GROWTH OF LUCIFERASE-TRANSFECTED BCG IN WHOLE BLOOD:

Use of a novel assay to study immune responses to mycobacteria in children

University of Cape Town

Nontobeko Gwendoline Tena

A thesis submitted to the Faculty of Medicine/Health Sciences, University of Cape Town, in fulfillment of the requirements for the degree of
Master of Science

Cape Town, 2003

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Abstract

Tuberculosis (TB) is a leading cause of death worldwide. Human immunodeficiency virus (HIV)-infected individuals have a significantly higher incidence of active TB, adding to the global burden. The rise in TB disease rates has been accompanied by the emergence of strains resistant to one or more of the commonly used therapeutic drugs. Differences in immune responses, possibly linked to age differences, are likely to determine who develops disease after infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) and who remains well. Animal models have been useful in elucidating the mechanisms for protection against mycobacterial infection but the mechanisms for a protective immune response are not well understood in the human host. Amongst other reasons, the more serious nature of the disease in children heightens the need for increased research into the problem of childhood TB.

The existing *Bacille Calmette-Guerin* (BCG) vaccine offers some protection against TB, but its efficacy varies for unknown reasons. In order to develop an effective vaccine, a comprehensive understanding of protective immunity in adults and children is needed. Studies involving host-pathogen interactions are complex and have been hampered by the slow growth of mycobacterial organisms. A novel *in vitro* whole blood assay which employs reporter-gene tagged BCG to study human immune responses to mycobacteria has recently been developed [71]. This assay combines the advantage of rapid enumeration of mycobacteria with the ability to study immune responses in small volumes of whole blood. It is therefore well suited for investigative work in paediatric populations. Here, we employ this novel assay to study immune responses to mycobacteria in children in regions of high incidences of childhood TB in Cape Town, South Africa.

We investigated the ability of whole blood to restrict growth of luciferase-transfected BCG (BCG *lux*) *in vitro* in healthy children of different age groups as well as HIV-positive children in order to study the influence of age and immune competence on growth-restricting properties of whole blood. Association between growth inhibition and cytokine production was also assessed. Although there was a trend towards

University of Cape Town

increased mycobacterial growth in the very young, healthy children, compared to older, healthy children, this did not reach significance.

However, children with HIV infection demonstrated a significantly decreased ability to control mycobacterial growth when compared with all healthy control children and with an age-matched group, $p<0.0001$. There was a significant difference in interferon gamma (IFNγ) production between age groups in healthy children ($p<0.006$) as well as between the healthy and HIV-infected groups, $p<0.0001$. There was no significant difference in levels of tumour necrosis factor alpha (TNFα).

The reduced ability to control mycobacterial growth in peripheral blood *in vitro* is consistent with the known susceptibility to clinical tuberculosis in HIV-infected populations. This model is therefore applicable to further studies of antimycobacterial responses such as in TB infection and disease. Our data suggest that the whole blood luminescent assay might also be a useful tool to assess changes in immune responses induced by vaccination.

University of Cape Town

Table of Contents

	Page
Title page	i
Abstract	ii-iii
Contents pages	iv-vii
List of figures	viii-ix
List of tables	x
Abbreviations	xi-xiii
Publications and presentations	xiv
Declaration	xv
Acknowledgements	xvi
Chapter 1: General Introduction	1
1.1 Tuberculosis	1
1.1.1 Global epidemiology of tuberculosis	1
1.1.2 Epidemiology of tuberculosis in South Africa	2
1.1.3 Pathogenesis	2
1.1.4 The organism	4
1.2 BCG	4
1.3 Immunity to tuberculosis	4
1.3.1 Host response to *Mycobacterium tuberculosis*	4
1.3.2 Cell mediated immunity	8
1.3.2.1 $CD4^+$ T cells	8
1.3.2.2 $CD8^+$ T cells	9
1.3.2.3 γδ Tcells	9
1.3.3 Correlates of protection	10
1.3.3.1 Interferon gamma	10
1.3.3.2 Interleukin-12	11
1.3.3.3 Tumour necrosis factor alpha	11
1.4 Childhood tuberculosis	11
1.5 Tuberculosis in the HIV-infected child	12

University of Cape Town

1.6 Reporter gene strategies 13
1.6.1 Biochemistry of bioluminescence 13
1.6.2 Common *lux* genes 13
1.6.3 Regulation of *lux* genes 14
1.6.4 Reporter constructs 14
1.7 Whole blood assays 15
1.8 Aim of project 17
1.9 Hypothesis and specific objectives 17
1.9.1 Specific objectives 17

Chapter 2: Materials and Methods 18
2.1 Human subjects 18
2.1.1 Healthy adult laboratory volunteers 18
2.1.2 Healthy children 18
2.1.3 HIV-infected children 19
2.2 Mantoux testing 19
2.3 Tissue culture 20
2.3.1 Materials and reagents 20
2.3.2 Blood sample collection 20
2.4 Recombinant mycobacteria 21
2.4.1 Mycobacterial strain 21
2.4.2 Plasmid construction 21
2.5 Bacterial culture 22
2.5.1 Media 22
2.5.1.1 Liquid media 22
2.5.1.2 Solid media 22
2.5.1.3 Supplements for mycobacterial media 22
2.5.2 Preparation and storage of mycobacteria 23
2.5.3 Preparation of mycobacterial culture for inoculation into whole blood 23
2.6 Bioluminescence assay 24
2.6.1 Protocol 24
2.6.2 Bioluminescence detection 24

University of Cape Town

2.7 CD4 Count determination 25
2.7.1 Materials and reagents 25
2.7.2 Immunofluorescent staining 26
2.7.3 Flow cytometric analysis 26
2.8 Analysis of supernatants by ELISA 26
2.8.1 Materials and reagents 27
2.8.2 Protocol 29
2.9 Statistical methods 30

Chapter 3: Validation of a Whole Blood Assay to Measure Growth of Mycobacteria 32
3.1 Introduction 32
3.2 Methods 33
3.3 Results 33
3.3.1 Relationship between luciferase activity and colony forming units 33
3.3.2 Intra-and Inter-assay variability 36
3.4 Discussion 39

Chapter 4: Growth of Mycobacteria in Whole Blood of Tuberculin Positive and Tuberculin Negative Volunteers 40
4.1 Introduction 40
4.2 Methods 41
4.3 Results 41
4.3.1 Growth of BCG *lux* in whole blood of tuberculin positive and tuberculin negative individuals 41
4.3.2 Associated cytokine production 42
4.3.3 Relationship between mycobacterial growth and cytokine production 45
4.4 Discussion 47

University of Cape Town

Chapter 5: Growth of Mycobacteria in Whole Blood of Healthy and HIV-Infected Children 48

5.1 Introduction 48

5.2 Methods 50

5.3 Results 50

5.3.1 Growth of mycobacteria in whole blood of healthy children 50

5.3.2 Associated cytokine production in whole blood of healthy children 53

5.3.3 Comparison of mycobacterial growth in whole blood of healthy and HIV-infected children 55

5.3.4 Comparison of cytokine production in whole blood of healthy and HIV-infected children 57

5.4 Discussion 59

Chapter 6: Concluding Discussion and Future Prospects 60

Appendices 63

1. Consent form for blood collection from healthy children 63
2. Consent form for blood collection from HIV-infected children 64
3. Tables of individual test values 65

References 69

University of Cape Town

List of Figures

		Page
1.	Global picture of tuberculosis	1
2.	Phagocytosis and immune recognition of *M. tuberculosis*	6
3.	*Mycobacterium tuberculosis* cell wall composition	7
4.	Diagram of the potential sequence of events in whole blood, illustrating the interaction between mycobacteria, macrophages and T cells	8
5.	Plasmid map of pMST1	21
6.	Growth of BCG *lux* measured by relative light units (○ RLU) and colony forming units (● CFU)	34
7.	Growth of BCG *lux* in whole blood from a tuberculin positive (● PPD +ve) and a tuberculin negative (○ PPD -ve) individual	34
8.	Growth of BCG *lux* in a single sample of blood obtained from a tuberculin positive individual	37
9.	Growth of BCG *lux* in whole blood obtained from a tuberculin positive individual and processed simultaneously by three different investigators at the same time	37
10.	(a) IFNγ and (b) TNFα produced during incubation of whole blood obtained from (■) tuberculin positive and (▲) negative individuals with BCG *lux*	44
11.	(a) IFNγ and (b) TNFα production from individuals showing restricted mycobacterial growth (<4.5) and individuals that failed to restrict mycobacterial growth (▲ >4.5)	46
12.	Growth of mycobacteria in whole blood of tuberculin positive and tuberculin negative healthy children (a) <1 year, (b) 1-4 years, (c) 5-9 years and (d) 10-15 years	52
13.	Correlation between (a) IFNγ levels and growth ratio and (b) IFNγ levels and age	54
14.	Comparison of mycobacterial growth in whole blood of (▲) HIV-infected children (n=22) and an age-matched (■) control group (n=24)	56
15.	Correlation between growth ratio and CD4 T cells	57

University of Cape Town

16. Comparison of (a) IFNγ and (b) TNFα production in whole blood of (■) healthy (n=24) and (▲) HIV-infected children (n=22) 58

University of Cape Town

List of Tables

		Page
1.	Comparison of growth ratios obtained for three tuberculin positive and three tuberculin negative individuals after using the old starting inoculum ($5X10^6$ RLU/ml) and the corrected starting inoculum ($1.5X10^8$ RLU/ml)	35
2.	Reproducibility of the whole blood assay	38
3.	Comparison of growth ratios between 0 and 96 hours of culture of mycobacteria in whole blood of tuberculin positive and tuberculin negative healthy adult volunteers	42
4.	The efficacy of BCG as determined in nine major trials	49
5.	Comparison of growth ratios obtained after culture of mycobacteria in whole blood of healthy children between the ages of 0-15	51
6.	Production of IFNγ and TNα in whole blood from healthy children during *in vitro* infection with BCG *lux*	53
7.	Clinical and immunological characteristics of HIV-infected children following the 1994 CDC classification system	55
8.	IFNγ and TNFα produced during incubation of whole blood obtained from tuberculin positive and negative individuals with BCG *lux*	65
9.	IFNγ and TNFα production from individuals showing restricted mycobacterial growth (growth ratio <4.5) and individuals that failed to restrict mycobacterial growth (growth ratio >4.5)	65
10.	IFNγ and TNFα produced during incubation of whole blood obtained from healthy children of different ages with BCG *lux*	66
11.	Growth ratios of HIV-infected children and an age-matched control group (1-4 years)	67
12.	IFNγ and TNFα produced during incubation of whole blood obtained from healthy children (1-4 years) and HIV-infected children with BCG *lux*	68

University of Cape Town

Abbreviations

α	Alpha
β	Beta
δ	Delta
γ	Gamma
µl	Microlitre
ADC	Albumin D-glucose Catalase
AIDS	Acquired Immuno Deficiency Syndrome
att	Attachment Site
AVP	Avidin Peroxidase
BCG	Bacillus Calmette-Guérin
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CD	Cluster Designation
CFU	Colony Forming Units
CR	Complement Receptor
CV	Coefficient Variation
dH_20	Distilled water
DNA	Deoxyribonucleic Acid
DOTS	Directly Observed Treatment Strategy
DTH	Delayed Type Hypersensitivity
ECD	Phycoerythrin-Texas Red®-X
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
E.coli	Escherichia coli
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FMN	Flavin Mononucleotide
$FMNH_2$	Reduced Flavin Mononucleotide
GR	Growth Ratio
HIV	Human Immunodeficiency Virus
HSL	Homoserine Lactone

University of Cape Town

IFNγ	Interferon Gamma
int	Integrase
IL	Interleukin
IUATLD	International Union against Tuberculosis and Lung Disease
LAM	Lipoarabinomannan
L	Litre
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acids
lux	Bacterial luciferase
M. africanum	*Mycobacterium tuberculosis*
M. fortuitum	*Mycobacterium fortuitum*
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
MAbs	Monoclonal Antibodies
macNOS	Macrophage NOS
MHC	Major Histocompatibility Complex
MDR	Multi-drug-Resistant
mg	Milligram
ml	Millilitre
min	Minute
mm	Millimetre
M	Molar
mM	Millimolar
MPO	Myeloperoxidase
MRC	Medical Research Council
NIAID	National Institute of Allergy and Infectious Diseases
N	Nolar
NK	Natural Killer
Nm	Nanometer
NO	Nitric Oxide
$NO^{\bullet}$	Nitric Oxide Radical
NOS2	Synthase
OADC	Oleic Acid Albumin D-glucose Catalase

University of Cape Town

OD	Optical Density
$OH^{\bullet}$	Hydroxyl Radical
OPD	o-Phenylenediamine Dihydrochloride
O_2	Oxygen
ori	Origin of Replication
PBS	Phosphate Buffered Saline
PC5	Phycoerthrin-Cy5
p	Plasmid
pg	Picogram
PPD	Purified Protein Derivative
RD1	Phycoerythrin
rev	Revolutions
RLU	Relative Light Units
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
SHP1	Phosphotyrosine Phosphatase
SD	Standard deviation
TB	Tuberculosis
TGFβ	Transforming Growth Factor Beta
TLR	Toll-Like Receptor
TNFα	Tumour Necrosis Factor Alpha
TU	Tuberculin Units
U	Units
USA	United States of America
V. fischeri	*Vibrio fischeri*
V. harveyi	*Vibrio harveyi*
WHO	World Health Organization

University of Cape Town

Publications and Presentations

Tena NG, Young DB, Eley B, Henderson HH, Nicol MP, Levin M, Kampmann B. Failure to control growth of mycobacteria in blood from children infected with HIV and its relationship to T cell function. J Infect Dis, 2003. 187 (10): p1544-51.

