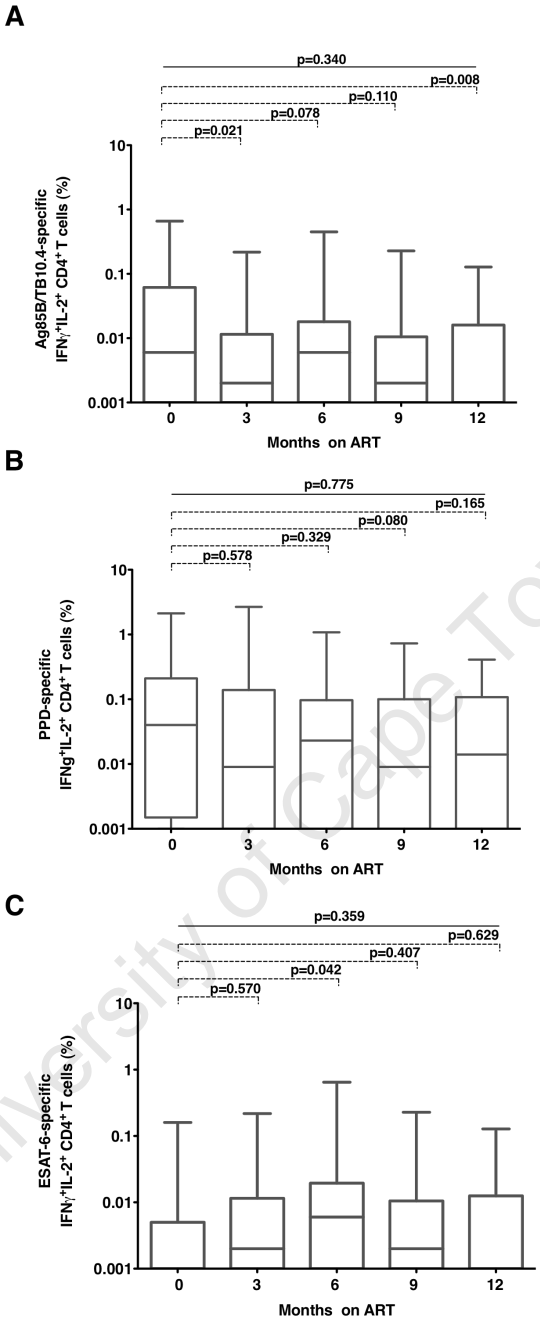

Figure 5.8: IFN-γ^{+}IL-2^{+} CD4 T cell responses during ART

Whole blood from HIV-infected children receiving ART was stimulated with (A) Ag85B/TB10.4, (B) PPD and (C) ESAT-6. After staining for intracellular cytokines CD4 T cells were assessed for differences in frequencies of IFN-γ^{+}IL-2^{+} cells at 0, 3, 6, 9 and 12 months. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

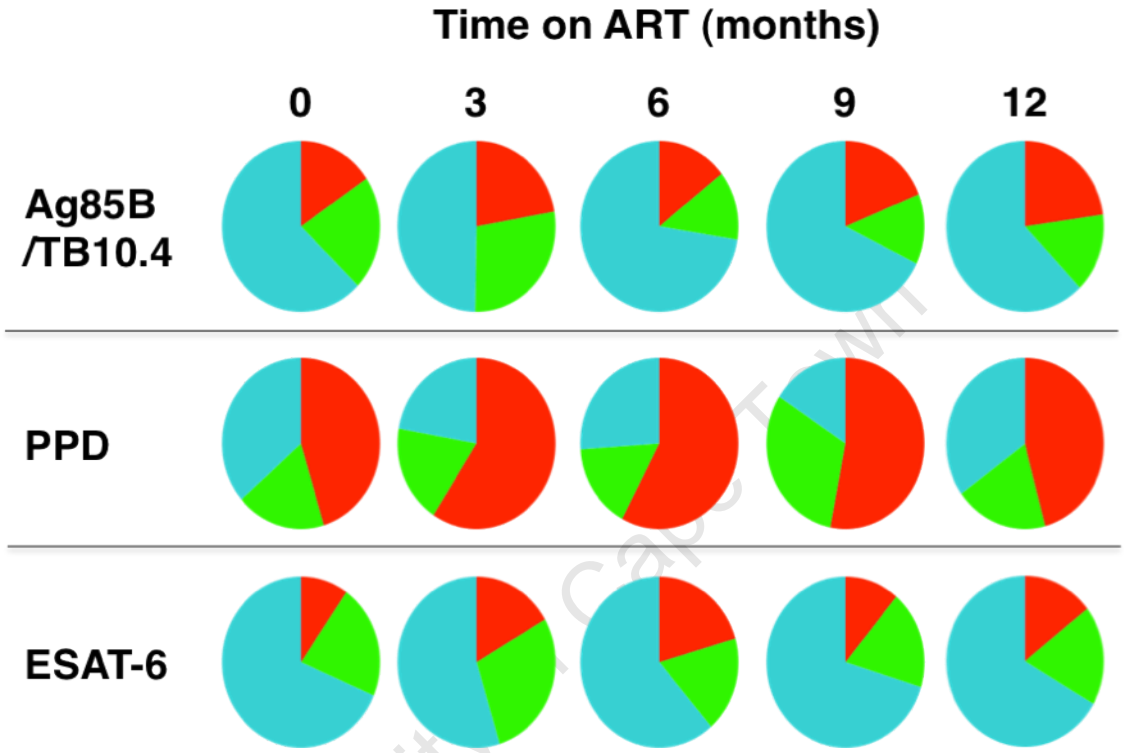

Figure 5.9: Analysis of the quality of mycobacteria-specific CD4 T cells in HIV-1 infected children

Pie charts represent the mean proportions of cells producing IFN-γ^{+}IL-2^{+} cells (red), IFN-γ^{+} only (green) and IL-2^{+} only (blue) of the total cytokine CD4 T cell response at baseline (T0), 3, 6, 9 and 12 months of ART. Whole blood was stimulated with Ag85B/TB10.4, PPD or ESAT-6.

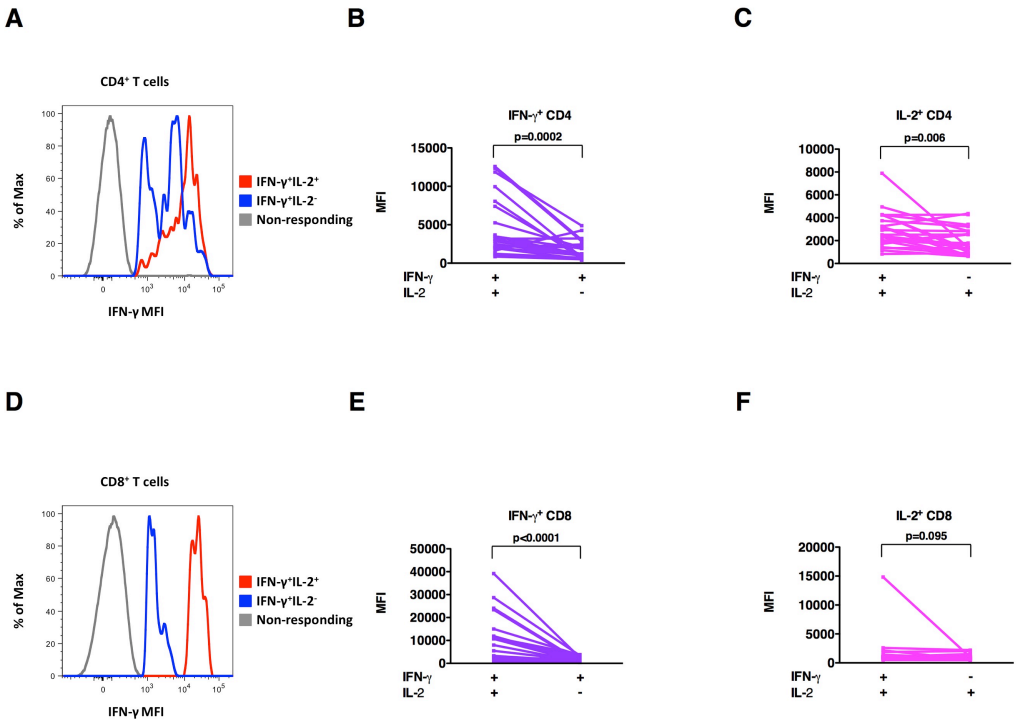

Figure 5.10: Median fluorescence intensity (MFI) for intracellular cytokines of Ag85B/TB10.4-specific CD4 and CD8 T cells

MFI for (A) IFN-γ was calculated for Ag85B/TB10.4-specific CD4+ T cells. IFN-γ MFI for each functional population is shown as a histogram and color-coded according to the number of positive functions. Representative data from a single HIV-infected child are shown. MFIs are shown for CD4 T cells producing (B) IFN-γ (purple) or (C) IL-2 (pink). IFN-γ MFI for Ag85B/TB10.4-specific CD8+ T cell responses is shown as a histogram in a single HIV-infected child. MFI for (D) Ag85B/TB10.4-CD8 T cells producing (E) IFN-γ (purple) or (F) IL-2 (pink) are also shown.