This work was presented in part at the Fifth International Conference on the Pathogenesis of Mycobacterial Infections, Saltsjobaden, Stockholm, Sweden, June 2002 (poster no.123).

University of Cape Town

Declaration

I, Nontobeko Gwendoline Tena, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise). It is being submitted for the degree of Master of Science in the University of Cape Town. It has not been submitted before for any degree or examination at this or any other University.

N. G. Tena

August 2003

University of Cape Town

Acknowledgements

I would like to thank my colleagues in the Department of Paediatrics at Red Cross Children's Hospital in Cape Town for their unlimited assistance.

I am grateful to the National Research Foundation for funding my MSc degree and the Wellcome trust for providing additional financial support giving me the opportunity to obtain training and work at Imperial College, St Mary's Hospital in London. I would also like to thank my colleagues in the Department of Paediatrics and Department of Medical Microbiology at the London laboratories for their assistance in my training.

My special thanks go to my supervisors, Professor Howard Henderson, Dr Brian Eley and Dr Beate Kampmann for entrusting me with this subject. I was privileged to experience constructive support throughout my thesis from each of them in their own personal way.

Finally thank you to my family for their encouragement and unconditional support throughout my thesis.

University of Cape Town

Chapter 1: General Introduction

1.1 Tuberculosis

1.1.1 Global epidemiology of tuberculosis

The World Health Organization (WHO) estimates that at least one-third of the world's population (or 2 billion people) is infected with TB [1]. Every year, eight million people worldwide develop active TB, of whom 95% are in the developing world [2], **figure 1**. Some industrialized countries have recently seen re-emergence of this health problem due to the increase in people moving across the continents [3]. TB kills more than two million people each year, more deaths than caused by malaria [4], and is considered as "the world's second commonest cause of death from infectious disease, after HIV/AIDS" [5].

University of Cape Town

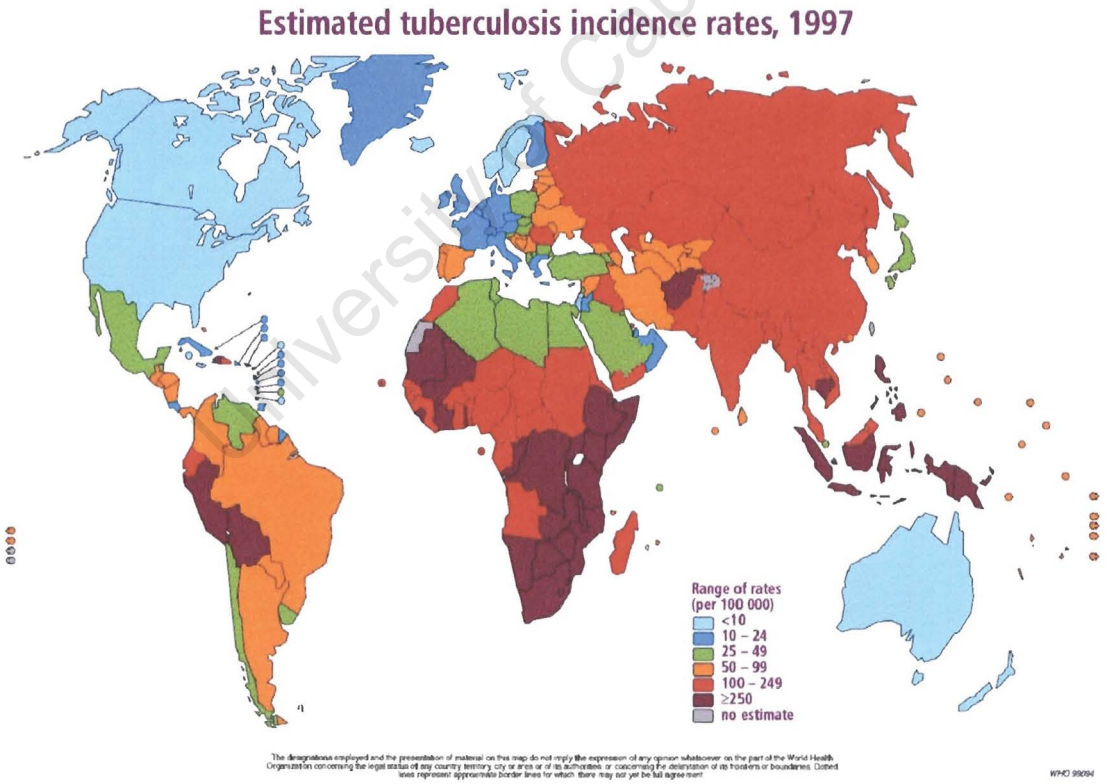


Figure 1. Global picture of tuberculosis

It has been estimated that up to 50 million people are infected with drug-resistant forms of TB. The development of drug resistance in the population has increased concern that TB may once again become an incurable disease. "Drug-resistant TB is caused by inconsistent or partial treatment, when patients do not take all their anti-TB medication regularly for the required period because they start to feel better, doctors and health workers prescribe the wrong treatment regimens or the drug supply is unreliable" [3]. Of particular concern is the development of multi-drug-resistant forms of the disease (MDR- TB), defined as forms resistant to two or more of the front line anti-TB drugs such as isoniazid and rifampicin. These forms of the disease are more often fatal and are difficult and expensive to treat [6].

1.1.2 Epidemiology of tuberculosis in South Africa

The appraisal of the TB situation by organisations like the WHO and IUATLD (International Union against Tuberculosis and Lung Disease) have shown that South Africa is burdened by one of the worst TB epidemics in the world. The disease rates are reported to be more than double those observed in other developing countries and up to 60 times higher than those currently seen in the USA or Western Europe [7]. There are many factors that have contributed to this epidemic. Amongst these, historical neglect and poor management systems have played a major role [8]. The problem is compounded by the legacy of fragmented health services in both the cities and the country. The co-existence of TB and HIV infection has led to escalating increases in TB rates in provinces such as KwaZulu-Natal, Mpumalanga and Gauteng. However, the TB control programme has reported dramatic improvements in cure rates in certain provinces such as Mpumalanga (despite relatively high HIV rates) and the Western Cape. This has been attributed to the disciplined implementation of the (Directly Observed Treatment Strategy) DOTS strategy of the WHO in these provinces. Despite improvement in the TB control programme since its introduction in the early 1990s stringent measures must be taken in implementing to ensure continued improvement in TB control.

1.1.3 Pathogenesis

Tuberculosis is transmitted in the form of aerosol droplets entering the lungs [9]. Pulmonary tuberculosis is the best known form of the disease and is characterised by a cough (often with bloody sputum), accompanied by weakness or fatigue, chest pain,

University of Cape Town

weight loss, fever and night sweats. The disease can also spread internally, via the blood, becoming miliary or disseminated tuberculosis, and infecting internal organs, skin, glands, bone and brain (tuberculous meningitis). Primary infection causes disease in 5% to 10% of adult patients and in an even higher percentage of children and persons with HIV infection [3]. Secondary tuberculosis (endogenous reactivation disease) is usually due to the reactivation of old lesions or gradual progression of primary tuberculosis into chronic form. However, recent evidence suggests that exogenous reinfection is responsible for some of the secondary tuberculosis [10]. *M tuberculosis* microfoci that have remained dormant after primary infection (latent tuberculosis) may undergo reactivation, and patients often are asymptomatic in the early stages. The chances of an individual with a latent form of *M. tuberculosis* developing active tuberculosis increases from roughly 10% over a lifetime to a 10% annual risk when coinfected with HIV [3].

1.1.4 The organism

It has been known since the late 19th century that tuberculosis in humans is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) [11], but in some areas, mainly Equatorial Africa, it is also caused by *M. africanum*. *M. tuberculosis* can infect a wide variety of animals but man is the principal host [12]. *M. tuberculosis* is a bacterium that is an obligate aerobe, rod-shaped, acid-fast bacilli (due to the ability of mycobacteria to retain the colour imparted by arylmethane dyes after treatment with weak mineral acids) [13] and has a characteristic mycolic acid cell wall. The mycobacterial genome is a single chromosome in a closed loop, carrying 4.4 million base pairs of genomic information encoding around 4000 genes. Several factors make *M. tuberculosis* a difficult organism to study in the laboratory, hindering tuberculosis research. The bacteria multiply very slowly, only once every 24 hours, and take a month to form a colony. Tuberculosis bacilli tend to form clumps, which makes working with them and counting them difficult. Most discouraging, *M. tuberculosis* is a dangerous, airborne organism and can be studied only in laboratories that have specialized safety equipment and facilities [9].

University of Cape Town

1.2 BCG

Bacillus Calmette-Guérin (BCG), a live attenuated strain of *M. bovis*, is the only currently available vaccine against tuberculosis and is the most widely administered of all vaccines in the WHO Expanded Programme for Immunization [14]. To generate this vaccine strain, *M. bovis* was passaged through 230 serial subcultures on slices of potato soaked in bile, over a 13-year period. It confers protection against disseminated and meningeal tuberculosis in children, which are associated with high mortality rates, and it has been shown to provide some protection against leprosy, but its efficacy in preventing pulmonary tuberculosis in adults has varied dramatically in various studies in different parts of the world-from 80% in the UK to 0% in Chingleput, India [15], [16]. The favoured explanation for this difference in efficacy is that infection with other harmless environmental mycobacteria can interfere with, or mask, BCG protection [17]. Because of case reports of disseminated BCG infection [18], this vaccine is contraindicated in HIV positive individuals [19] and other immunocompromised individuals [20], [21], [22], and safer and more efficacious vaccines are clearly needed. An increasing effort is being made to design anti-tuberculosis vaccines (saprophytes, subunit, plasmid DNA, and recombinant vaccines) according to our current understanding of anti-tuberculosis immunity, in large part, from animal models [23], [24], [25].

1.3 Immunity to tuberculosis

1.3.1 Host response to *Mycobacterium tuberculosis*

The immune response to *M. tuberculosis* can be characterized as being both beneficial and damaging to the survival of the host, with the former contributing to control of mycobacterial replication and the later being attributed to the promotion of tuberculosis [26]. After the initial infection of *M.tuberculosis* the mycobacteria are taken up by alveolar macrophages (professional phagocytes) in the lung. The normal function of macrophages is to ingest and destroy intracellular bacteria. But although macrophages are able to ingest mycobacteria they are not always successful at destroying them. When macrophages fail to destroy the mycobacteria the host's immune systems next line of defence is to form granulomas around the infected macrophages. Phagocytes and lymphocytes surround the infected macrophages

University of Cape Town

sealing them inside a barrier known as a granuloma. Cytokines (especially IFNγ) influence the macrophages to transform into epithelioid cells. These epithelioid cells produce greater mediators of inflammation than their predecessors, the macrophages and T-lymphocytes, which further promotes the formation of granulomas. IFNγ also promotes the fusion of epithelioid cells into multinucleated giant cells (Langerhans cells). Hence, a tubercle is formed around the pathogen consisting of epithelioid cells, giant cells and a peripheral zone containing lymphocytes responsible for macrophage activation. This is the latent form of the disease. Although antibodies are made against several mycobacterial products, including cell-wall components, it is universally accepted that the role of humoral substances in immunity to tuberculosis has not been clearly demonstrated [27].

M. tuberculosis enters through any of several cellular molecules abundantly present on the surface of macrophages, **figure 2**, such as the complement receptors (CR), CR1, CR3 and CR4, [28], [29], fibronectin receptors [30], mannose receptors, [31], and others, to gain access to the intracellular compartments of these professional phagocytic cells [32]. Recent studies have identified mammalian homologues of the *Drosophila* Toll protein; proteins now referred to as Toll-like receptors (TLRs) [33], [34], [35]. Mammalian TLRs appear to represent a conserved family of innate immune recognition receptors that are activated by chemically diverse microbial products. In the context of CD14, TLR2 binds lipoarabinomannan, a heterodimer of TLR2 and TLR6 binds a 19-kDa *M. tuberculosis* lipoprotein, TLR4 binds to an undefined heat-labile cell-associated factor, and (possibly) TLR9 binds to *M. tuberculosis* DNA, figure 2, [36]. These receptors are coupled to a signalling pathway that is conserved in mammals, insects and plants, resulting in the activation of genes that mediate innate immune defenses. Recognition of microbial products by TLRs expressed on dendritic cells triggers functional maturation of dendritic cells and leads to initiation of antigen-specific adaptive immune responses [37]. This method of entry evades the normal macrophage ingestion process; thereby avoiding respiratory 'burst' (one of the mechanisms by which macrophages usually kill bacteria).

University of Cape Town

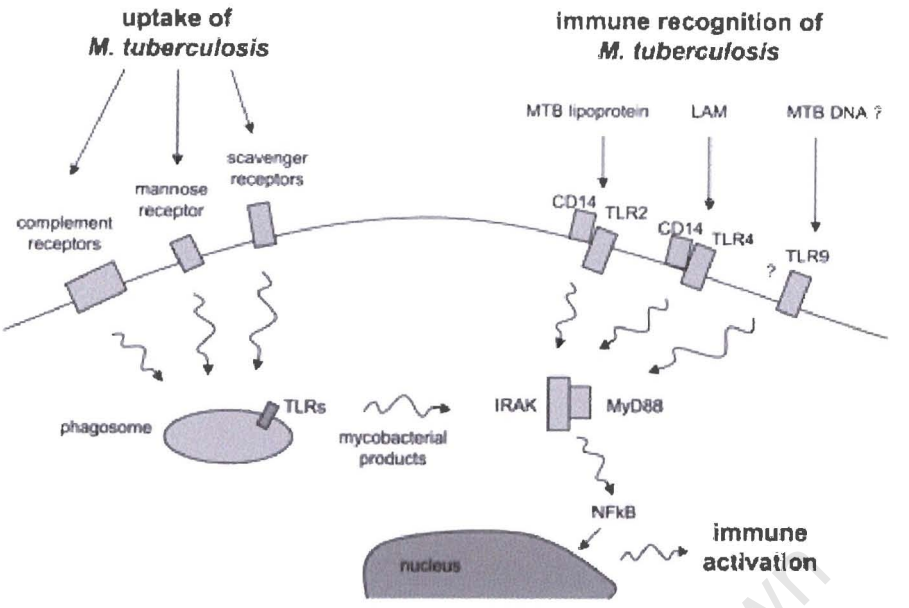


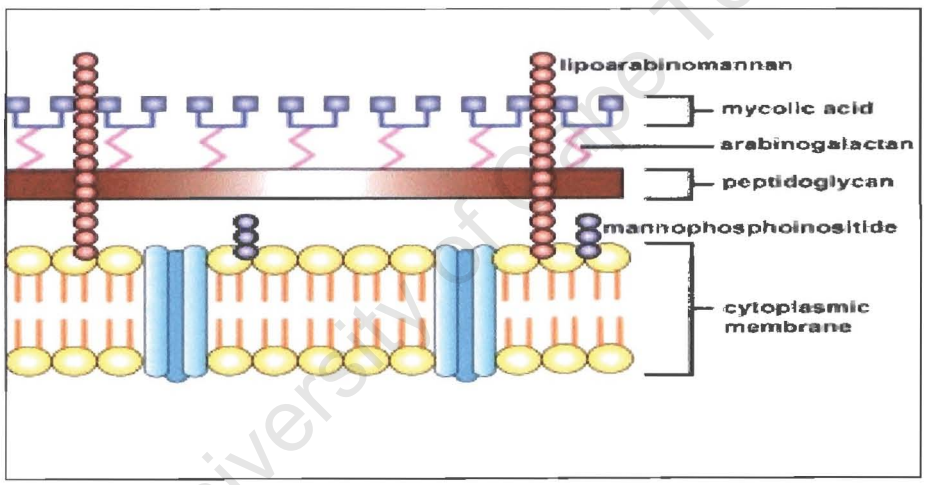
Figure 2. Phagocytosis and immune recognition of *M. tuberculosis*

University of Cape Town

Respiratory burst is a process that produces reactive oxygen intermediates (ROIs) such as H_2O_2, hydroxyl radicals (·OH) and toxic reactive nitrogen intermediates (RNIs) such as NO radicals (·NO radicals). These intermediates along with a cocktail of proteinases and other enzymes are used to acidify lysosomes (a complex vacuolar organelle of the late endocytic pathway) within the macrophages, which usually fuse with and kill any ingested bacteria [38]. Within the lysosomal vacuoles are potent hydrolytic enzymes capable of degrading a whole range of macromolecules [39]. Phagocytes generate ROIs and RNIs by myeloperoxidase (MPO), nitric oxide (NO) synthase (NOS2 [the macrophage NOS also known as macNOS or iNOS]) or NADPH oxidase. These enzymes are activated by signalling systems, which recognise microbial products (e.g. lipopolysaccharide, LPS, and lipoproteins) and cytokines (e.g. IFN-γ and IL-8). There are several known interactions between these antimicrobial effector pathways [40]. Microbes are equipped with a variety of defence mechanisms (resistance genes) that help them to survive the oxidative and nitrosative stress.

Despite induction of several potent macrophage-activating molecules such as tumour necrosis factor alpha (TNF α) and interleukin-1 (IL-1) by *M.tuberculosis*, intracellular

replication ensues because potent macrophage deactivating molecules such as transforming growth factor beta (TGF β) and (IL-10) are also induced by *M. tuberculosis*; these counteract the macrophage activating cytokines (TNF α and IFN γ) and microbicidal molecules (ROI and RNI). Lipoarabinomannan (LAM), one of the polymers that form the mycobacterial cell envelope, **figure 3,** is also thought to dampen down the host immune response by disrupting the protein phosphorylation-mediated signalling mechanisms of the macrophage. LAM is capped with a mannose residue, which both inhibits tyrosine phosphorylation in the macrophage and alters the acidity of the phosphotyrosine phosphatase, SHP1, an enzyme that dephosphorylates tyrosine residues, thereby disrupting macrophage signal transduction. This results in reduced stimulation of cytokines such as (TNF α and IL-1).



University of Cape Town

Figure 3. *Mycobacterium tuberculosis* cell wall composition

The organism's ability to alter intracellular iron concentrations, through mycobactins, may allow modulation of the production of macrophage activating cytokines [32]. *M.tuberculosis* evasion of these powerful intracellular mechanisms subsequently results in uncontrolled intracellular replication, which eventually may culminate in the rupture of infected macrophages and spread of infection to other cells. This allows for the initiation of both extracellular growth, and tissue damage culminating in caseation necrosis. Consequently, lymphohematogenous spread allows the seeding of both pulmonary and extrapulmonary sites.

1.3.2 Cell mediated immunity

The protective immune response to tuberculosis involves the activation of infected macrophages by antigen-specific T cells and the subsequent killing of intracellular tubercle bacilli, **figure 4**. Studies using gene knockout mice have shown that IFNγ is the essential activating cytokine [41], [42], [43] and that $CD4^+$ and $CD8^+$ T cells are both involved in protection. A role for γδ T cells is supported by functional studies demonstrating their capacity to display cytotoxicity and produce IFNγ in response to *M. tuberculosis* infected macrophages [44].

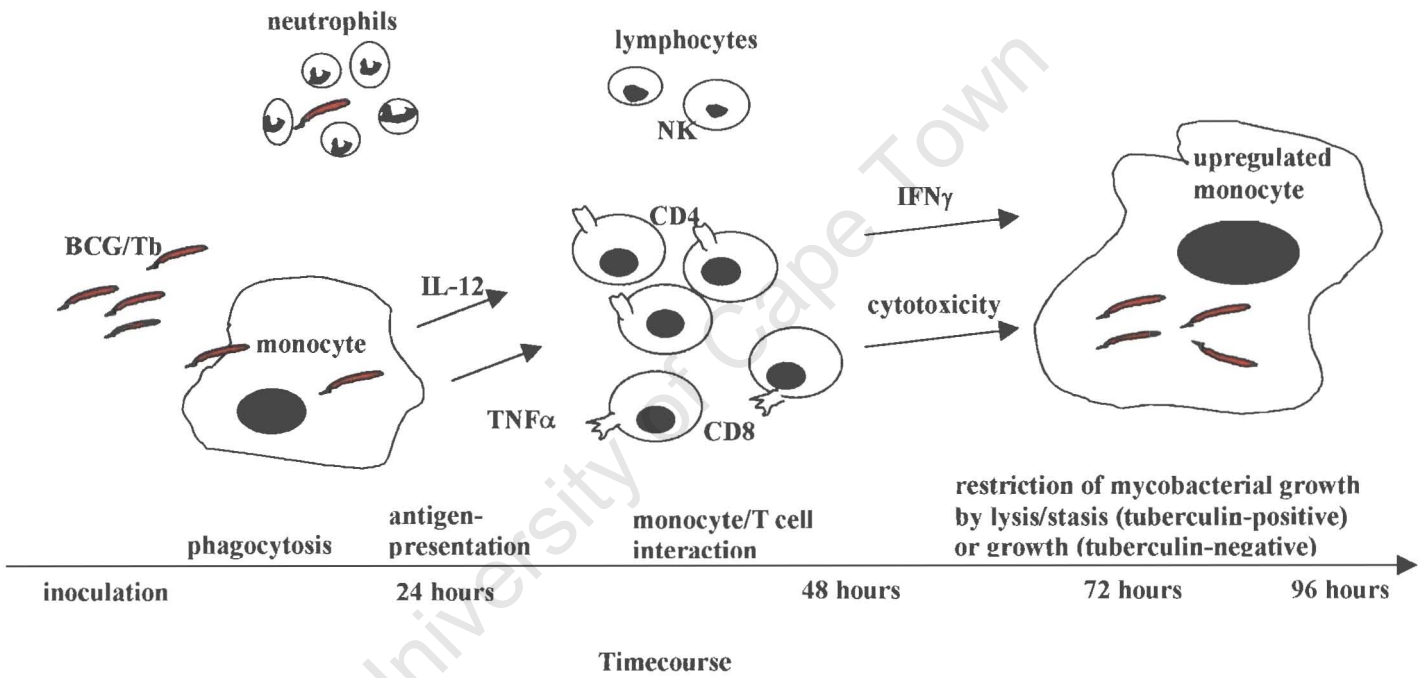


Figure 4. Diagram of the potential sequence of events in whole blood, illustrating the interaction between mycobacteria, macrophages and T cells

1.3.2.1 $CD4^+$ T cells

$CD4^+$ T cells reactive with mycobacterial antigens produce cytokines, chiefly of the Th1 pattern, and also engage in major histocompatibility complex (MHC) class II-restricted killing of antigen-presenting cells infected with *M. tuberculosis* [41]. Among these cytokines IFNγ seems to be the crucial effector molecule in mice [45], [43], [42], as well as in humans [46], [47]. In mice, IFNγ mediates its protective

University of Cape Town

effect predominantly by the induction of RNI, which are necessary for the killing of the intracellular mycobacteria [48], [49]. In contrast, the contribution of RNI to immunity against intracellular pathogens in humans still remains a matter of discussion. Although $CD8^+$ T cells also secrete this cytokine, $CD4^+$ T cells probably constitute the major source of this protein.

1.3.2.2 $CD8^+$ T cells

In contrast to $CD4^+$ T cells, the role of $CD8^+$ T cells in the immune response to *M. tuberculosis* has received little attention. However, in recent years isolation of $CD8^+$ T cells [50], [51] specific for mycobacterial antigens from infected hosts support the notion that $CD8^+$ T cell responses as well as $CD4^+$ T cell [41] responses must be stimulated to provide protective immunity. $CD8^+$ T cells in mycobacterial infections are both classically and nonclassically restricted. In contrast to the peptide epitopes presented by MHC class Ia molecules (classical), CD1 molecules (nonclassical) present lipids or glycolipids to T cells [52]. There appear to be two primary effector functions for $CD8^+$ T cells in tuberculosis: lysis of infected target cells such as monocytes and macrophages, and production of cytokines, namely IFNγ. When macrophages release the intracellular bacteria, activated effector cells that have been recruited through a variety of signals can then kill those bacteria. The role of IFNγ is generally believed to be macrophage activation. $CD8^+$ T cells also possess direct microbicidal activity. A granule-associated T-cell protein called granulysin can be secreted by $CD8^+$ T cells, and can kill extracellular *M. tuberculosis* directly. In concert with perforin, another T-cell product, it may also kill intracellular mycobacteria.