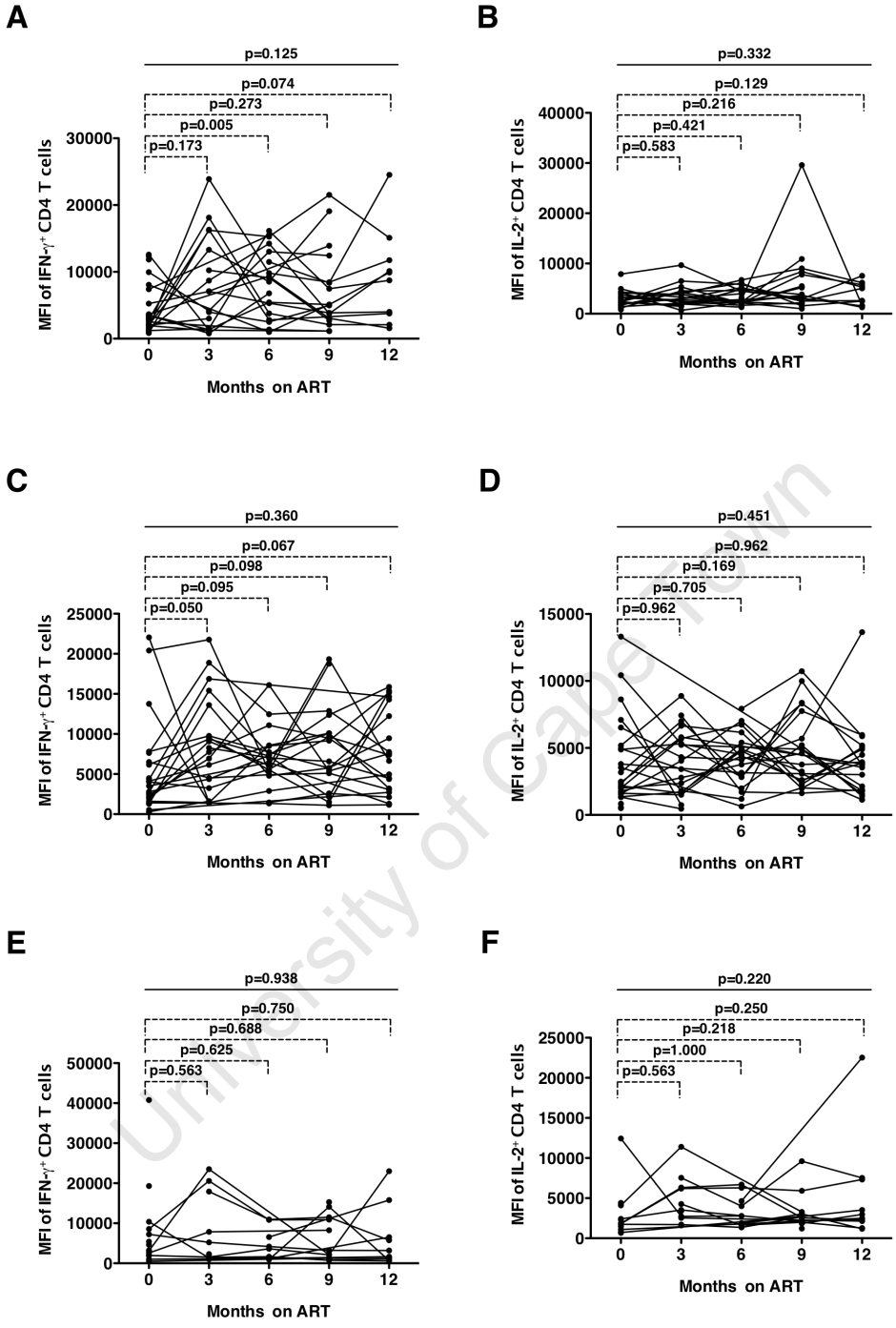

Figure 5.11: Longitudinal changes in MFIs for intracellular cytokines of mycobacteria-specific CD4 T cells

Changes in MFI for IFN-γ or IL-2 of polyfunctional cells at 0, 3, 6, 9 and 12 months after stimulation with (A and B) Ag85B/TB10.4, (C and D) PPD or (E and F) ESAT-6.

5.3.4 Memory T cell phenotype during ART

ART is known to restore the production of naïve T cells by the thymus and reduce antigen load and is therefore likely to have effects on the overall phenotypic distribution of the circulating T cell population (Vigano *et al.*, 2000; Ometto *et al.*, 2002). We assessed changes in memory phenotype of Ag85B/TB10.4-specific T cells, during the course of ART. Expression of the phenotypic markers, CD45RA, CD27 and CCR7, previously discussed in chapter 4, were measured.

In the total (non-specific) CD4 T cell population, the proportion of cells expressing the naïve phenotype $CD45RA^{+}CCR7^{+}CD27^{+}$ increased significantly over the first 3 months of ART ($p<0.0001$, **Figure 5.12**). This naïve population continued to increase to a median of approximately 60% by 6 months, and remained at this level 12 months into ART.

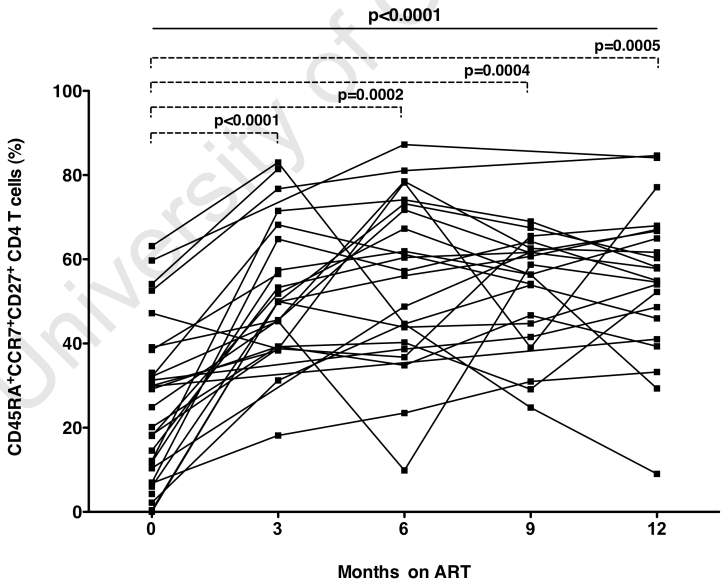

Figure 5.12: Non-specific naïve CD4 T cell population
The $CD45RA^{+}CCR7^{+}CD27^{+}$ naïve CD4 T cell population, determined by flow cytometry, significantly expanded during the first 12 months of ART. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

Central memory cytokine producing Ag85B/TB10.4-specific CD4 T cells, which lack expression of CD45RA ($CD45RA^-CCR7^+CD27^+$), did not change (p=0.301, **Figure 5.13**). The proportion of effector memory Ag85B/TB10.4-specific CD4 T cells ($CD45RA^-CCR7^-CD27^+$), decreased significantly from baseline to 13.34% (**Figure 5.14 A**), 3 months into ART. $CD45RA^-CCR7^-CD27^-$ CD4 T cells also decreased significantly from baseline, to 3 months, (p=0.006, **Figure 5.14 B**).

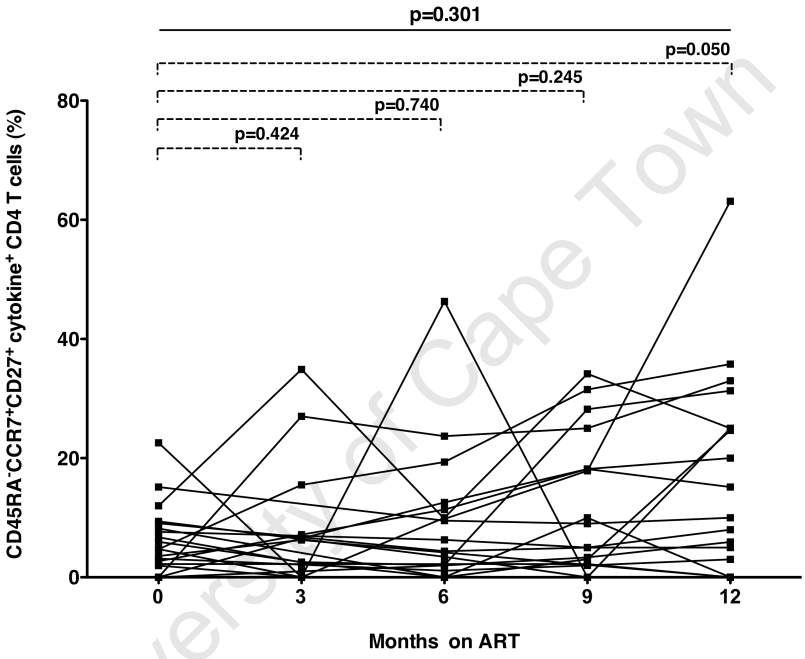

Figure 5.13: Ag85B/TB10.4-specific central memory CD4 T cells
The $CD45RA^-CCR7^+CD27^+$ central memory phenotype showed an increasing trend but this did not reach statistical significance. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

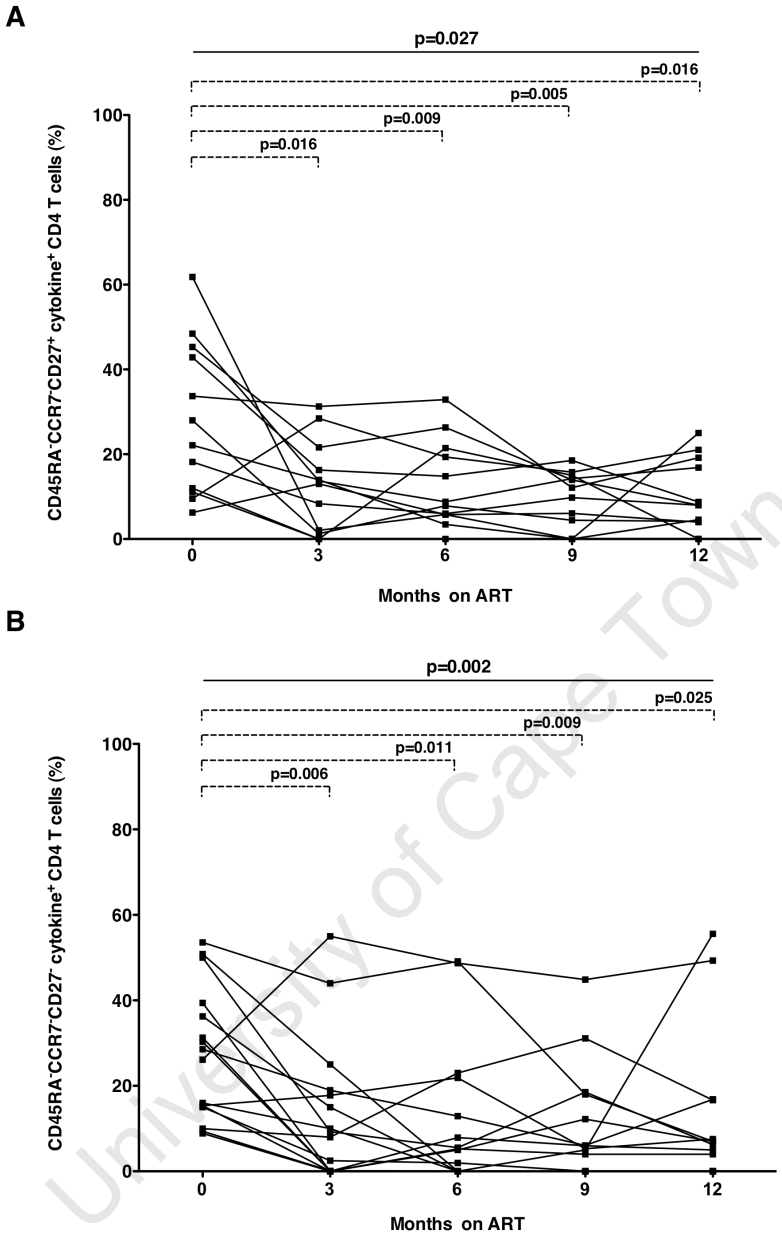

Figure 5.14: Ag85B/TB10.4-specific effector memory CD4 T cells

Proportional decline of effector memory populations: (A) $CD45RA^{-}CCR7^{-}CD27^{+}$ and (B) $CD45RA^{-}CCR7^{-}CD27^{-}$, from baseline (T0) to 3 months of ART. This was sustained over 12 months of therapy. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

$CD45RA^+CCR7^+CD27^+$-expressing naïve, non-cytokine producing CD8 T cells increased steadily, and showed a significant difference, 6 months into ART (p=0.020, **Figure 5.15**). Although at a lower rate than naïve CD4 T cells, the naïve CD8 T cells continued to increase 12 months into ART (p=0.002).

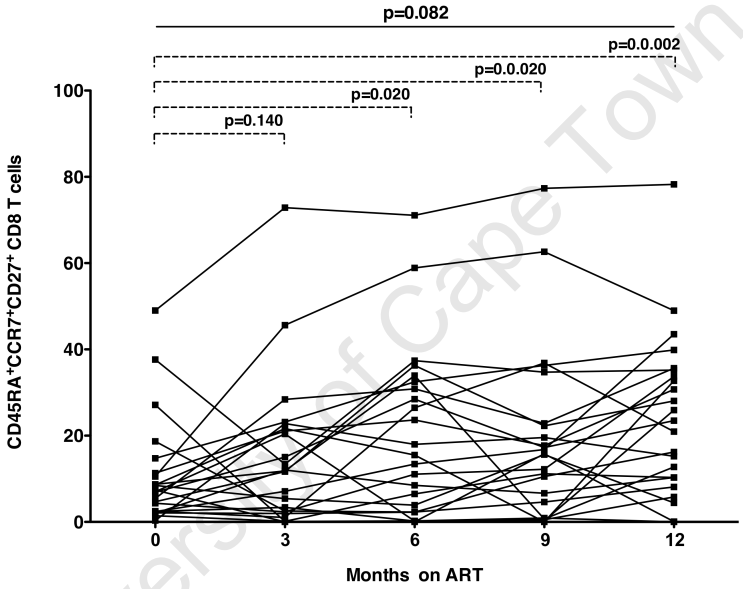

Figure 5.15: Non-specific naïve CD8 T cell population

The $CD45RA^+CCR7^+CD27^+$ naïve CD8 T cell population, determined by flow cytometry, significantly expanded during the first 12 months of ART. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

The Ag85B/TB10.4-specific CD8 T cell central memory population characterized by $CD45RA^{-}CCR7^{+}CD27^{+}$ remained low throughout the first 12 months of ART (data not shown). CD8 T cells expressing the effector memory phenotype $CD45RA^{-}CCR7^{-}CD27^{+}$ or $CD45RA^{-}CCR7^{-}CD27^{-}$ significantly decreased 3 months into ART (p=0.003 and p=0.004, **Figure 5.16 A and B**, respectively). The significant decrease was sustained in the first 12 months of therapy. The terminally differentiated phenotype characterized by expression of $CD45RA^{+}CCR7^{-}CD27^{-}$ did not change in the first 12 months ART, (p=0.540, **Figure 5.17**).

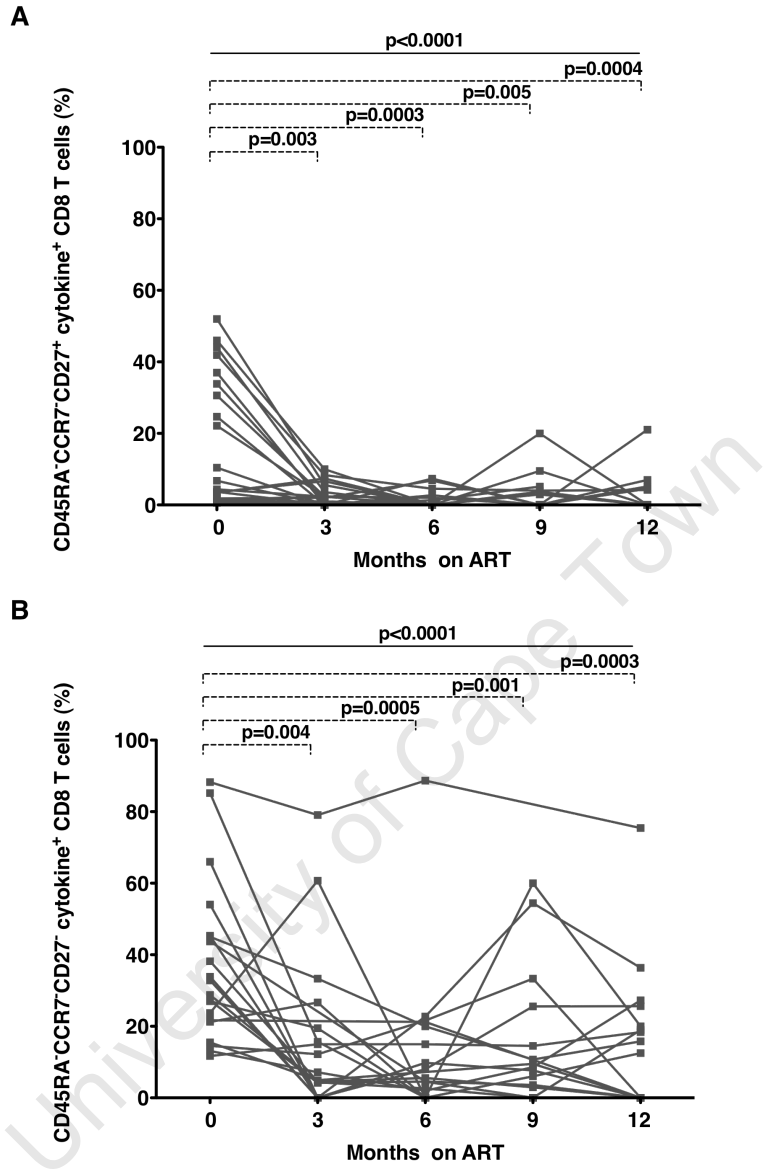

Figure 5.16: Ag85B/TB10.4-specific effector memory CD8 T cells

The effector memory CD8 T cell populations: (A) $CD45RA^{-}CCR7^{-}CD27^{+}$ and (B) $CD45RA^{-}CCR7^{-}CD27^{-}$, decreased significantly over the course of the 12 months of ART. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

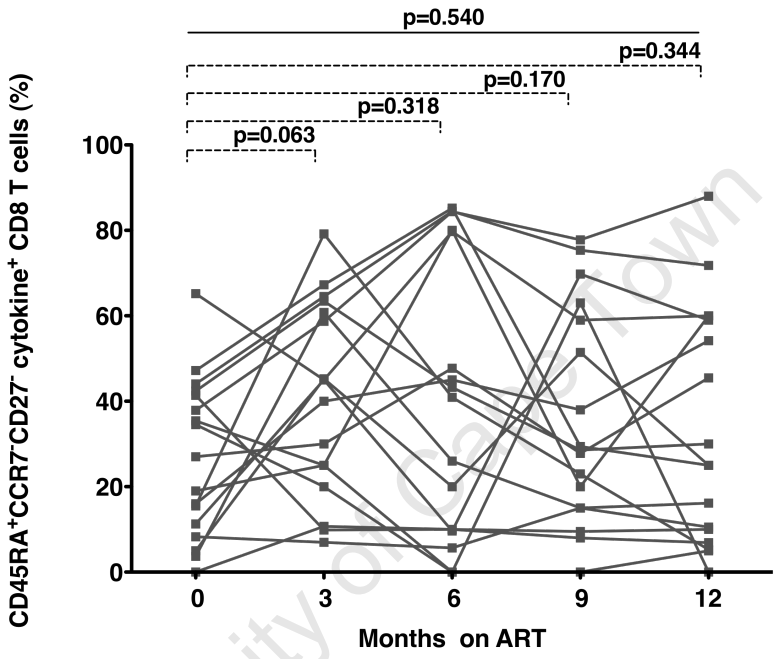

Figure 5.17: Ag85B/TB10.4-specific terminally differentiated effector memory CD8 T cells

There was no significant change in the percentages of terminally differentiated CD8 T cells. The Kruskal-Wallis test (solid line) was used to analyse the overall change. Differences between time-points were analysed with the Wilcoxon matched pairs test (dashed line). A $p<0.05$ indicates significance.

5.4.Discussion

Tuberculosis (TB) remains a leading cause of death for children in TB endemic countries, especially in children with HIV infection. ART has completely transformed both life expectancy and quality of life for HIV-infected individuals, by reducing the immunodeficiency characteristic of AIDS. However, there is heterogeneity in the extent to which immune recovery is realised. Little is known about reconstitution of the mycobacteria-specific T cell memory response in children during ART. This study provides the first detailed analysis of these T cell sub-populations in children on ART. We assessed the longitudinal changes in mycobacteria-specific CD4 and CD8 T cell responses in HIV-1-infected children on ART. Three main findings emerged from these data: 1. mycobacteria-specific CD4 and CD8 T cell frequencies declined on ART; 2. effector memory specific CD4 and CD8 T cell populations decreased during ART; 3. non-specific naïve CD4 and CD8 T cells increased dramatically during ART.

Overall Ag85B/TB10.4-specific CD4 and CD8 T cells declined in frequency during the first year of ART. PPD-specific responses remained unchanged and ESAT-6- specific responses remained very low before and during the first year of ART. However, most children retained their ability to respond to SEB, a superantigen with potent cytokine-inducing potential. This suggests a selective defect in the response to recall antigens, while responses to the superantigen are maintained. Although the inter-individual responses varied greatly, the overall percentages of effector memory specific CD4 and CD8 T cell populations had significantly declined by 3 months of ART. The decrease was observed in Ag85B/TB10.4-specific IFN-γ producing CD4 and CD8 T cells. Mycobacteria specific responses were not uniform among participants. The staining, acquisition and analysis of longitudinal samples, of individual patients were performed in batch. The gating strategy used was the same for all samples and was consistent across all time-points for each individual. Furthermore the changes in the responses in some individuals at 3-9 months could not be explained by differential cell viability or assay variability, since the consistent response to the positive control (shown in Figure 5.2) does not

support this hypothesis. The data presented here suggest that once HIV has induced depletions within the T cell pool, ART does not result in an immediate and complete restoration of mycobacteria-specific responses. The changes in CD8 responses appeared more dramatic than the changes in the CD4 responses. A discrepancy in the rate of replacement of these two subsets could have contributed to this. However, we did not address this experimentally, since we did not include assays to detect recent thymic emigrants (Douek *et al.*, 1998; De Rossi *et al.*, 2002) in our cohort of HIV-infected children.