1.3.2.3 γδ Tcells

$CD4^+$ and $CD8^+$ T cells, which constitute more than 90% of all peripheral T cells in humans and mice, use the αβ chain T cell receptor (TCR) for antigen recognition. A small T cell subgroup (< 5%) exists which utilises the alternate TCR of a γδ chain and are generally double negative (neither CD4 nor CD8 markers are present). These γδ T cells occur in small numbers in peripheral blood but have also been found to be highly responsive to mycobacterial antigens in both murine and human studies [53], [54] Mice deficient in γδ T cells suffer from exacerbated tuberculosis. The findings

University of Cape Town

suggest an early role of γδ T cells in protection against experimental tuberculosis. The activation of γδ T cells in humans depends on antigen-presentation to these cells via mononuclear phagocytes, which are infected with live *M. tuberculosis* [55]. In humans and primates, γδ T cells specifically respond to non-peptidic phosphoantigens from *M. tuberculosis* without the need for any MHC presentation [56]. Like CD8 αβ T cells, the γδT cell exerts potent cytotoxic effector activity for *M. tuberculosis* and produces large amounts of IFNγ [44]. Although the functional role of γδ T cells in human immunity remains uncertain, it is generally believed that they contribute to control of primary infection before the αβ response has become established [57].

1.3.3 Correlates of protection

The majority of people exposed to tuberculosis mount a protective immune response that controls the infection at the initial stages and protects against re-infection. However, our knowledge about human correlates of protection against tuberculosis remains limited. Although no definite correlate has been identified the importance of cytokines such as IFNγ, TNFα and IL-12 in the modulation of the immune response is beyond doubt.

1.3.3.1 Interferon gamma

Studies in genetically modified mice indicate that IFNγ is essential for protective immunity against *M. tuberculosis* [43]. In this model the effects of IFNγ are mediated in large part by nitric oxide pathways. However, the murine model is only partially reflective of human tuberculosis as $NO^{\bullet}$ has not been identified as a free radical involved in mycobacterial killing. Individuals with an IFNγ receptor abnormality show marked susceptibility to infection with atypical mycobacteria [46] disseminated infection with the BCG strain of *M. bovis* [58], [22] and infection with *M. tuberculosis*. This pro-inflammatory cytokine has multiple beneficial actions, many of which are centered on its effects on macrophage biology. IFNγ has several other effects on macrophages, including the increased expression of major histocompatibility complex (MHC) class II molecules, thus leading to increased antigen presentation and further amplification of the immune response. IFNγ also upregulates expression and secretion of TNFα from macrophages.

University of Cape Town

1.3.3.2 Interleukin-12

Interleukin-12 (IL-12) is a cytokine of prime interest as it is an efficient stimulator of cells that produce IFNγ. In the murine model of tuberculosis, treatment with exogenous Il-12 increases resistance to tuberculosis [59]. Il-12 enhances IFNγ production by antigen-specific CD4 cells and shifts the immune response towards a TH1 type response. Treatment with IL-12 increases the accumulation of macrophages in infected tissues. In vivo studies have confirmed that IL-12 is a crucial cytokine in both murine and human immune responses: mice with a genetic defect of the IL-12 receptor [59], [60] as well as humans with defects in the IL-12 receptor gene [61],[62] or IL-12 gene itself [63] are highly susceptible to infection with intracellular pathogens, in particular mycobacteria, emphasising the fundamental importance of this cytokine for the protective immune response against mycobacteria.

1.3.3.3 Tumour necrosis factor alpha

Although it has been long considered a crucial component of protection in tuberculosis, TNFα has also been suggested as the mediator of tissue damage during tuberculosis. Its expression by macrophages is induced by *M. tuberculosis*. TNFα-receptor gene deficient mice are extremely susceptible to tuberculosis [64]. These mice show delayed expression of RNIs, which may be ascribed to the absence of TNFα activation of macrophages. The lack of TNFα mediated effects also results in the lack of epithelioid cells within granulomas, although it does not affect the total number of granulomas formed [64].

University of Cape Town

1.4 Childhood tuberculosis

Of the 3 million deaths due to TB, more than 250 000 are said to occur in children. The pathogenesis of TB in children and protective immune responses have not been properly investigated to date. The source of transmission of TB, to a child is usually an adult (usually a family member) with sputum smear-positive pulmonary TB [65]. Children rarely have sputum smear-positive TB, so they are rarely infectious. Treatment is necessary as pulmonary TB can lead to the more serious forms of the disease such as disseminated TB and TB meningitis. The risk of progression of

infection to disease is greatest in children <1 year old and decreases with increasing age [66] probably reflecting the immature immune response of young children [67]. The diagnosis of childhood TB is difficult, due to the low numbers of bacteria, lack of sputum positivity and frequently negative Mantoux [68], [69]. Improved diagnostic methods are urgently required for childhood disease.

1.5 Tuberculosis in the HIV-infected child

Immune suppression, notably that due to HIV infection, greatly enhances the risk of primary infection progressing to active TB and increases the chance of terminating *M. tuberculosis* latency [26]. HIV infection increases replication of *M. tuberculosis* in the human host and vice versa. Although most HIV-related TB is of the reactivation type, primary disease of all clinical types also occurs, particularly in children. HIV-related TB differs from the classical types in that, as immune responses are suppressed, there is limited walling-off of the lesions. Extrapulmonary TB, for example, lymph node TB, tuberculous meningitis and abdominal TB are said to be present in 25% of children with TB. Patients with dual infection have decreased $CD4^+$ T lymphocytes and show increased suppression of IFNγ production and an increase in TNFα and TGFβ. Increased production of these cytokines during HIV-tuberculosis coinfection may contribute to increased viral replication and dissemination.

The loss of $CD4^+$ T lymphocytes also promotes the development of the AIDS complex and further susceptibility to opportunistic infections. HIV infection and AIDS causes considerable diagnostic problems and unfortunately, the tuberculin test, which is considered as one of the mainstays of the diagnosis of tuberculosis in childhood is frequently negative. Hence, it has been decided that 5 mm induration on Mantoux testing can be accepted as a 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. The period of treatment for TB in HIV-infected children remains controversial. However, a minimum period of 9 months is recommended [70]. Strict supervision with chest radiographs and follow-up cultures for *M. tuberculosis* is highly recommended. Despite the controversies surrounding

University of Cape Town

BCG vaccination [18], [19] in the face of the AIDS epidemic, its use in HIV-infected children, but not those with active AIDS is still recommended by the WHO.

1.6 Reporter gene strategies

It has been difficult to study host-pathogen interactions *in vitro*, as *M.* tuberculosis grows extremely slowly (requiring 2-6 week culture periods by conventional methods) and multiple components of the immune system have to interact. Kampmann *et al.*, [71] recently described a new bioluminescence assay, which employs reporter gene strategies for rapid enumeration of mycobacteria in whole blood. Bioluminescence is the biological production of light that can be observed in many organisms such as bacteria, fish, fungi, insects, algae and squid [72]. This assay employs a luciferase expression vector, which in a viable organism will produce a bioluminescent signal that can be read by a luminometer (a light-detecting instrument).

1.6.1 Biochemistry of bioluminescence

Luciferase is an enzyme that catalyses a bioluminescent reaction. Bacterial luciferase (*lux*) is found in bioluminescent species and catalyses oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde, (tetradecanal considered to be a natural substrate), with molecular oxygen [73], [74].

$$[FMNH_2 + RCHO + O_2 \longrightarrow FMN + RCOOH + H_2O + Light\ (490nm)]$$

Since $FMNH_2$ production depends on a functional electron transport system, only viable cells will produce light.

1.6.2 Common *lux* genes

It has been found that the light emitting reactions are quite distinct for different organisms, with the only common component being molecular oxygen. Therefore, significant differences have been found between the structures of the luciferase and the corresponding genes from one luminescent organism to another. Genes that code for the subunits of the luciferase enzyme (lux AB) and for the enzymes that convert the standard physiological metabolites into the high-energy substrates for this reaction

University of Cape Town

(lux CDE) have been cloned and sequenced from the lux operons of luminescent bacteria from three genera: *Vibrio*, *Xenorhabdus* and *Photobacterium* [74]. In all Lux systems, there appears to be conservation of the order of these genes i.e. *lux* CDABE together with extensive DNA homology. Beside these common genes, other *lux* genes are found in different species. For example, the *lux*G and *lux*H genes from *V. harveyi* lie immediately downstream of the *lux*E gene, and are thought to encode proteins involved in the synthesis of reduced flavin [72]. Whether these proteins are involved in light production, or are only required in particular physiological conditions, remain unclear. A number of additional *lux* genes of unknown function have been described and reviewed by Meighen [74].

1.6.3 Regulation of *lux* genes

Regulation of bioluminescence in most bacteria depends on cell density and requires autoinduction [72]. The factors involved in this regulation, termed autoinducers, have been identified as homoserine lactone (HSL) derived molecules that are excreted into the media and allow bacteria to sense the culture density and, therefore, to communicate between themselves. These inducers are produced at a low constitutive rate during the early stages of growth but become effective when their concentrations increase in the culture as cells enter stationary phase. HSLs are species specific and are not generally able to cause induction of luminescence in other species. The genes responsible for the regulation of light emission have been identified in *V. fischeri* and *V. harveyi*. Despite structural similarities in the HSLs found in the two species, the gene system in *V. fischeri* and *V. harveyi* have different operons and therefore different evolutionary histones.

University of Cape Town

1.6.4 Reporter constructs

Advances in biotechnology have led to the development of systems for the introduction of genes into mycobacteria. Escherichia coli-mycobacterium shuttle vectors, termed shuttle plasmids, have been constructed [75]. Shuttle plasmids are multifunctional vectors that can replicate in E.coli and mycobacteria. The most commonly used systems for transient transformation employ plasmid vectors carrying the origin of replication, oriM, from a naturally occurring mycobacterial plasmid, pAL5000 from *M. fortuitum* [76]. Such plasmids also harbour an origin of replication for E.coli, oriC. Stable transformants have been generated using plasmids containing

the chromosomal attachment site (attP) and the integrase (int) gene from the mycobacteriophage L5 [77]. These constructs carry the hygromycin resistance gene as the selectable marker. Garbe *et al.* have demonstrated that the hygromycin resistance gene from *Streptomyces hygroscopicus* is more efficiently expressed in mycobacteria than the E.coli-derived aminoglycoside phosphotransferase gene involved in kanamycin resistance [78].

Bioluminescence has been employed as a reporter in a number of systems to detect the presence of low-level bacterial contamination or infection and as a promoter probe to measure specific gene expression [79]. In recent years, reporter constructs employing the *M. bovis* BCG heat shock promotor hsp60 and the luciferase genes of *V. harveyi* have been developed [80]. These constructs carry hygromycin as a selectable marker. Bioluminescent strains of mycobacteria have also been found to be useful in high-throughput screens for drug susceptibility [81], [82] in animal models to study host-pathogen interactions [83] and more recently in the context of human cell-based systems to study mycobacterial growth [84], [71]. The interaction of whole blood and mycobacteria presented in this thesis employs the advantages of the reporter gene strategies.

University of Cape Town

1.7 Whole blood assays

Because of ethical and practical considerations, it is necessary to develop *in vitro* systems to study host pathogen interactions. Any *in vitro* system should aim for the most accurate reflection of *in vivo* events in order for meaningful conclusions to be drawn. The more complex these interactions appear, the more it will be likely that an *in vitro* system based on isolated components of immune responses will fail to deliver. In case of human *in vitro* studies of host-mycobacteria interaction, cell culture systems based on human monocytes have been developed [84]. In these systems, it has become obvious that restriction of mycobacterial growth relies on the interaction of the monocytes with other cell populations such as T cells, and cell culture systems combining both cell types are now available [85]. Although these *in vitro* systems have made an important contribution in our understanding of the relevant cell populations involved in restriction of mycobacterial growth, they have failed to distinguish between individuals able to contain mycobacterial growth and those who

permit mycobacterial growth. The development of an *in vitro* system able to deliver such distinctions is crucial for the understanding of what distinguishes a protective from a non- protective immune response in an infected human host.

In the murine model using whole organs such as spleen, liver or lung tissue it has been possible to distinguish between mice vaccinated with BCG and then challenged with *M. tuberculosis*, which are able to contain growth of the mycobacteria, and unvaccinated mice which fail to do so [83]. Current screening strategies for new vaccines against tuberculosis rely on such animal systems [86]. The complex interactions between mycobacteria and different cell types in a solid organ in the murine immune system confirm the observation, that an *in vitro* system able to incorporate these features would be preferable to study the human immune responses, as opposed to systems using isolated cell fractions such as PBMC. Although tuberculosis is not primarily a disease spread haematogenously, positive blood cultures have been found in patients [87], [88] and haematogenous spread of mycobacteria certainly occurs in disseminated disease and in children, who can present with a less well contained spectrum of clinical disease, such as tuberculous meningitis, miliary tuberculosis or osteomyelitis [89].

To use whole blood as an *in vitro* system to study immune responses to tuberculosis appears therefore very attractive, as all relevant cell types as well as humoral factors are potentially represented and can contribute to the interaction with mycobacteria. The advantage of whole blood assays is not only that they are comparable to the conventional assay using purified PBMC's [90] but they also demonstrate less interassay variability and can be performed on small blood samples without sophisticated processing of blood. This is particularly relevant when studying paediatric populations and for the laboratory set up of many developing countries [91].