The extent of immune reconstitution was assessed in HIV-infected children in the first year of ART. We observed significantly increased CD4 T cell percentages and decreased viral load as early as three months after initiation of treatment. This was sustained during the first year of follow-up and likely reflects the early effect of ART on immune restoration. Previous studies in adults and children have shown that recovery of CD4 cells on ART is associated with significant expansion of naïve CD4 T cell populations (Autran *et al.*, 1997; De Rossi *et al.*, 2002; Wilkinson *et al.*, 2009).

Consistent with the previous studies, increased proportions of CD4 T cells were of a 'naïve' $CD45RA^{+}CCR7^{+}$ phenotype, during ART. These T cells are not antigen experienced, and do not possess the capacity to respond quickly to antigen re-exposure. Therefore they do not produce IFN-γ in response to mycobacteria. Two mechanisms have been suggested for this high recovery in the naïve populations: greater thymic output of naïve T cells or higher peripheral T cell turnover. The rise in CD4 T cells in children on ART is predominantly confined to $CD45RA^{+}$ cells (Bohler *et al.*, 1999). Thymic output of T cells is recognised to be greater in children compared with adults (Ometto *et al.*, 2002). T cells recently generated in the thymus are quantified by measuring T cell receptor excision circles (TRECs) (Ribeiro and de Boer, 2008). Studies quantifying thymic output using TREC assays confirm that a high proportion of cells are recent thymic emigrants (Douek *et al.*, 1998; De Rossi *et al.*, 2002). Immune reconstitution using ART might also lead to an expansion in the periphery of existing resting/naïve T cells, as shown in adults

(Emery and Lane, 1997; Lange *et al.*, 2002), but the timing and extent have not yet been fully established.

The early effects of ART have previously been described in HIV-infected children and adolescents (Bohler *et al.*, 1999). In the study by Böhler *et al.,* ART caused a significant increase in absolute lymphocyte and CD4 T cell counts and a decrease in activation-induced apoptosis of CD4 and CD8 T cells to near-normal levels, even in patients without complete suppression of viral replication. Resting/naïve cells contributed significantly to the rise in CD4 T cells especially in infants and young children. An early rise in resting/naïve CD4 T cells (and, to a lesser extent, also in CD8 T cells) during ART was observed in these patients. It is well known that routine childhood vaccinations do not appear to elicit equally long lasting memory responses in children with HIV compared to HIV-negative children. It is therefore recommended to revaccinate HIV-infected children once ART has established successful immune reconstitution (Moss *et al.*, 2007; Sutcliffe and Moss, 2010).

ART treated individuals with substantially restored CD4 T cells may still have impaired antimycobacterial immunity. This might be caused by the failure of ART to restore central memory T cell populations (Elrefaei *et al.*, 2004), which are capable of rapid expansion in response to antigen re-stimulation, and are critical for the development of T cell memory. Care must therefore be taken when following-up latently infected individuals on ART because they are at a high risk of TB reactivation or disease development. During latency mycobacteria are sequestered within the granuloma and are poised for uncontrolled replication in conditions of immunosuppression. Wilkinson *et al.* showed a significant increase in the central memory population in HIV-infected adults on ART (Wilkinson *et al.*, 2009). In addition, the potential for early ART to preserve antimycobacterial immune responses should be explored. Jansen *et al.* demonstrated that early introduction of ART in acute HIV-1 infection resulted in higher numbers of HIV-specific IFN-γ and IL-2-producing CD4 T cells (Jansen *et al.*, 2005). Central memory cells are associated with long-term immunological memory. There was no change in proportions of central memory cells in children in the first 12 months of

therapy. It is likely that the dynamics of T cell turnover differ between adults and children with HIV starting ART, with the initial changes in children primarily associated with naïve T cells (De Rossi *et al.*, 2002).

The study by *Wilkinson et al.* reported that frequencies of PPD-specific IFN-γ-producing effector cells, measured by ICS, proportionally declined as CD4 T cell numbers recovered during ART (Wilkinson *et al.*, 2009). However, the number of IFN-γ-producing cells, measured by ELISpot assay (spot forming cells, SFC) increased with increasing numbers of absolute CD4 T cells (Wilkinson *et al.*, 2009). A discrepancy between ICS and ELISpot can be observed because ICS reports the data as a frequency of CD4 T cells. So, changes in T cell numbers are not taken into account by ICS. By contrast, ELISpot assays report data as SFC per million PBMC, so if the relative proportion of CD4 cells out of a million PBMC increases during ART, this may be detected as an increase in responses. Our results are in concordance with the flow cytometric measurements of this work in adults, since we also observed a significant decrease in the effector memory populations, accompanied by a decrease in IFN-γ production after stimulation with Ag85B/TB10.4. This is attributed, to the effector cell population proportionally declining within the expanding naïve CD4 T cell pool (Wilkinson KA, 2009). Due to limited volumes of blood available, unfortunately, we could not confirm the relative expansion of IFN-γ producing effector CD4 T cells, measured by ELISpot, as described by Wilkinson et al.

We were also interested in the quality of the T cell pool in both the CD4 and CD8 T cell compartment, since polyfunctional cells have been linked to protective immunity against *Leishmania major* (Darrah *et al.*, 2007) and *M. tuberculosis* (Forbes *et al.*, 2008). IFN-γ^{+}IL-2^{+} T cells produced significantly increased amounts of cytokine compared with cells producing only one cytokine. However, no significant qualitative differences were found under ART. ART leads to a decline in susceptibility to disease with *M. tuberculosis*. A clear correlation with polyfunctional T cell responses has not yet been demonstrated. Kalsdorf *et al.* observed lower frequencies of cells co-

expressing IFN-γ, TNF-α and IL-2 in HIV-1-infected adults than in uninfected controls (Kalsdorf *et al.*, 2009). However, another paper from the same group illustrated the lack of a correlation between polyfunctional T cells and protection against TB in BCG-vaccinated infants. Infants who developed TB were compared with age-matched controls who did not develop TB (Kagina *et al.*, 2010). No difference in the frequencies of polyfunctional T cells was found between the two groups. Further studies are required to elucidate the role of these polyfunctional T cells in immunity to TB.

We conducted further analysis to determine changes in the quality of the antigen-specific response, by analysing changes in proportions of effector cell populations and also the median fluorescent intensity (MFI). The MFI indicates the amount of cytokine produced on a per cell basis (Darrah *et al.*, 2007). These parameters were not significantly different. Wilkinson *et al* used IFN-γ Elispot assay to determine qualitative changes. We were not able to assess effector function using this assay as well, due to constraints on blood volumes in children.

A limitation of this study is the absence of healthy control samples to compare the mycobacteria response at 12 months of ART. A second limitation is the absence of non-tuberculous antigens to rule out global impairment of the T cell response during ART (Sutherland *et al.*, 2006). For example, cytomegalovirus-specific (CMV) CD4 T cells with IFN-γ-secreting capacity have been detected in patients who controlled cytomegalovirus disease after ART initiation (Jacobson *et al.*, 2001).

In conclusion, ART results in significant immune restoration in HIV-infected children, but the degree of antigen-specific restoration may be limited. It was interesting to note that most changes in immunity were detected in the first three months of ART and were then maintained. Phenotypic and functional analysis of CD4 T cells might help to guide decisions regarding discontinuation of prophylactic medication against opportunistic infections in patients with undetectable viral load under ART. The availability of a larger

population of naïve T cells under ART is encouraging in the context of vaccination of HIV-infected children, since antigen-specific effector and memory T cells can be generated from this pool of T cells. This has important implications for the timing of vaccination in HIV-infected children.

CHAPTER 6

Effects of ART on mycobacterial growth inhibition in HIV-infected children

6.1 Introduction

6.2 Methods

6.3 Results

6.3.1 Participant follow-up

6.3.2. Increased mycobacterial growth inhibition in children on ART

6.3.3 Correlation of mycobacteria-specific T cell responses with mycobacterial growth inhibition

6.4.Discussion

6.1 Introduction

Exposure to mycobacterial pathogens results in no infection, containment of infection or progression to active disease (acute or chronic) (Kaufmann and McMichael, 2005). A better understanding of the immunological determinants of these outcomes of mycobacterial exposure would be valuable in the development of new vaccines. A cell-mediated immune response (CMI) is essential for control of *M. tuberculosis* infection (Cooper and Flynn, 1995). Containment of mycobacterial growth can be measured in animal models, enumerating CFU in target organs. However, quantification of mycobacterial load in humans is very difficult. Although assays that measure host cell-mediated immunity to mycobacteria exist (such as ELISA, Elispot, ICS and proliferation assays), these do not measure antigen load or mycobacterial survival.

HIV infection can be understood as a model of impaired ability to contain mycobacterial infection. HIV-infected individuals have increased risk of TB disease as a consequence of their impaired cell-mediated immune responses, resulting in insufficient ability to contain the growth of *M. tuberculosis* (Imperiali *et al.*, 2001; Bezuidenhout *et al.*, 2009), more rapid progression of clinical symptoms (Daley *et al.*, 1992), and high mortality (Harries *et al.*, 2001). Introduction of ART improves CD4 cell numbers and dramatically decreases viral load, resulting in reduced incidence of TB disease in HIV infected patients (Kirk *et al.*, 2000; Girardi *et al.*, 2000; Badri *et al.*, 2002). Most studies of antigen-specific immune reconstitution during ART have concentrated on measuring lymphoproliferative responses or the specific production of cytokines in whole blood or PBMC, measured via ELISA, RNA expression or ICS (Imami *et al.*, 1999; Lange *et al.*, 2002). However, these assays measure the host immune response and do not provide a read-out of the effect on mycobacterial growth.

Kampmann *et al.* developed an *in vitro* model that measures the growth of reporter-gene tagged mycobacteria (bacillus Calmette-Guérin; BCG lux) in whole blood as a functional read-out of antimycobacterial immune responses

(Kampmann *et al.*, 2000). In our previous studies using this model we have established an association between *in vitro* growth of mycobacteria and observations of anti-mycobacterial host immune response. For example, tuberculin skin test (TST)-positive individuals are better able to restrict mycobacterial growth compared to TST-negative individuals (Kampmann *et al.*, 2000). Further, increased inhibition of mycobacterial growth is related to the production of IFN-γ and the presence of antigen-experienced CD4 T cells. Anti-mycobacterial growth inhibition is enhanced following BCG vaccination in adults (Cheon *et al.*, 2002; Hoft *et al.*, 2002) and children (Kampmann *et al.*, 2004). Children infected with HIV do not restrict growth of BCG as successfully as age-matched non-HIV infected children (Tena *et al.*, 2003). Introduction of ART can improve growth restriction of BCG in this in vitro assay (Kampmann *et al.*, 2006).