University of Cape Town

1.8 Aim of project

The aim of the project was to determine the effect of age and immune competence of children on the *in vitro* growth restricting properties of whole blood when cocultured with luciferase-transfected BCG (BCG *lux*).

1.9 Hypothesis and Specific Objectives

The hypothesis tested in the study was that age and immune competence influences the ability of whole blood to control the rate of mycobacterial growth *in vitro*.

1.9.1 Specific objectives:

1. To establish the whole blood luciferase assay at Red Cross Children's Hospital
2. To determine intra- and inter- assay variability
3. To study the effect of age of healthy children on the ability of whole blood to restrict the growth of mycobacteria
4. To study the ability of whole blood from HIV-infected children to restrict mycobacterial growth
5. To compare these data with that from age matched healthy children
6. To look for an association between cytokine production and growth restriction in healthy and HIV-infected children

University of Cape Town

Chapter 2: Materials and Methods

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

2.1 Human subjects

Ethical approval for this study was obtained from the Ethics Research Committee of the University of Cape Town. Healthy adult volunteers and healthy children of different ages, as well as a small group of HIV-infected children were evaluated.

2.1.1 Healthy adult laboratory volunteers

Ten healthy adult laboratory volunteers were recruited to the study and tested for delayed type hypersensitivity (DTH) skin test response to tuberculin purified protein derivative (PPD) after the initial collection of blood samples. All subjects had given verbal consent to PPD skin testing (Mantoux test) and blood sampling. The volunteers were designated as tuberculin-positive or tuberculin-negative on the basis of size of their skin test response. The six tuberculin-positive and four tuberculin-negative adult volunteers had skin responses of more than 15 mm and less than 5 mm respectively. Blood collected from the adult volunteers was used in the *in vitro* luminescence assay to validate the reproducibility of the assay and establish the assay in the Cape Town laboratory.

University of Cape Town

2.1.2 Healthy children

A total of 88 healthy children under 15 years of age were recruited from two local communities in Cape Town with a high incidence of tuberculosis. All children showed normal growth and development and were free of intercurrent infections for at least 6 weeks preceding the study. None of the children had received treatment for tuberculosis. For purposes of analysis four age groups were considered: (i) <1 year, (ii) 1-4 years, (iii) 5-9 years and (iv) 10-15 years, consisting of 18, 24, 23 and 23 children respectively. All children had received their BCG vaccinations and were screened with a Mantoux test for evidence of prior mycobacterial exposure. Parental consent for all tests was obtained for all healthy children participating in this study

(see appendix 1). Local anaesthetic cream was applied prior to venous puncture to minimise discomfort and the blood drawn was used to assess growth of BCG *lux* in the whole blood of this group. There were no risks encountered by the trial subjects. Any children who were extremely anxious and unwilling were excluded, even after parental consent had been obtained. All parents were adequately counselled and all children with a positive tuberculin skin test were referred for clinical and or radiological evaluation and anti tuberculin prophylaxsis or treatment.

2.1.3 HIV-infected children

A cohort of 22 HIV-infected children aged 1-10 years (mean: 59 months, range: 9-120 months) was recruited from the Infectious Diseases Clinic at Red Cross Children's Hospital in Cape Town. All of these children had perinatally acquired HIV infection and were under regular care at Red Cross Children's Hospital. Confirmation of HIV-infection, as well as clinical and immunological staging of these children was undertaken according to the diagnostic criteria of the 1994 revised Centers for disease control and Prevention (CDC) classification system for perinatally acquired HIV-infection in children under 13 years of age [92]. None of the children had received anti-retroviral therapy, as this was not currently available to children in the public health care sector in South Africa. All HIV-infected children received trimepthoprim (150 mg/m^2/day) / sulphamethoxazole (750 mg/m^2/day) prophylaxis for prevention of *Pneumocystis carinii* pneumonia. $CD4^+$ T cell counts were measured in 19/22 HIV-infected children using 4-colour flow cytometry according to the CDC recommendations [93]. A Mantoux test was done on all children to determine prior exposure to mycobacterial antigen. Blood collected from the HIV-infected children was used in the *in vitro* luminescence assay to study mycobacterial growth in whole blood of HIV-infected children as opposed to mycobacterial growth in the whole blood of the healthy children control group. Parental consent for all tests was obtained for all HIV-infected children participating in this study (see appendix 2).

2.2 Mantoux testing

The tuberculin status of all participants of this study was determined by Mantoux testing. The test was performed by intradermal injection of 2 TU (tuberculin units) of RT-23 PPD (Biovac, Cat. No. 14022ZA) into the left forearm. The degree of

University of Cape Town

induration was measured after 72 hours by determining the transverse diameter in millimetres, excluding any surrounding erythematous zone. To interpret the test result the following criteria, based on the diameter of the induration, were applied: <5 mm (negative), 5-10 mm (reactive but not positive) and 15 mm or more (positive), for the healthy adult volunteers and healthy children controls. In the HIV-infected children, tuberculin reactions measuring 5 mm or more were taken as a positive result.

2.3 Tissue culture

Throughout all tissue culture procedures, sterile conditions were maintained.

2.3.1 Materials and reagents

RPMI 1640: RPMI 1640 culture medium (Life Technologies Ltd., Gibco, Paisley, UK, Cat. No. 31870-025) without L-glutamine or Hepes was stored at 4°C.

L-glutamine: 1% L-glutamine (ICN Biomedicals, USA, Cat. No. 101806) was stored at 4°C.

Heparin: 1000 000 units of Sodium heparin (Sigma, Cat. No. H3149) was diluted 1 in 1000 giving a working concentration of 1000 U/ml. This anticoagulant was stored at 4°C

(NB. No antibiotics were added to the tissue culture medium used for diluting whole blood used for inoculations with mycobacteria.)

2.3.2 Blood sample collection

Peripheral blood obtained aseptically by venipuncture was collected from each subject and transferred into a 30 ml Sterilin universal disposable container with preservative-free sodium-heparin (10 U/ml of blood). A total of 6 ml was collected from healthy adult volunteers and healthy children and used in the luminescence assay. A total of 7 ml was collected from the HIV infected children: 6 ml for the luminescence assay, 500 µl EDTA blood for CD4 count determination, and 500 µl EDTA blood for full blood count and differential count. The full blood count and differential count were routinely done in the department of Haematology at Red Cross Children's Hospital.

University of Cape Town

2.4 Recombinant mycobacteria

2.4.1 Mycobacterial strain

The mycobacterial species used in this thesis was *M. bovis* BCG Montreal (ATCC 35735). It was agreed that it was justifiable to use the avirulent *M. bovis* BCG strain as opposed to the virulent *M. tuberculosis* H37Rv strain since previous work by one of our collaborating colleagues with the whole blood luciferase assay showed concordant results [71]. Our lack of access to a P3 containment facility for working with hazardous material also prompted this decision.

2.4.1.1 Plasmid construction

The mycobacterial reporter construct for *M. bovis* BCG Montreal was developed by Dr. P. Ó Gaora in the Department of Medical Microbiology at Imperial College, London [80]. Molecular biology techniques were carried out according to standard procedures [94]. The *lux A* and *luxB* genes of *Vibrio harveyi* were cloned under the control of the BCG hsp60 promoter on a *XbaI-NheI* fragment from pPA3 [75] into pPOLYG [80] a modification of p16R1 [78] to generate pSMT1, **figure 5**. This Plasmid is a shuttle vector in *E.coli* and mycobacteria and employs hygromycin as a selectable marker. Plasmid DNA was prepared from recombinant *E.coli* DH5α by using Qiagen columns according to manufacturer's recommendations and was used to electroporate mycobacterial species. Recombinant colonies were tested for luminescence and were maintained at -80°C in 25% glycerol.

University of Cape Town

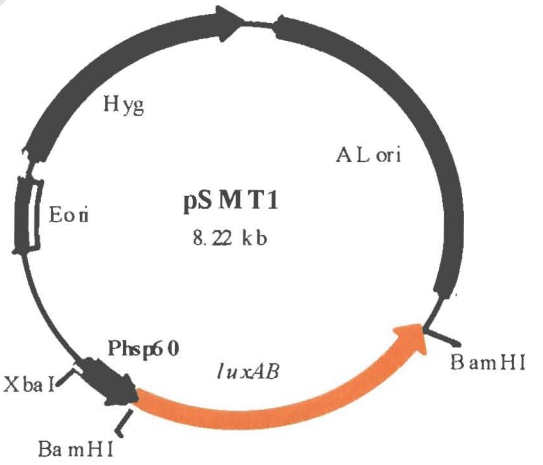


Figure 5. Plasmid map of pMST1

2.5 Bacterial Culture

2.5.1 Media

2.5.1.1 Liquid media

Middlebrook 7H9 broth: 4.7 g of powdered 7H9 broth (Becton Dickinson, Difco, USA, Cat. No. 71317) was dissolved in 900 ml dH_20 (containing 2 ml glycerol [BDH, Merck, Cat. No. 10118] and 500 µl Tween 80 [Sigma, Cat. No. P1754]). Aliquots of the broth were prepared in 180 ml volumes and autoclaved for 15 min at 121°C. The prepared media was supplemented with 10% (20 ml) ADC {see 2.5.1.3} and 10 mg hygromycin B (200 µl of 50 mg/ml stock) before use as liquid growth medium for BCG *lux* and stored at 4°C.

2.5.1.2 Solid media

Middlebrook 7H11 agar: 10.5 g of powdered 7H11 agar (Becton Dickinson, Difco, USA Cat. No. 83817) 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 {see 2.5.1.3}, 10 mg of amphotericin B (50 mg/ml) and 25 mg hygromycin B (500 µl of 50 mg/ml stock) before use as solid growth medium for BCG *lux*. The medium was then poured into plates under sterile conditions and left to set.

2.5.1.3 Supplements for mycobacterial media

ADC: 50 g bovine albumin fraction V (ICN Biomedicals, USA, Cat. No. 160069), 20 g D-glucose (Sigma, Cat. No. G7528), 8.5 g sodium chloride (Saarchem, Merck, Cat. No. 582232) and 0.04 g catalase (ICN Biomedicals, USA, Cat. No. 100428) were dissolved in 500 ml dH_20 and the solution was made up to 1 litre with dH_20. The prepared supplement was filter sterilised in a flow cabinet and aliquoted into 100 ml volumes using a 0.22 µm filtration system (Microsep, (Pty) Ltd, Millipore, UK, Cat. No. SCGPU01RE) and stored at 4°C.

OADC: 50 g bovine albumin fraction V, 20 g D-glucose, 8.5 g sodium chloride and 0.04 g catalase were dissolved in 500 ml dH_20 as described above for ADC. 0.5 g oleic acid (Sigma, Aldrich Chem. Co., USA, Cat. No. 23397-8) was dissolved in 41.5

University of Cape Town

ml of 20 mM sodium hydroxide (Saarchem, Merck, Cat. No. 5823200EM) and this solution was added to the main solution and the solution made up to 1 litre with dH_20. Again, the prepared supplement was filter sterilised in a flow cabinet and aliquoted into 100 ml volumes using a 0.22 µm filtration system and stored at 4°C.

Hygromycin B: 50 mg/ml stock solution (Boehringer Mannheim, Roche Diagnostics, Germany, Cat. No. 843555) was used at a working concentration of 50 µg/ml and stored at 4°C.

Amphotericin B: 50 mg stock (Sigma, Cat. No. A9528) was used at a working concentration of 50 µg/ml and stored at 4°C.

2.5.2 Preparation and storage of mycobacteria

Recombinant mycobacteria (BCG *lux*) were grown into the logarithmic phase over 72 hours in a shaking incubator at 37°C in 7H9/ADC/hygromycin medium. The culture was dispensed into 1 ml vials; glycerol was added to a final concentration of 25% and the vials frozen at -80°C. Each vial was shown to contain 1.5 x 10^6 CFU/ml viable mycobacteria, by plating of an aliquot on 7H11/OADC/hygromycin/amphotericin medium.

2.5.3 Preparation of mycobacterial culture for inoculation into whole blood

Prior to each experiment, a vial of stored BCG *lux* was thawed, added to 10 ml of 7H9/ADC/hygromycin medium in a sterile disposable 250 ml Erlenmeyer Flask (Corning, USA, Cat. No. 430183) and incubated with shaking at 37°C for 72 hours. Mycobacteria were then centrifuged for 10 minutes at 3200 rpm and washed once with sterile PBS. After the wash spin (again for 10 minutes at 3200 rpm to recover the BCG *lux*) the mycobacteria were then resuspended in enough sterile PBS to obtain a concentration of 2 x 10^8 RLU/ml prior to inoculation.

University of Cape Town

2.6 Bioluminescence Assay

2.6.1 Protocol

The blood samples were set up in the whole blood luminescence assay within two hours of blood collection. The 6 ml heparinised whole blood samples obtained from the subjects were diluted 1 in 2 with serum-free medium (RPMI 1640 supplemented with 1% L-glutamine). The blood was then aliquoted into 900 µl volumes in 7 ml screw-top, endotoxin-free Bijou tubes (Merck, Sterilin, Cat. No. DA275046024) to give a final volume of 1 ml per tube following inoculation with 100 µl luciferase-labelled Bacille Calmette-Guerin (BCG *lux*) at 2 x 10^8 RLU/ml. Cells were stimulated in triplicate with the BCG *lux* at three time points: 0, 72 and 96 hours (plus one unstimulated control sample at each time point). The samples were then incubated at 37°C on a platform shaker (Merck, Stuart Scientific, STR6, Cat. No. 330/011/400) set at 20 rev/min except for the 0 hour time point samples that was analysed on the day of blood collection.

The 0 hour samples were centrifuged for 10 minutes at 3200 rpm and 400 µl supernatant was collected from each tube and stored at -80°C for subsequent cytokine measurement by ELISA. Supernatants were harvested for cytokine measurement at each time point. To readjust the volume, 400 µl of sterile PBS was added to the samples, then the samples were transferred to 25 ml Sterilin universals and 10 ml dH_2O was added to lyse the red blood cells. The samples were incubated for 10 minutes at room temperature then centrifuged for 10 minutes at 3200 rpm to recover the BCG *lux*. The supernatant was discarded, glass beads (Sigma, Cat. No. GSIM632649/2) were added to the pellet (to reduce mycobacterial clumping) and the pellet was vortexed to disperse the BCG *lux*. The BCG *lux* pellet was resuspended in 1 ml of sterile PBS. Samples were diluted in duplicate by 10- and 100-fold (to minimise any reduction of luminescence in opaque samples [quenching]) prior to luminescence measurement.

2.6.2 Bioluminescence detection

Luminescence was measured using an EG&G Berthold AutoLumat LB 953 tube luminometer (Berthold, Germany, Cat. No. 109151). This design uses an injectable

University of Cape Town

port to inject 0.1 ml of the substrate, 1% n-decyl aldehyde, (Sigma, Cat. No. D7384) in ethanol into each 5 ml, 75 X 12 mm luminometer tube (Sarstedt, Röhren, Germany, Cat. No. 09778). Raw data was collected in duplicates over 20 seconds from samples prepared in triplicates and the mean calculated. Luminescence was measured at 0, 72 and 96 hours 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:

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

2.7 CD4 count determination

CD4 counts were measured in the HIV-infected children by flow cytometry to determine the severity of their acquired immunosupression.

2.7.1 Materials and reagents

Falcon tubes: 5 ml, 12 X 75-mm, sterile, polystyrene round bottom tubes, (Beckton Dickinson, France, Cat. No. 352054) were used.

Cyto-Stat® tetraCHROME™: CD45-FITC (Fluorescein isothiocyanate) /CD4-RD1 (Phycoerythrin) /CD8-ECD (Phycoerythrin-Texas Red®-X) /CD3-PC5 (Phycoerthrin-Cy5), (Beckman Coulter, France, Cat. No. 6607013) and CD45-FITC (Fluorescein isothiocyanate) /CD56-RD1 (Phycoerythrin) /CD19-ECD (Phycoerythrin-Texas Red®-X) /CD3-PC5 (Phycoerthrin-Cy5), (Beckman Coulter, France, Cat. No. 6607073) four-colour fluorescent monoclonal antibodies were stored at 4°C.

OptiLyse® C: Erythrocyte lysing solution was stored at room temperature (Immunotech, Beckman Coulter, France, Cat. No. 1401).

University of Cape Town

PBS: 10 X PBS (see 2.8.1) was diluted 1 in 10 with dH_2O to give a 1 X PBS solution, autoclaved for 15 minutes at 121°C and filter sterilized using a 0.22 µm filter. The 1 X PBS solution was stored at room temperature.

2.7.2 Immunofluorescent staining

A mixture of 5 µl CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and 5 µl CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5, fluorescent-conjugated monoclonal antibodies (mAbs) were placed into two separate Falcon tubes and 100 µl of EDTA unstimulated whole blood was added to each tube. The mixture was vortexed to mix well then incubated for 10 minutes at room temperature in the dark. Optilyse C (500 µl) was added to both tubes to lyse the red blood cells (and fix the white blood cells). The mixture was again vortexed and incubated for 10 minutes at room temperature in the dark. Filter sterile PBS (500 µl) was added to both tubes to dilute the cells. The samples were then vortexed, and depending on time constraints were either, incubated for 4 hours in the dark at room temperature and analysed on the same day or incubated over night at 4°C and analysed the following day.

2.7.3 Flow cytometric analysis

The samples were analysed on a Coulter flow cytometer. A manual gate was drawn around the lymphocyte population and a total of 5000 events were counted. Lymphocyte subset analysis for $CD4^+$, $CD8^+$ T cells, B ($CD19^+$) cells, NK ($CD56^+$) cells and $CD3^+$ T cells and an absolute count for each population of interest was calculated with the full blood count results.

University of Cape Town

2.8 Analysis of supernatants by ELISA

Supernatants for cytokine analysis were thawed and serial dilutions prepared in PBS/Tween/10% foetal calf serum. Interferon γ and tumour necrosis factor α were measured by ELISA using antibody-pairs provided by Pharmingen (Becton Dickinson, Cowley, Oxford, UK). Standard curves were generated using reference cytokines provided by Pharmingen.

2.8.1 Materials and Reagents

Bicarbonate Buffer: 8.4g Na_2HCO_3 (Merck, Cat. No. 6329) was dissolved in 1L dH_2O to make a 0.1 M coating buffer solution, pH 8.4. The buffer was autoclaved at 121°C and stored at 4°C.

10 X PBS: 58 g Na_2HPO_4 (Saarchem, Merck, Cat. No. 5822870), 10 g KH_2PO_4 (Saarchem, Merck, Cat. No. 5043600), 10 g KCL (AEC, Cat. No. 7520598) and 400 g NaCl (Saarchem, Merck, Cat. No. 5822320), were dissolved in 5 L dH_2O, autoclaved at 121°C and stored at room temperature.

PBS/Tween: 0.05% (2.5 ml) Tween 20 (Sigma, Cat. No. P1379) was added to 500 ml of 10 X PBS and the solution made up to 5 L with dH_2O, pH 7.4. The solution was stored at room temperature.

Foetal Calf Serum (FCS): FCS (Delta Bioproducts, SA, Cat. No. 14501AI) was heat inactivated for 30 minutes at 56°C in a water bath, filter sterilised through a 0.22 µm filter (Micron Separations Inc., Osmonics, Cat. No. FSF46220) into 20 ml sterile glass bottles and stored at -20°C.

PBS/Tween/10% FCS: The 10% (volume/volume) filtered FCS in PBS/Tween was freshly prepared prior to use and used as diluent.

PBS/10% FCS: The 10% (volume/volume) filtered FCS in PBS (no Tween 20) was freshly prepared prior to use and used as blocker.

Citric Acid Buffer: 5.19 g citric acid (BDH, Merck, Cat. No. 10081) and 7.23 g Na_2HPO_4 (Saarchem, Merck, Cat. No. 58228) were dissolved in 1 L dH_2O, pH 5. The buffer was autoclaved at 121°C and stored at room temperature.

30% Hydrogen peroxide: The 30% (mass/volume) H_2O_2 solution was stored at 4°C.

University of Cape Town

2N Sulphuric Acid: 53.3 ml of H_2SO_4 (Saarchem, Merck, Cat. No. 5885120) was added to 1 L dH_2O to make a 2N stopping solution. The solution was stored at room temperature.

Avidin peroxidase (AVP): Lyophilized AVP powder (Sigma, Cat. No. A3151) was reconstituted in 2 ml sterile 1 X PBS to give a stock concentration of 2.5 mg/ml and stored at -80°C. The stock AVP was diluted 1 in 500 to give a final concentration of 5 µg/ml for IFNγ and 1 in 2000 to give a final concentration of 1.25 µg/ml for TNFα.

o-Phenylenediamine dihydrochloride (OPD): OPD (Sigma, Poole, UK, Cat. No. P-1526) stock was stored at -20°C and used at a concentration of 2.5 mg/ml of citric acid buffer.

ELISA plates: 96-well, flat bottom, high affinity Nunc plates, (Greiner, Dursley, UK).

Capture Antibodies: 1.0 mg/ml purified mouse anti-human TNF-α (BD PharMingen, Oxford, UK, Cat. No. 18631D) and 1.0 mg/ml IFN-γ (BD PharMingen, Oxford, UK, Cat. No.18891D) monoclonal antibodies were stored at 4^0C. Both antibodies were diluted 1 in 500 (2 µg/ml) in 0.1 M bicarbonate buffer and used as capture antibodies.

Detection Antibodies: 0.5 mg/ml biotinylated mouse anti-human TNF-α (BD PharMingen, Oxford, UK, cat. No. 18642D) and IFN-γ (BD PharMingen, Oxford, UK, 554550) monoclonal antibodies were stored at 4^0C. Both antibodies were diluted 1 in 500 (1 µg/ml) in diluent and used as detection antibodies.

TNF-α Standard: Recombinant human TNF-α (10 µg at 200 µg/ml stock), (BD PharMingen, Oxford, UK, Cat. No. 554618) was reconstituted in 10 ml sterile PBS containing 10 mg/ml bovine serum albumin (BSA), (Roche, Cat. No. 735086) giving a storage concentration of 1 µg/ml, aliquoted into 20 µl volumes and stored at -80°C. Prior to use the 1 µg/ml stock was diluted 1 in 250 (10 µl in 2.5 ml diluent) giving a final concentration of 4000 pg/ml for the highest TNF-α standard.

University of Cape Town

IFN-γ Standard: Recombinant human IFN-γ (50 µg at 250 µg/ml stock), (BD PharMingen, Oxford, UK, Cat. No. 554617) was reconstituted in 2 ml sterile PBS containing 10 mg/ml bovine serum albumin (BSA), (Roche, Cat. No. 735086) giving a storage concentration of 25 µg/ml, aliquoted into 13 µl volumes and stored at -80°C. Prior to use the 25 µg/ml stock was diluted 1 in 25 (10 µl in 240 µl diluent), then 1 in 200 (10 µl in 2 ml diluent) giving a final concentration of 5000 pg/ml for the highest IFN-γ standard.

TNF-α WHO control: The WHO International Standard for TNF-α (WHO, National Institute for Biological Standards and Control, Herts, UK, Cat. No. 87/650) was reconstituted in 500 µl sterile dH_2O and the volume made up to 1 ml by adding 500 µl PBS. The solution was stored at -80°C at a storage concentration of 1 µg/ml (40 000 U/ml). Prior to use the WHO control was diluted 1 in 500 (5 µl in 2.5 ml diluent) giving a working concentration of 2000 pg/ml.

IFN-γ WHO control: The WHO International Standard for IFN-γ (WHO, National Institute for Biological Standards and Control, Herts, UK, Cat. No. Gxg01-902-535) was reconstituted in 500 µl sterile dH_2O and the volume made up to 1 ml by adding 500 µl PBS. The solution was stored at -80°C at a storage concentration of 1 µg/ml (40 000 U/ml). Prior to use the WHO control was diluted 1 in 200 (5 µl in 995 µl diluent), then 1 in 2.5 (200 µl in 300 µl diluent) giving a working concentration of 2000 pg/ml.

University of Cape Town

2.8.2 Protocol

Concentrations of TNFα and IFNγ in supernatants collected at specific time points during the whole blood experiments were measured using sandwich ELISA antibody pairs according to manufacturer's recommendations. Flat bottomed 96-well high affinity Nunc ELISA plates were coated with 50 µl per well of the capture antibody diluted in sodium bicarbonate buffer to a final concentration of 2 µg/ml. Plates were covered and kept at 4^0C over night. Plates were washed three times in PBS/Tween and subsequently blocked for 1.5-2 hours with 200 µl/well of PBS/10% FCS without Tween. After blocking, they were washed three times, again with PBS/Tween before

application of samples and standards. Recombinant human cytokines were used as reference standards. For each experiment, 1 vial of each recombinant cytokine was defrosted and diluted as described above. The top standard was applied in duplicate and serially diluted across the ELISA plate. The same batch of standards was used for all experiments. Supernatants generated during whole blood experiments were stored at -80^0C until use. Aliquots were thawed at room temperature, and serially diluted in volumes of 100 µl/well in duplicates after mixing on a vortex mixer. ELISA plates were then sealed and incubated over night at 4^0C.