Growth inhibition as a functional read-out of antimycobacterial immune responses is attractive as a tool to measure immunological correlates, including vaccine-induced immune responses. The whole blood *M. tuberculosis* luciferase (MTB lux) assay used in this study employs mycobacterial growth restriction *in vitro* as the final read-out of immunological effector functions. Our recent work using this model has shown that HIV-infected children are more permissive of BCG growth *in vitro* than healthy children, reflecting the high susceptibility to tuberculosis in HIV infection (Tena *et al.*, 2003). We have also shown that ART leads to rapid and sustained improvement of the ability to restrict growth of BCG *in vitro* (Kampmann *et al.*, 2006). We now wished to confirm the relevance of our earlier observations using the avirulent BCG lux strain with the virulent organism, *M. tuberculosis* H37Rv.

In this chapter we describe findings from measuring *M. tuberculosis* growth inhibition in HIV-infected children using the virulent MTB H37Rv lux strain. We also examined the role of antigen-specific T cell responses on MTB inhibition.

6.2 Methods

The MTB lux assay was performed in the same samples obtained from the cohort of HIV-infected children as described in chapter 4 and 5. The whole-blood assay utilising luciferase-tagged MTB H37Rv has been described in chapter 2. Briefly, heparinised venous blood, diluted 1 in 2 with RPMI, was inoculated in triplicate with 1 x 10^7 RLU/ml of MTB lux and incubated for 96h with continuous mixing. An unstimulated control sample incubated with whole blood and RPMI only was included at each time point. Growth of MTB lux in mycobacterial culture medium only was also measured after 96h. Mycobacterial viability was measured before incubation at the time of inoculation (T0) and at 96h (T96) after the lysis of red blood cells, using a single tube luminometer (Turner Design 20/20, Germany). The growth ratio was calculated as follows:

$$\text{Growth ratio} = \frac{\text{RLU of BCG lux at T96}}{\text{RLU of BCG lux at T0}}$$

Mycobacterial growth inhibition was measured prior to ART and at 3, 6, 9 and 12 months of therapy. All work was carried out in a BSL3 laboratory.

6.3 Results

6.3.1 Participant follow-up

The whole blood mycobacterial growth inhibition assay was performed on blood obtained from 19 of the 30 (63.33%), HIV-infected children enrolled into the longitudinal study. This was because the BSL3 unit was not available at the time of commencing enrollment. In 17/19 children the whole blood lux assay was completed at each time point (every 3 months) for the entire study duration of 12 months. Two children did not complete the study: one child died from septicaemia and the second child was lost to follow-up. To the best of our knowledge, none of the children had been exposed to acute tuberculosis in his or her household prior to commencing the study or during the follow-up visits. None of the children were receiving anti-TB treatment or any other forms of antibiotics known to be active against mycobacteria.

6.3.2. Increased mycobacterial growth inhibition in children on ART

Prior to commencing ART we established the baseline effect of HIV infection on mycobacterial immunity expressed in the whole blood assay. **Table 6.1** summarises the details of baseline CD4 cell percentage, age, log viral load and growth ratios.

The median age of the HIV-infected children whose blood was obtained for the whole blood lux assay was 4 years (IQR: 1-7 years). The median log viral load was 5 $\log_{10}$ RNA copies/ml (IQR: 4.70-6.04 $\log_{10}$ RNA copies/ml) at baseline. The baseline median growth ratio was 8.94 (IQR: 6.92–12.21, **Figure 6.1 A**) and median CD4 T cell count was 17.30% (IQR: 13.00-23.40%, Figure 6.1 B). The age, viral load and CD4 percentage measurements were all similar to those obtained in the cross-sectional study. We observed significant negative correlation between the growth ratio and CD4% prior to ART (p=0.004) (**Figure 6.2 A**). There was no association between viral load and growth ratios at baseline (Figure 6.2 D). Age was also not correlated to growth ratios (data not shown).

Next we assessed the ability of whole blood from HIV-infected children on ART to inhibit growth of MTB lux. The introduction of ART was followed by reconstitution of anti-mycobacterial immune responses, measured as the decreased growth of mycobacteria (**Figure 6.1 A**). Mycobacterial growth inhibition increased significantly by 6 months of ART (p=0.001, **Figure 6.1. A**), continued to increase at 9 months and this growth ratio stayed the same at 12 months into ART. We observed a strong association of the ability to inhibit mycobacterial growth and rising CD4 percentage during the first 12 months of ART. This is reflected in the highly significant restoration of CD4 T cell percentages as shown in **Figure 6.1 B**. There was substantial correlation between CD4 percentage and the ability to restrict growth at all time-points during the first 12 months of ART, (data shown for baseline, 6 and 12 months, **Figure 6.2 A-C**).

In order to establish whether levels of HIV viral load before receiving ART were predictive of the growth ratios at any particular time-point, we calculated correlation coefficients between viral load at baseline and subsequent growth ratios. No correlations were found at any time-point (**Figure 6.2 D-F**).

Study ID	Age (years)	Viral load (log_{10} RNA copies/ml)	CD4 Cell Count (%)	Growth ratio MTB lux
H1	0.42	6.28	32.10	5.16
H2	4	5	7.34	13.58
H3	2	N/A	18.00	8.00
H4	4	6	18.10	7.48
H5	0.42	4.79	12.20	6.85
H6	11	8.08	20.80	6.92
H7	0.58	6.48	13.00	6.78
H8	5	5	10.50	12.21
H9	1	3.37	19.10	7.71
H10	4	2.83	14.00	12.48
H11	5	2.58	24.50	6.07
H12	2	4.96	14.50	12.12
H13	8	6.08	17.30	11.19
H14	0.67	n/a	23.40	8.94
H15	6	4.60	17.10	11.67
H16	12	4.97	15.20	12.98
H17	7	5	7.76	15.25
H18	8	5.91	19.00	10.81
H19	4	4.85	34.60	7.26

Table 6.1: Summary of patient details: study number, age, viral load, percentage CD4 T-cell count and growth of MTB lux in whole blood at the time of enrolment into the study, (N/A: not available)

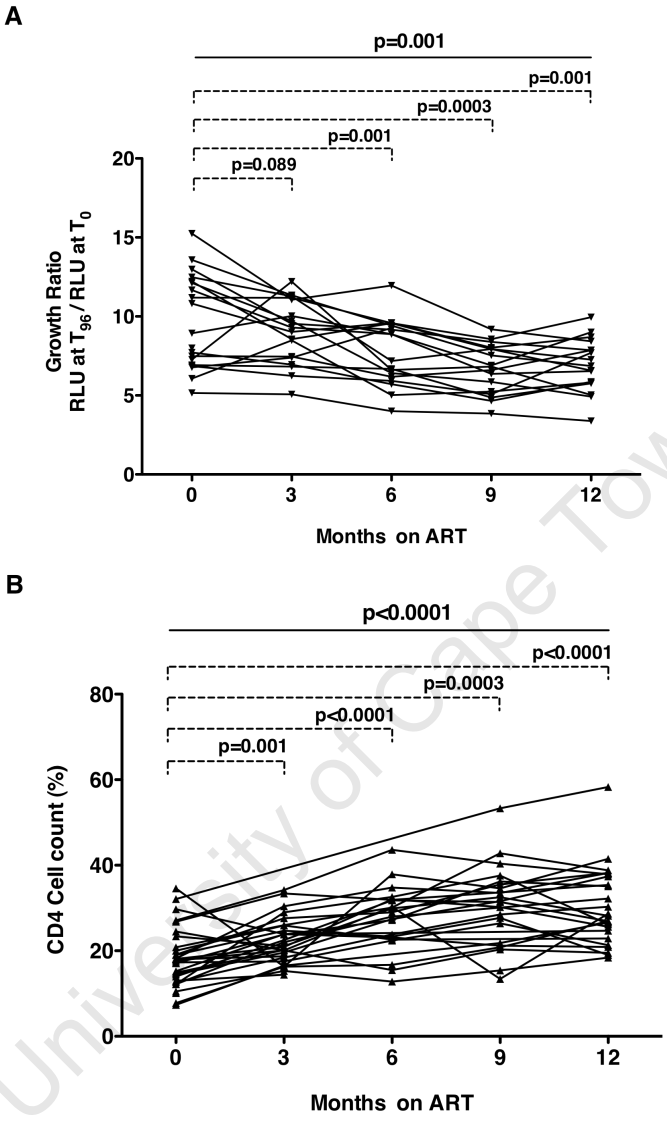

Figure 6.1: Longitudinal changes in mycobacterial growth inhibition and CD4 percentage

(A) Growth of *M. tuberculosis* lux in whole blood was measured at time of enrolment and subsequently at 3-monthly intervals after the introduction of ART. (B) The percentage CD4 T-cell count was measured at the time of enrolment into the study (T0), and then at 3, 6, 9 and 12 months