After 4 washes with PBS/Tween the biotin labelled detection antibodies were diluted in PBS/Tween/10%FCS to a final concentration of 1 µg/ml and applied to the plates in a volume of 100 µl/well. After 1 hour, the plates were washed 6 times and avidin peroxidase diluted to the appropriate concentration for TNFα and IFNγ (as described above) and applied to all wells in a volume of 100 µl for 30 minutes. After 8 washes, ELISAs were developed in the dark using 2.5 mg/ml of O-phenylenediamine dihydrochloride in citrate buffer containing 30% hydrogen peroxide (diluted 1 in 1000), in volumes of 100 µl/well. The reaction was stopped after 15 minutes for TNFα, and after 20 minutes for IFNγ, using 100 µl/well of 2N H_2SO_4 and the plates read using a Versamax microplate reader (Molecular Devices Ltd., Crawley, UK, Cat No.0200-2100). Cytokine concentrations were calculated using ELISA software (Versamax, Software) from a standard curve run in duplicate on every plate. Cytokine concentrations are reported as the concentration with the background (PBS/Tween) subtracted. The absorbance was measured at an OD of 490nm in a Versamax microplate reader. The lower range of detection was 19.531 pg/ml; the highest detectable levels were 5000 pg/ml, using the above antibody-pairs and standards. Repeat ELISAs were prepared for any results that were found to be out of range and having CVs above 15%. In addition, TNF-α and IFN-γ WHO reference standards were used as internal control.

University of Cape Town

2.9 Statistical Methods

All data were analysed using GraphPad Prism (GraphPad Software, San Diego, USA). Non-parametric statistical tests were used to avoid assumption of normally distributed

data sets. To determine whether two sets of data were significantly different for a given variable, the Wilcoxon signed rank test and the Mann-Whitney rank sum test were used. A p-value of < 0.05 was regarded as significant.

University of Cape Town

Chapter 3: Validation of a Whole Blood Assay to Measure Growth of Mycobacteria

3.1 Introduction

Conventional methods of monitoring mycobacterial viability involve culture of the organism of interest in liquid media followed by plating of serial dilutions of the culture on solid media and subsequent enumeration of colony forming units after weeks of incubation. Slow growing bacteria such as *Mycobacterium tuberculosis* require 4 to 6 weeks to form colonies on agar plates with a doubling time of 18 to 24 hours. This pathogenic organism tends to form clumps, which makes working and counting of mycobacteria difficult.

The availability of recombinant strains of *Mycobacterium bovis* BCG expressing an integrated prokaryotic luciferase gene has permitted a strategy for mycobacterial enumeration employing the non-pathogenic BCG strain for the rapid enumeration of bacterial numbers. The BCG *lux* was obtained from the Department of Microbiology at Imperial College. Previous work with this construct (plasmid pSMT1), developed by P. Ó Gaora [80] has demonstrated a linear relationship between the conventional colony forming units (CFU) and relative light units (RLU) [83], [71]. Initially the assay was performed with the assumption that 10 RLU correspond to 1 CFU as was shown by previous work carried out at the St Mary's laboratory. Subsequent experiments at the Cape Town laboratory demonstrated variation in luminometer sensitivity. This identified a need for further evaluation of consistency and sensitivity of the assay.

In this chapter, we reassessed the relationship between RLU and CFU as well as the reproducibility of the whole blood assay.

University of Cape Town

3.2 Methods

Cultures of BCG *lux* were grown in 7H9 medium under standard conditions as previously described in section 2.5.2. Serial dilutions of the BCG *lux* were prepared after growth of the cultures and enumeration of bacteria was done by both relative light units (RLU) and colony forming units (CFU) on agar plates for growth curves to asses viability of the organism. The enumeration of bacteria in whole blood was done by RLU only.

3.3 Results

3.3.1 Relationship between luciferase activity and colony forming units

Initial experiments compared growth in liquid mycobacterial medium, either measured as RLU or diluted and plated out onto 7H11 agar plates for CFU to be counted after 3-4 weeks of incubation, **figure 6**. Growth was compared over a timecourse, incorporating the exponential and stationary phase of growth. Readings from the Automat luminometer were taken at the same time as dilutions were plated out for CFU counts. A parallel increase of bioluminescence and CFU was observed, confirming RLU determinations as a good indicator for viable bacteria.

Parallel experiments were set up comparing growth of BCG *lux* in whole blood of tuberculin positive and tuberculin negative individuals employing luminescence as readout since colonies take up to a month to develop. Growth curves based on RLU values were identical for tuberculin positive and tuberculin negative individuals, **figure 7**.

University of Cape Town

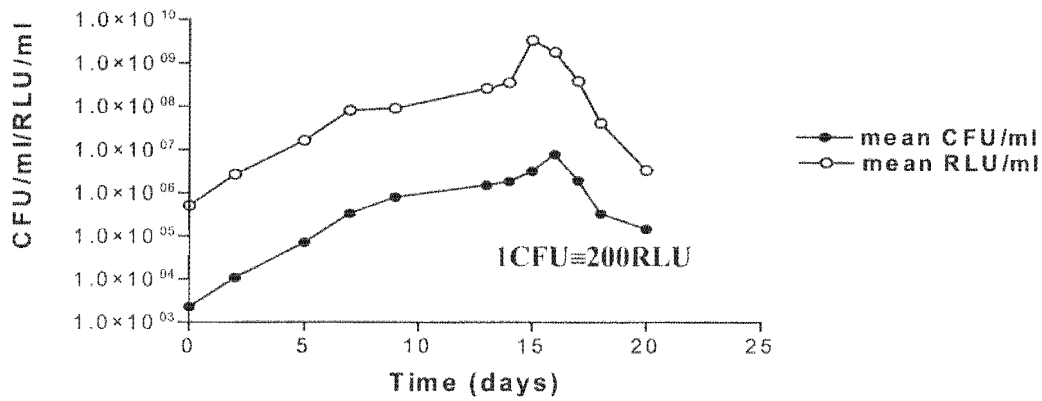


Figure 6. Growth of BCG *lux* measured by relative light units (○ RLU) and colony forming units (● CFU). Growth in mycobacterial culture medium: 10^8 RLU (10^5 CFU) of BCG lux was inoculated into 7H9 mycobacterial culture medium, and the number of mycobacteria was measured over 20 days. At each time point, 100 µl was removed from the culture, and RLUs and CFUs were assessed in triplicate samples. These growth curves were prepared from the mean of three experiments.

University of Cape Town

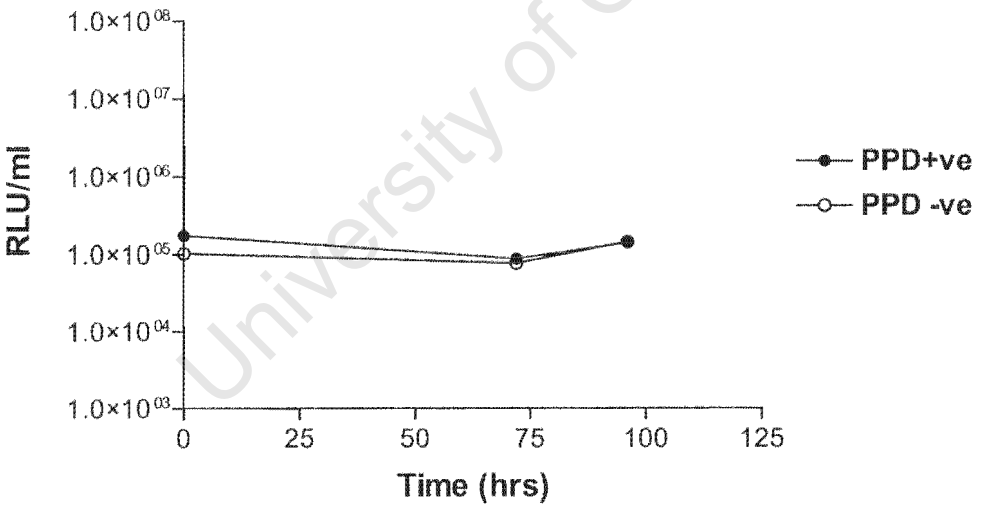


Figure 7. Growth of BCG *lux* in whole blood from a tuberculin positive (● PPD +ve) and a tuberculin negative (○ PPD -ve) individual. Whole blood obtained from a tuberculin positive individual and a tuberculin negative was inoculated with BCG lux and mycobacterial numbers were measured over 96 hours. At each time point, the RLUs were assessed in triplicate samples.

This was unexpected as previous work by Kampmann *et al.*, [71] demonstrated a significant difference between growth of mycobacteria in whole blood of tuberculin positive individuals compared to tuberculin negative individuals. Analysis of the CFU data for the growth of mycobacteria in 7H9 media revealed the root of this discrepancy. The RLU: CFU ratio was higher than expected, with combined results from three separate experiments demonstrating a ratio of 200 RLU per CFU, figure 6. Compared to previous work [71] this represents a different ratio of RLU to CFU, which was due to using different luminometers. The starting inoculum was therefore corrected from $5X10^6$ RLU/ml to $1.5X10^8$ RLU/ml. The corrected inoculum of $1.5X10^8$ RLU/ml gave a clear distinction in growth ratios between tuberculin positive and tuberculin negative individuals as demonstrated in **Table 1.**

Table 1. Comparison of growth ratios obtained for three tuberculin positive and three tuberculin negative individuals after using the old starting inoculum ($5X10^6$ RLU/ml) and the corrected starting inoculum ($1.5X10^8$ RLU/ml)

Tuberculin positive individuals	**Tuberculin negative individuals**
5.88 (1.13)	6.02 (1.10)
2.42 (1.19)	4.13 (1.06)
1.41 (1.53)	9.86 (1.90)

NOTE. Growth ratios obtained using the old inoculum in brackets. The growth ratios were measured after a 96-hour incubation period of whole blood with mycobacteria.

University of Cape Town

The bacterial load in the inoculum was a parameter in need of standardization to ensure consistency of results between experiments. Initial studies with the recombinant BCG strain were performed in Tween 80 supplemented Middlebrook 7H9 broth, and although endpoints were clearly discernible, clumping of bacterial cells was noticeable and introduced a problematic variable. Addition of glass beads to dissipate any aggregated cells in the last stage of the assay (see section 2.6.1) resulted in more homogeneous bacterial suspensions throughout the course of an experiment and decreased variability of the starting inoculum.

3.3.2 Intra-and Inter-assay variability

There was minimal intra-assay variability when a single sample of blood was obtained from an individual and aliquoted into several experiments processed simultaneously (mean GR=6.3; SD=0.173; CV=3%), **figure 8**. The variability increased when blood was obtained from the same individual and processed on different occasions. However, the inter-assay variability fell within the acceptable range (20-30% for biological assays) for the individuals assayed with only two exceptions falling outside of the acceptable range as demonstrated in **Table 2**. This data was generated over a period of time using BCG *lux* sub-cultures grown from the same original frozen stock. Although the sub-cultures were grown from the same original stock, this variability could be attributed to differences in growth patterns of BCG *lux* over time. Other sources of variability might be found within the blood donors. Individual differences in immune responses from day to day could account for a significant portion of variance in growth ratios if samples were taken at different times in the day or setting up of the assay was delayed.

In experiments comparing the variability between different investigators using the blood of the same person and the same stock of BCG *lux* on the same day, there was less than 10% variability between the calculated growth ratios (mean GR=6.4; SD=0.503; CV=8%), **figure 9**.

University of Cape Town

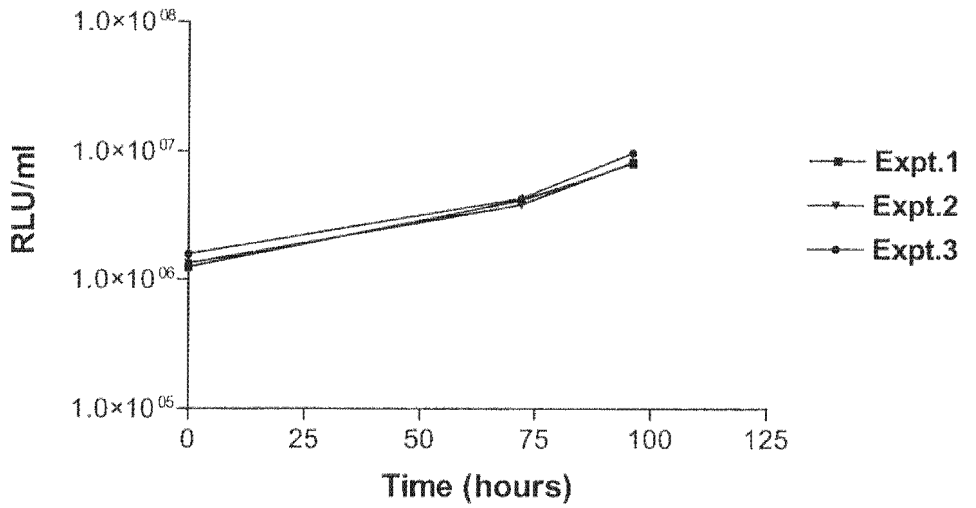


Figure 8. Growth of BCG *lux* in a single sample of blood obtained from a tuberculin positive individual. The sample was divided into three experiments and processed simultaneously. Whole blood obtained from a tuberculin positive individual was inoculated with BCG lux and mycobacterial numbers were measured over 96 hours. At each time point, the RLUs were assessed in triplicate samples.