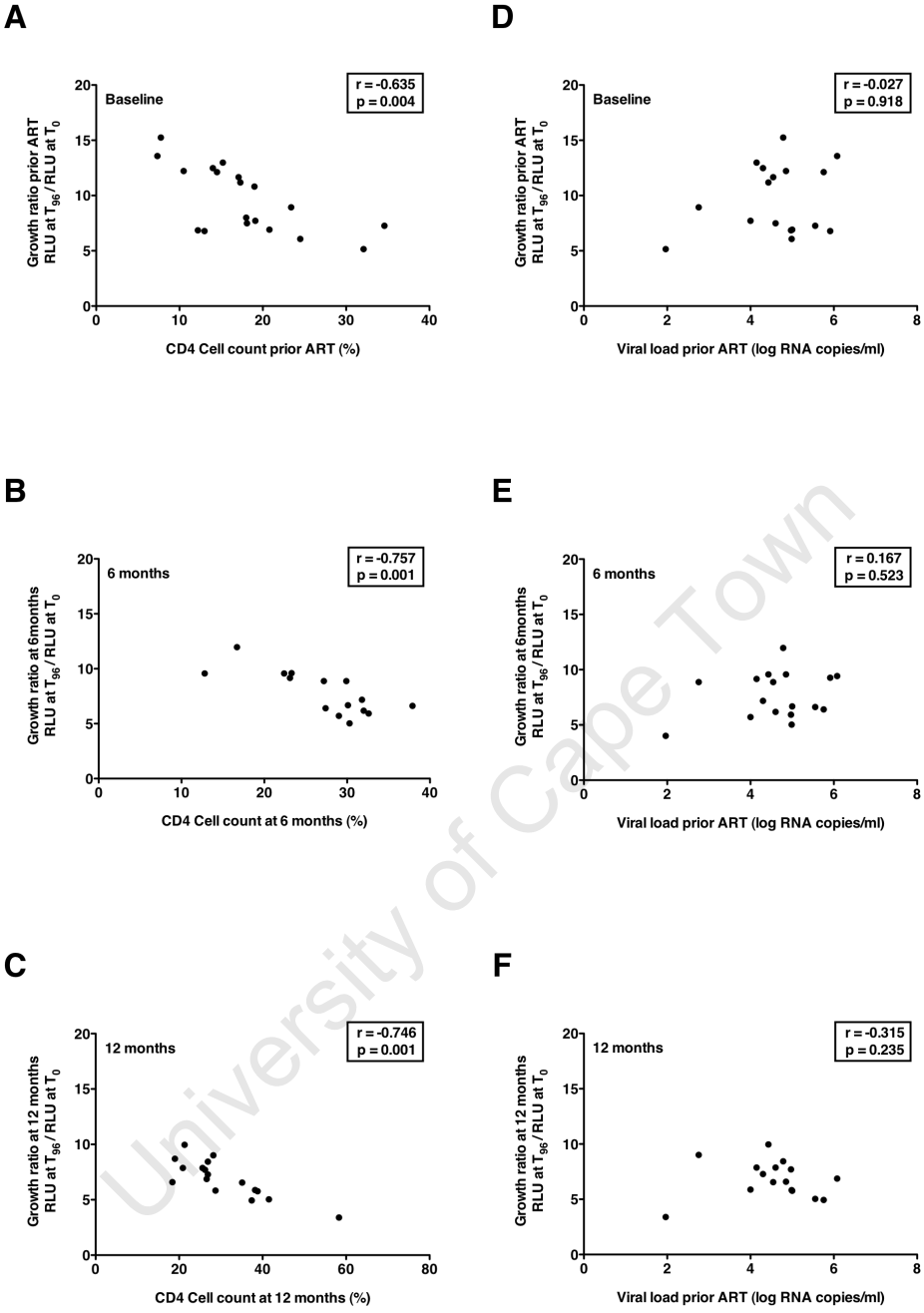

Figure 6.2: Relationship between mycobacterial growth inhibition and CD4 percentage or viral load

There was a significant inverse correlation between growth ratios and CD4 percentages at (A) baseline (n=19), (B) 6 (n=15) and (C) 12 (n=17) months into ART. There was no correlation between growth ratios and viral load at (D) baseline (n=19), (E) 6 (n=15) and (F) 12 (n=17) months into ART. The CD4 percentages and viral loads were not available for some children at 6 and 12 months.

6.3.3 Correlation of mycobacteria-specific T cell responses with mycobacterial growth inhibition

In order to assess the effect of mycobacteria-specific T cells on the ability to control mycobacterial growth we compared functional CD4 and CD8 T cell responses (defined as those that produce IFNγ and/or IL-2 in response to mycobacterial antigens) to growth ratios before ART (T0) and after 3, 6, 9 and 12 months of therapy (only data for baseline and 12 months are shown).

The frequency of functional subsets of mycobacteria-specific T cells was compared with their ability to restrict the growth of mycobacteria in vitro. Ag85B/TB10.4-specific total cytokine, or IFN-γ or IL-2 producing CD4 T cells did not correlate with MTB lux growth inhibition prior to commencing ART (**Figure 6.3 A-C**). The antigen specific T cell response was then normalised to the CD4 percentage by multiplication of the antigen-specific response with the CD4% (in decimal format). However, there was still no association between the ability to inhibit mycobaterial growth and Ag85B/TB10.4-specific T cell response at T0 (**Figure 6.3 D-F**). This was also true when comparing Ag85B/TB10.4-specific T cell responses at 3, 6, and 9 months with growth ratios (**Figure 6.4 A-E**, only data obtained at 12 month is shown). However, there was a significant correlation between Ag85B/TB10.4-specific total IL-2 expressing CD4 T cells and mycobacterial growth at 12 months of ART (**Figure 6.4 F**).

PPD- specific total cytokine, or IFN-γ or IL-2 producing CD4 T cells did not correlate with MTB lux growth inhibition before ART (**Figure 6.5 A-C**). There was no association between the baseline PPD-specific T cell response (normalised to the CD4 percentage) and mycobacterial growth (**Figure 6.5 D-F**). There was also no association between the ability to inhibit mycobaterial growth and PPD-specific T cell response (**Figure 6.6 A-F**), whether normalised to CD4 percentage or not.

ESAT-6 specific responses did not correlate with mycobacterial growth

restriction prior to commencing ART or at 3, 6, 9 and 12 months into ART (data not shown). This was also true for mycobacteria-specific CD8 T cell responses for all antigens tested (data not shown).

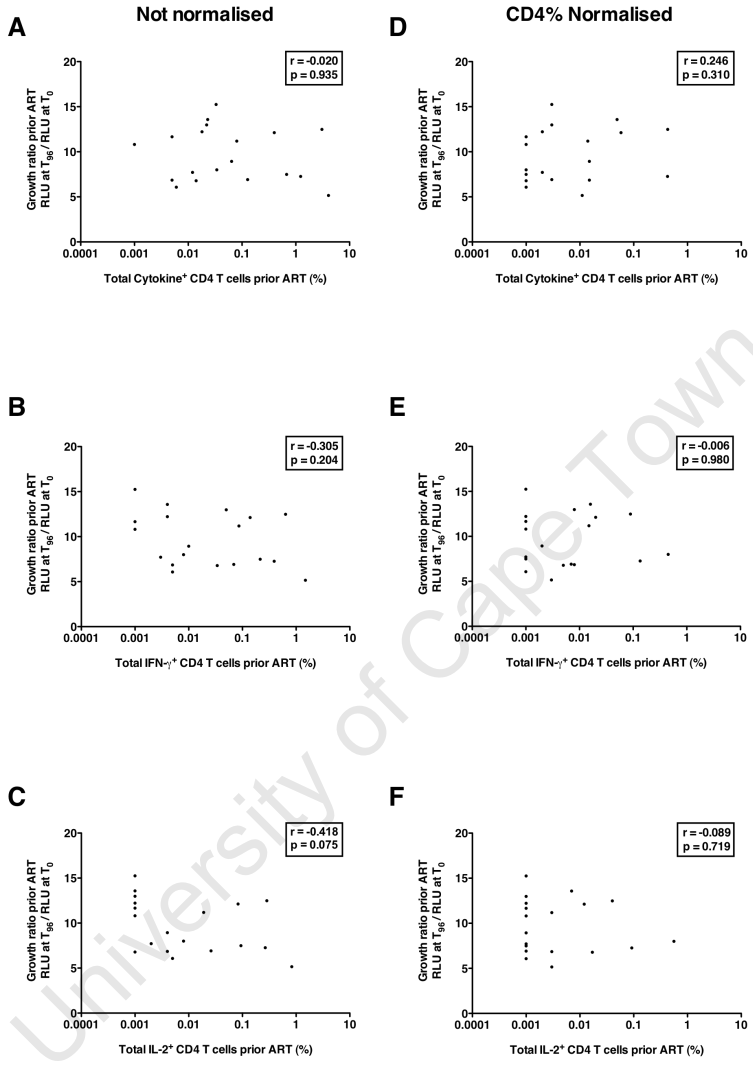

Figure 6.3: Association between Ag85B/TB10.4-specific CD4 T cells and growth ratios before ART

The relationship between (A) total cytokine$^+$, (B) IFN-γ^+ or (C) IL-2$^+$ Ag85B/TB10.4-specific CD4 T cells and the ability to inhibit mycobacterial growth was assessed in HIV-infected children before commencing ART. There was no correlation between antigen specific CD4 T cells and growth ratios at baseline. There was also no association between the CD4 percentage normalised (D) total cytokine$^+$, (E) IFN-γ^+ or (F) IL-2$^+$ Ag85B/TB10.4-specific CD4 T cell response and the ability to inhibit mycobacterial growth.

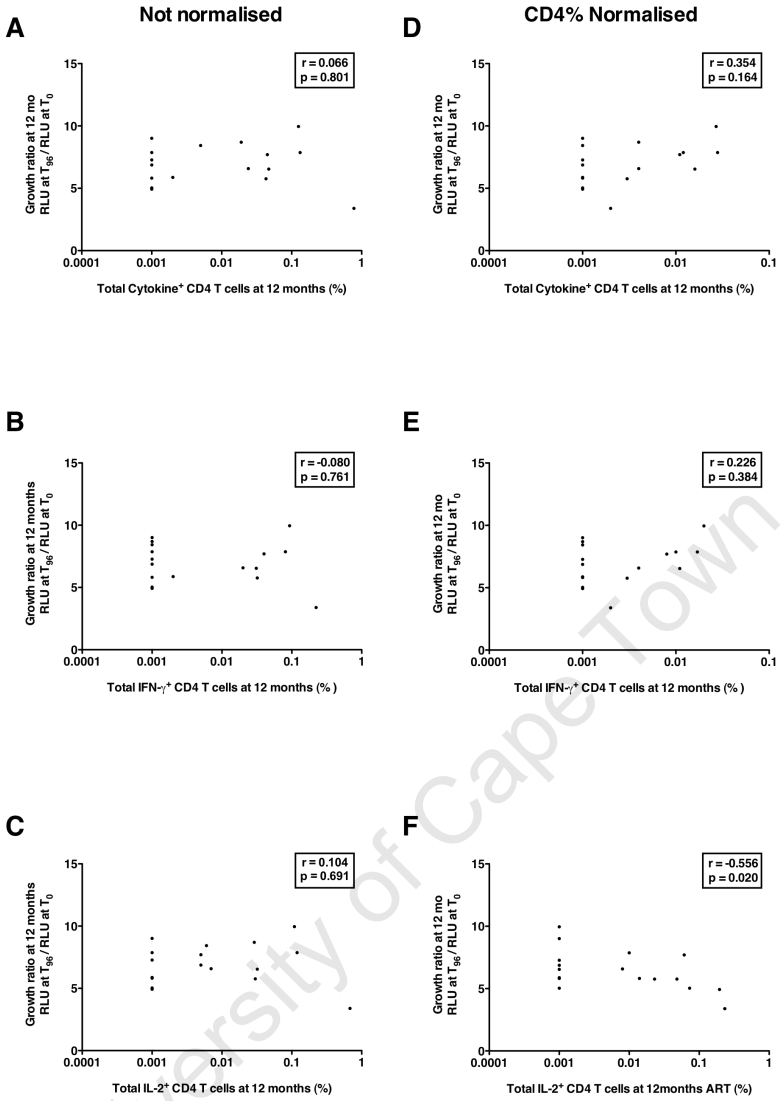

Figure 6.4: Relationsip between Ag85B/TB10.4-specific CD4 T cells and growth ratios after 12 months of ART

The correlation between (A) total cytokine$^+$, (B) IFN-γ^+ or (C) IL-2$^+$ Ag85B/TB10.4-specific CD4 T cells and the ability to inhibit mycobacterial growth was assessed in HIV-infected children at 12 months of ART. There was no correlation between antigen specific CD4 T cells and growth ratios at 12 months. There also was no association between (D) total cytokine$^+$ or (E) IFN-γ^+ Ag85B/TB10.4-specific CD4 T cells and the ability to inhibit mycobacterial growth, after normalizing the antigen specific response to CD4 percentages. However, there was significant correlation between the IL-2$^+$ Ag85B/TB10.4-specific CD4 T cell response (normalized to CD4 percentage) and mycobacterial growth at 12 months.

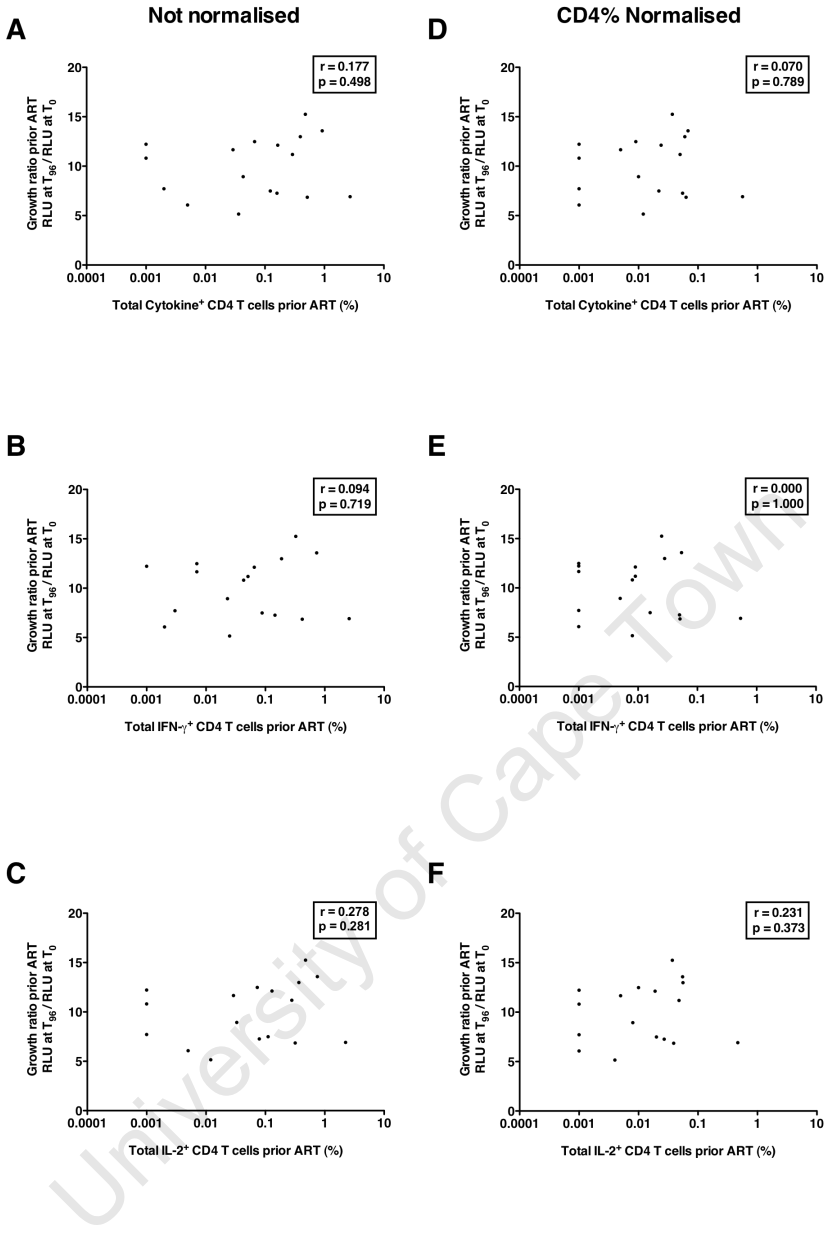

Figure 6.5: Relationship between PPD-specific CD4 T cells and growth ratios before ART

The correlation between (A) total cytokine$^+$, (B) IFN-γ^+ or (C) IL-2$^+$ PPD -specific CD4 T cells and the ability to inhibit mycobacterial growth was assessed in HIV-infected children before ART. There was no correlation between PPD-specific CD4 T cells and growth ratios at baseline. There was also no association between the CD4 percentage normalised (D) total cytokine$^+$, (E) IFN-γ^+ or (F) IL-2$^+$ PPD-specific CD4 T cell response and the ability to inhibit mycobacterial growth.

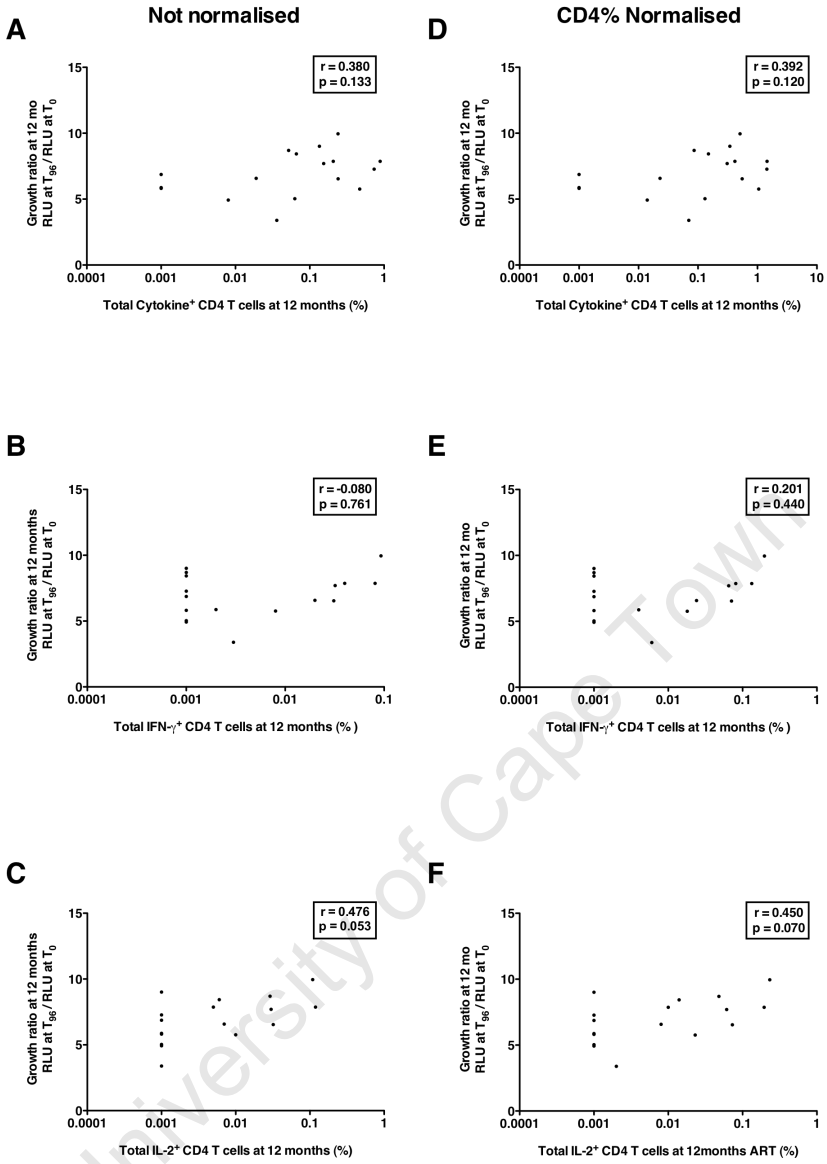

Figure 6.6: Correlations between PPD-specific CD4 T cells and growth ratios after 12 months of ART

The correlation between (A) total cytokine$^+$, (B) IFN-γ^+ or (C) IL-2$^+$ Ag85B/TB10.4-specific CD4 T cells and the ability to inhibit mycobacterial growth was assessed in HIV-infected children at 12 months of ART. There was no association between PPD-specific CD4 T cells and growth ratios at 12. There also was no association between (D) total cytokine$^+$, (E) IFN-γ^+ or (F) IL-2$^+$ PPD-specific CD4 T cells and the ability to inhibit mycobacterial growth, after normalizing the antigen specific response to CD4 percentages.

6.4.Discussion

Cell-mediated immunity is of key importance to combat mycobacterial infections, such as *M. tuberculosis* (Orme *et al.*, 1993; Cooper and Flynn, 1995). However, the mechanisms of protection have not been fully described. Earlier in vitro studies have concentrated on antigen-specific lymphoproliferative responses, IFN-γ production, and cytotoxic effector functions of immune cells (Andersen *et al.*, 1991; Barnes *et al.*, 1993; Tsukaguchi *et al.*, 1995). Recently, Kampmann *et al.* developed a whole blood in vitro model to study human antimycobacterial immunity (Kampmann *et al.*, 2000). This functional whole blood reporter-gene assay (lux assay) measures the combined anti-mycobacterial immune response in whole blood and employs mycobacterial growth restriction as the read-out of the final effector function. The virulent MTB lux showed the same decreasing trend as with the avirulent BCG lux (Kampmann *et al.*, 2006). The initial decline in mycobacterial growth was less rapid with the MTB lux compared to that previously observed with BCG lux (Kampmann *et al.*, 2006), but was significant by 6 months and sustained from then on. The more delayed effect might be due to the more virulent strain of mycobacteria, which requires more vigorous immune responses than BCG.

As our previous work with this assay has shown, it provides an additional and complimentary method to measurements of CMI by combining measurements of host responses with their effect on mycobacterial survival *in vitro*. It has potential use in the evaluation of TB vaccine candidates. In this study, we observed a significant reduction in the ability of virulent MTB lux to grow in the blood of HIV-infected children once receiving ART, which was maintained over the 12 months of observation. This was accompanied by a significant increase in absolute CD4 T cell numbers and percentages as well as by a significant decrease in the HIV viral load, due to ART. However, mycobacteria-specific T cell responses, (those that produce IFN-γ and IL-2) did not correlate with the ability to restrict growth prior to and during the first 12months of ART. Interestingly, the percentage of IL-2-producing numbers of

CD4 T cells did correlate with growth inhibition by 12 months. *Mycobacterium tuberculosis* has a broad repertoire of antigens. Thus, although highly recognized by the immune system, Ag85B and TB10.4 might not represent antigens associated with virulence, since they are also found in BCG and environmental mycobacteria. The responses to these selected antigens is likely to be different from the responses to the whole organism, which is what is measured in the lux assay.

In agreement with our work with BCG lux this study also showed a significant correlation between CD4 T cell numbers and growth ratios when using MTB lux. Our previous work using this model has confirmed the critical role of CD4 T cells in mediating this growth restriction, because the in vitro depletion of CD4 T cells using magnetic beads leads to enhanced growth of mycobacteria, in line with the in-vivo depletion of CD4 T cells in HIV-infected children (Kampmann *et al.*, 2000; Tena *et al.*, 2003) associated with the highly increased susceptibility to tuberculosis. Interestingly, the control of mycobacterial growth and reconstitution of CD4 T cell numbers did not result in increased levels of IFN-γ in our previous study, using BCG lux, suggesting the involvement of additional pathways of TB immunity. We previously only measured secreted levels of IFN-γ using ELISA technique, which cannot identify the source of the cytokine. In this study, we wished to additionally identify the source of the cytokine IFN-γ and also measure antigen-specific IL-2 as a marker associated with central memory.

The extent of reconstitution of cellular immunity through ART is not fully understood (Lawn *et al.*, 2005), and very few studies have been conducted in HIV-infected children on ART (Resino *et al.*, 2003; Resino *et al.*, 2004). Most of these studies have used non-specific antigens. Work in adults attributes antigen-specific immune recovery to the presence of memory CD4 T-cells. However, the scale of immune-recovery differs depending on the nadir CD4 cell count and the antigens investigated (Lange *et al.*, 2002; Wendland *et al.*, 1999).

As previously observed in our studies using the BCG lux model, ART reversed the CD4 T cell deficiency caused by HIV infection, but cytokine production by CD4 and CD8 T cells in response to mycobacterial antigens related poorly to the ability to control mycobacterial growth. This probably reflects the only partial recovery of the mycobacterial immune response after initiation of ART in HIV infection, or the time period of sustained viral suppression needed to achieve more comprehensive immune recovery is much longer than the 12 months of follow-up in our current study. The details of restoration of immune function in HIV-infected adults on ART are still poorly understood and even less data exist in children. It has been suggested that in order for the recall response to recover after initiation of ART, HIV viral load should remain persistently low for a prolonged period (Li *et al.*, 1998). However, substantial decrease in HIV replication did not translate to increased antigen-specific responses in the HIV-infected children in our study. Only a small percentage of individuals show reactivity to tuberculin after initiation of ART (Wendland *et al.*, 1999; Girardi *et al.*, 2002).

We did not conduct a tuberculin skin test in our population. Recovery of IFN-γ production, a more common measure of lymphocyte responsiveness, is partial and delayed. Immune reconstitution using ART is likely to lead to a re-expansion of surviving memory T cells, as shown in adults (Emery and Lane, 1997; Lange *et al.*, 2002), but the timing and extent have not yet been fully established. In contrast to adults, the increase in CD4 T cells in children in response to ART is seen predominantly with the CD45RA (naive) phenotype (De Rossi *et al.*, 2002). Thymic output of T cells is recognized to be greater in children, and TCR excision circle assays confirm that a high proportion of cells are recent thymic emigrants. These T cells are not antigen experienced, and might not necessarily produce IFN-γ in response to mycobacteria. This is in line with our flow cytometric measurements reported in chapter 5: the immune reconstitution we observed was largely due to naïve CD4 T cells and we did not see a significantly enhanced effector memory response.

Interestingly though, the growth inhibition in the lux assay was maintained

throughout the study period and did correlate well with absolute CD4 counts and percentages, but not with antigen-specific T cell populations. However, growth inhibition did correlate with IL-2 producing CD4 T cells, when adjusted for total percentage number of CD4 T cells. As far as IFN-γ is concerned, this observation can be interpreted in several ways: either the increasing numbers of naïve CD4 T cells made it impossible to appreciate the very small changes within the antigen-specific populations measurable by the flow cytometric technique, or antigen-specific T cell responses were not adequately reflected by enumerating IFN-γ and IL-2 as the only intracellular cytokines. Alternatively, frequency of antigen-specific T cells correlate badly with growth inhibition and other immune mechanisms play a role, which we did not capture in our experimental set-up, beyond the total numbers of CD4 T cells.

This is of some concern, since many investigators measuring “vaccine take” in vitro rely exclusively on measurements of the increased induction of polyfunctional antigen-specific CD4 and CD8 T cell responses in their immuno-assays. However, our study did not show that such measurements correlate with the key effector function of growth inhibition in our model.

However, the observation that by 12 months percentages of IL-2 producing CD4 T cells in response to Ag85/TB10.4 did show a correlation with growth restriction is in line with the anticipated recovery of the central memory compartment under ART. Wilkinson et al recently showed that although the relative percentages of cytokine-producing CD4 T cells decreased in HIV-infected adults receiving ART, the absolute number of IL-2 secreting T cells increased, as measured via Elispot. Our data now illustrate a similar finding and relate this to anti-mycobacterial activity in vitro, which has not been shown previously.

Our findings highlight the complex interplay of different mechanisms of immunity involved in the containment of mycobacteria and may reflect the differences in components of the immune response that are measurable in any of these assays and may vary according to time intervals. Individual

cytokines alone might not be good indicator of protective immunity. Rather a combination of factors including those that mediate reduced mycobacterial growth may better reflect this complexity. Researchers have reported IFN-γ-independent mechanisms of *M. tuberculosis* control in murine models *in vitro* and *in vivo* (Cowley and Elkins, 2003). Other mechanisms of growth inhibition could involve cytotoxic or apoptotic functions, mediated by granzymes/perforin and FAS/FAS ligand interactions, respectively (Oddo *et al.*, 1998; Serbina *et al.*, 2000).

Future studies aiming to identify the "right" correlates of protection to be induced by novel TB vaccines should possibly use a combined approach of measurements of antigen-specific host responses as well as functional growth inhibition assays to identify the most suitable candidates to move forward in vaccine trials. Moreover, an *in vitro* system may also facilitate a systematic evaluation of mycobacterial antigen for their ability to induce effective in vitro immunity, and thus provide a screening method for evaluating vaccine candidates, as we had already shown for the BCG vaccine (Kampmann *et al.*, 2004).

CHAPTER 7:

General discussion

The findings of this thesis are relevant for the assessment of novel TB vaccines, as they provide background data of sensitization to mycobacterial antigens from a TB-endemic population of children with and without HIV-infection. These are the types of cohorts in which current and future trials of novel TB vaccines are placed, and it is important to know about the existing background responses, which might play a role in the assessments of "vaccine take".

In this thesis, we show evidence of the successful development and application of the powerful tools of multiparameter flow cytometry to small whole blood samples collected from childhood populations. We were successful in the development of a complicated multi-colour, multiparameter panel for flow cytometric analysis of rare events, combined with the ability to phenotype the memory T cells. This panel took a long time to develop but is now in use by our own group and has inspired the use of similar fluorochromes by other users in the group. As always, the limitations lie with the still limited amount of parameters, which can be measured simultaneously when using flow cytometry, the long process of optimising the panel and the more complicated data analysis required when many parameters are included. This is an innate problem with flow cytometry, which is offset by the ability of the tool to characterise phenotype and function at the single cell level.

We were able to measure all parameters in comparatively small volumes of blood, which are ideal for studies in children, as conducted for this thesis.
By applying the flow cytometric methods in this study we found that a substantial proportion of children have mycobacteria-specific T cell populations, expressing IFN-γ and/or IL-2, which potentially could be augmented by new vaccines in a heterologous boosting strategy. We have

examined these responses in 3 different groups of children and have found sizeable responses, even in HIV-infected individuals. Our data illustrate the enormous inter-individual variability between donors, which led to the observation that there were no significantly different responses measurable between HIV-infected children and healthy, HIV-negative children. This clearly differed from our hypothesis. A limitation is the study size and potentially age of the children, although the ages were carefully matched and no correlation was found between age and antigen-specific responses in either HIV-positive or negative children. It is likely that the antigen-specific responses mirror natural exposure and that the exposure is similar in all cohorts, as they reside in the same TB-endemic environment.

Few studies have conducted careful phenotyping of mycobacteria-specific memory responses in children living in TB endemic settings to date. We have gathered data on antigen-specific and non-specific CD4 and CD8 populations, which show that the overall distribution of the memory phenotypes did not differ between the groups, but that the cytokine-producing CD4 and CD8 T cells had a clear effector memory phenotype. This confirms findings in adults and also in vaccine-induced responses.

The additional experimental approach we chose was to combine the flow cytometric, single cell measurements with a functional assay of anti-mycobacterial immunity, which we have previously used. Our previous results had already validated this model in the context of BCG vaccination and immune reconstitution in children receiving ART, but we now wished to test the model using the virulent H37Rv strain of *M. tuberculosis* (MTB lux), again genetically modified with the luciferase genes.

We were able to show concordant results compared with the use of BCG lux. In addition we could correlate the mycobacterial growth restriction with total numbers of CD4 T cells, but not with antigen-specific or polyfunctional responses. The exception was the observation that at 12 months, mycobacterial growth restriction correlated with production of IL-2 by antigen-specific CD4 T cells. This is encouraging, since the recovery of central

memory has been observed in adult HIV-infected patients receiving ART (Wilkinson *et al.*, 2009), but this has not been correlated with functional ability to contain mycobacteria *in vitro*. This observation only occurred at the later time-point, possibly indicating the need for prolonged immune reconstitution with ART to achieve better immunological control.

However, we did not observe a significant correlation between polyfunctional T cell responses and mycobacterial growth restriction, suggesting that polyfunctional T cells may not mediate a detectable *in vitro* protective effect. Polyfunctional T cells are frequently measured to gauge "vaccine take" or immunogenicity of candidate TB vaccines with the hypothesis that these cells may be the best estimate of protective T cell responses. Our data support a recent paper from our group that identified the lack of such a correlation between polyfunctional T cells and protection against TB in BCG-vaccinated infants, where infants who developed TB were compared with matched controls who did not develop TB (Kagina *et al.*, 2010). No difference in the frequencies of polyfunctional T cells was found between the 2 groups.

Unfortunately, we still do not know if measurable parameters routinely evaluated in TB vaccine development will correlate with lower risk of TB in children in either natural exposure or following vaccination. We acknowledge that we have not shown protection against tuberculosis in our studies, but it might still be important to include additional assays such as the lux assay, which have a functional read-out of anti-mycobacterial immunity in future studies of novel TB vaccines.

In summary, this work has led to a comprehensive characterisation of mycobacteria-specific CD4 and CD8 responses in HIV-infected and uninfected children, including the assessment of memory, functional anti-mycobacterial capabilities and the impact of ART. We hope that in the future, safe, novel TB vaccines are tested rapidly in HIV-infected children, since they remain particularly vulnerable to developing TB. The best time to vaccinate this group might indeed be prior to depletion of their cellular immune responses and again after ART-induced recovery of their naïve and central

memory populations. This group of children might also benefit from revaccination, as ART might not fully restore vaccine-induced immunity established before the start of therapy. However, levels of immunity or memory to pathogens might need to be monitored and some children might need additional vaccine doses to restore or maintain immunological memory to pathogens.

We believe that our work has contributed to knowledge in this field.

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