University of Cape Town

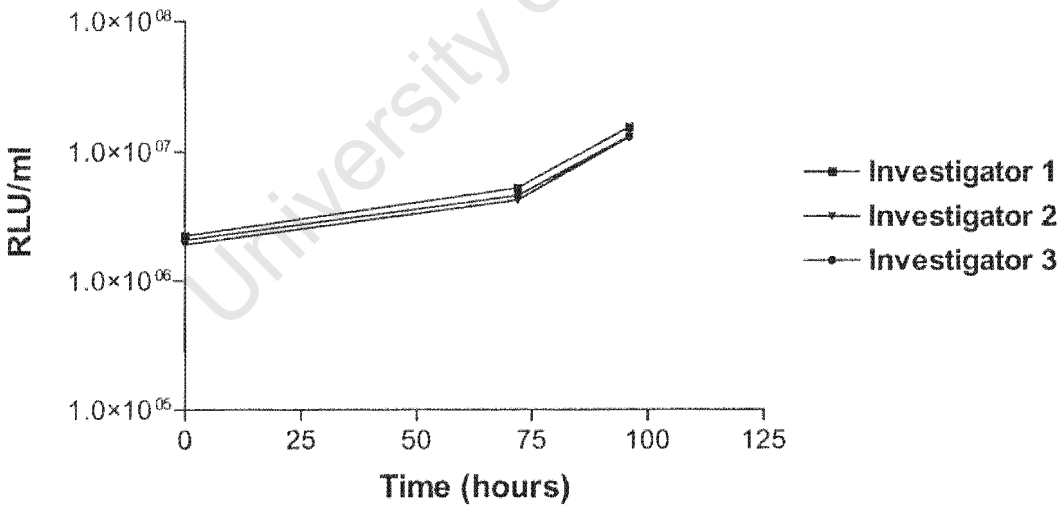


Figure 9. Growth of BCG *lux* in whole blood obtained from a tuberculin positive individual and processed simultaneously by three different investigators at the same time. Whole blood obtained from a tuberculin positive individual was inoculated with BCG lux and mycobacterial numbers were measured over 96 hours. At each time point, the RLUs were assessed in triplicate samples.

Table 2. Reproducibility of the whole blood assay

Adult Control	Growth Ratio	SD	CV (%)
1	5.9	0.638	11
2	9.9	0.644	6
3	3.0	1.046	34
4	7	0.287	4
5	8.1	0.379	5
6	5.2	0.424	8
7	2.1	0.932	44
8	8.3	1.155	14
9	3.8	0.563	15
10	6.3	0.581	9

NOTE. Mean growth ratios of 5 experimental sessions for each adult control

University of Cape Town

3.4 Discussion

Our study demonstrates that a luciferase assay with recombinant mycobacteria containing the lux gene can rapidly and reliably determine numbers of viable mycobacteria. Bioluminescence sensitivity allows for the rapid measurement of very small numbers of bacteria, whereas, it takes weeks to obtain results when using conventional methods. This method lends itself to screening a large number of samples in a quick and cost effective manner. In the first part of the study, we standardized the luciferase assay by examining the growth curves of viable mycobacteria. Using these growth curves, we determined the necessity of comparing RLU and CFU readings, as luminometers might differ between institutions. The establishment of the RLU to CFU ratios was essential to determine the ideal size of inoculum for the whole blood assay. It was apparent from the growth curves (in mycobacterial media and in whole blood) that the bacterial number in the initial inoculum was too low to tease out any differences in mycobacterial growth between tuberculin positive and tuberculin negative individuals. The bacterial inoculum was therefore adjusted taking into account that 1CFU≡200RLU.

The findings of this study also showed that the nature of the bacteria is also important for the establishment of the correct RLU/CFU ratio since homogeneous bacterial suspensions resulted in decreased variability of the starting inoculum. Reproducibility of the whole blood assay is important to the validation and interpretation of results. As with any study utilising samples of biological origin there is substantial variability between individual's results. Although a certain amount of variability remained, attempts to minimise this variability were made by optimising the assay as much as possible by preparing sub-cultures from the same frozen stock of BCG *lux* in order to eliminate potential differences in growth of the organism and to evaluate sizes of the starting inoculum to ensure that more or less the same amount of stimulant was used in the whole blood assay.

University of Cape Town

Chapter 4: Growth of Mycobacteria in Whole Blood of Tuberculin Positive and Tuberculin Negative Volunteers

4.1 Introduction

The study of host-pathogen interactions involving mycobacteria are complex and the general understanding is that these interactions determine the outcome of mycobacterial infections. Exposure to mycobacterial pathogens results in either containment of infection or progression to active disease. What determines these differences in outcome and decides between susceptibility and resistance has been the subject of extensive studies in murine models and also in the human host. Although uncertainty remains about the desirable immune responses it is certain that the host's ability to control mycobacterial growth after exposure and infection ultimately decides whether containment or multiplication of organisms can occur.

Differences in growth of mycobacteria in whole blood have been demonstrated between individuals who have not been exposed to *M. tuberculosis* (as demonstrated by a negative Mantoux skin test to mycobacterial antigen) and individuals, who have been exposed (with a positive Mantoux skin test), but remained completely well. Kampmann *et al.*, [71] have demonstrated that tuberculin positive individuals can significantly control growth of mycobacteria in whole blood compared to tuberculin negative individuals.

We have sought to confirm this finding in whole blood obtained from tuberculin positive and tuberculin negative healthy adult volunteers and have also assessed the associated cytokine production. The attractiveness of whole blood assays is that they can be performed on small blood samples without sophisticated processing of blood making them more suitable for application to field based studies.

University of Cape Town

4.2 Methods

Standard methods described in section 2.5.3 and 2.6.2 were used to culture and enumerate BCG *lux* respectively. Cytokine production generated during the interaction of mycobacteria and whole blood in the *in vitro* assay was measured according to the method described in section 2.8.2. The whole blood assay was set up according to the protocol described in section 2.6.1.

4.3 Results

4.3.1 Growth of BCG *lux* in whole blood of tuberculin positive and tuberculin negative individuals

To validate the use of mycobacterial viability as a means of assessing immune status the growth of BCG *lux* was compared in whole blood of tuberculin positive and tuberculin negative individuals. Blood samples were collected from 10 healthy adult volunteers who on the basis of their tuberculin reactivity were shown to be either Mantoux skin test positive or negative. Cutaneous responses were measured 72 hours after intradermal inoculation with 2 TU of PPD; an induration of <5 mm was considered as a negative response, and an induration of >15 mm as a positive response. All of the healthy adult volunteers were found to have a skin test response of either 0 mm (no response) or > 15 mm. Six of the healthy volunteers were found to be tuberculin positive (> 15 mm) and four were tuberculin negative (< 5 mm) as illustrated in **Table 3**.

University of Cape Town

Table 3. Comparison of growth ratios between 0 and 96 hours of culture of mycobacteria in whole blood of tuberculin positive and tuberculin negative healthy adult volunteers

Adult Control	Tuberculin Status	Growth Ratio
1	positive	5.9
2	positive	3.0
3	positive	7.0
4	positive	5.2
5	positive	2.1
6	positive	3.8
7	negative	9.9
8	negative	8.1
9	negative	8.3
10	negative	6.3

NOTE. Tuberculin positive (median: 4.5*, range: 2.1-7.0)
Tuberculin negative individuals (median: 8.2, range: 6.3-9.9)
*P=0.0190, Mann-Whitney test

University of Cape Town

Growth of luminescent mycobacteria was significantly lower (p=0.0190, Mann-Whitney test) in the blood samples of the tuberculin positive individuals (median: 4.5, range: 2.1-7.0) than in the blood samples of the tuberculin negative individuals (median: 8.2, range: 6.3-9.9). Initially luminescence was measured at 0, 72 and 96 hours. The differences in RLU readings at 72 and 96 hours were negligible, we therefore abandoned the 72 hour timepoint for luminescence readings and only collected supernatant for cytokine measurements at this time point.

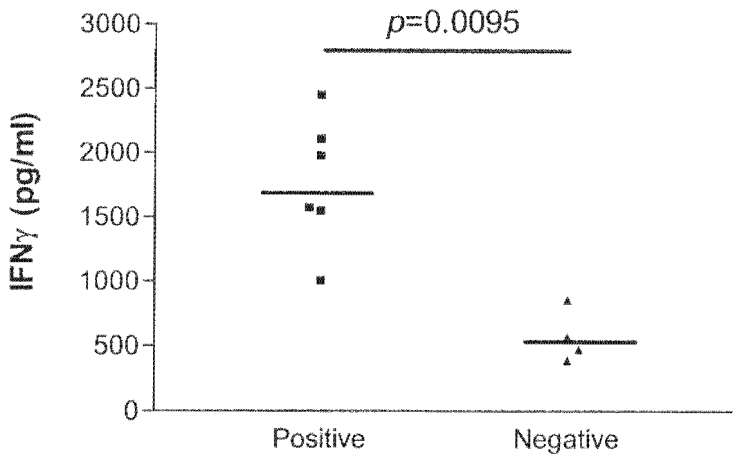
4.3.2 Associated cytokine production

IFNγ and TNFα have been shown to be important for controlling mycobacterial growth in murine models. We therefore studied the production of IFNγ and TNFα in supernatants obtained from the whole blood samples during growth of BCG *lux*. TNFα peaked at 72 hours, while IFNγ peaked at 96 hours. Significantly more IFNγ (p=0.0095, Mann-Whitney test) was produced during growth of BCG *lux* in whole blood of tuberculin positive individuals as compared with tuberculin negative

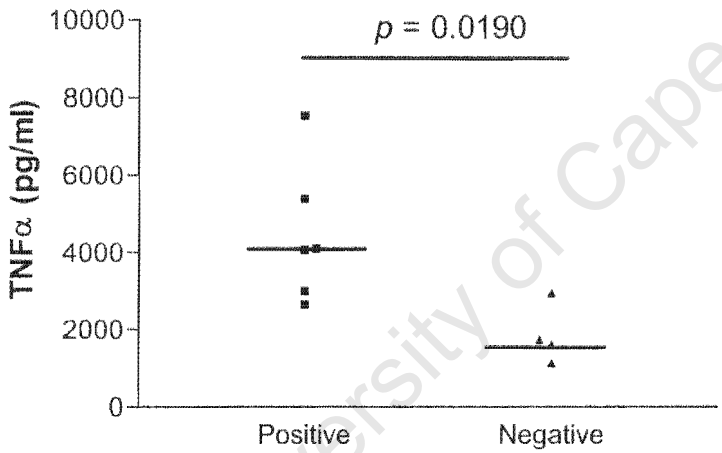
individuals, **figure 10a**. TNFα production was also significantly higher during growth of BCG *lux* in whole blood of tuberculin positive individuals (p=0.0190, Mann-Whitney test) than in whole blood of tuberculin negative individuals, **figure 10b**.

University of Cape Town

a) IFNγ measured after a 96-hour incubation period



b) TNFα measured after a 72-hour incubation period



■ Tuberculin positive ▲ Tuberculin negative

University of Cape Town

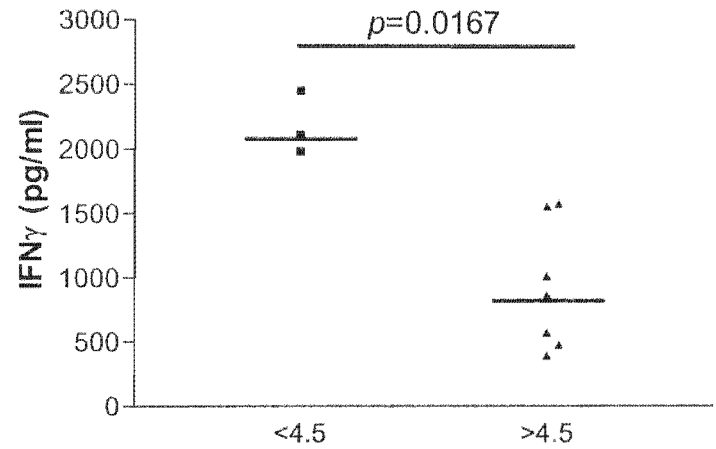
Figure 10. (a) IFNγ and (b) TNFα produced during incubation of whole blood obtained from (■) tuberculin positive and (▲) tuberculin negative individuals with BCG *lux* (see appendix 3, **Table 8** for individual test values). TNFα and IFNγ was measured by ELISA at 72 and 96 hours respectively. Horizontal lines show the median for the group. Mann-Whitney test analysis (unpaired groups) was carried out to determine the significance of the differences between the two groups. Tuberculin positive individuals produced significantly more IFNγ (median: 1780 pg/ml, p=0.0095) than tuberculin negative individuals (median: 521 pg/ml). TNFα levels also reached statistical significance between the two groups (tuberculin positive-median: 4080 pg/ml, tuberculin negative-median: 1659 pg/ml, p=0.0190).

4.3.3 Relationship between mycobacterial growth and cytokine production

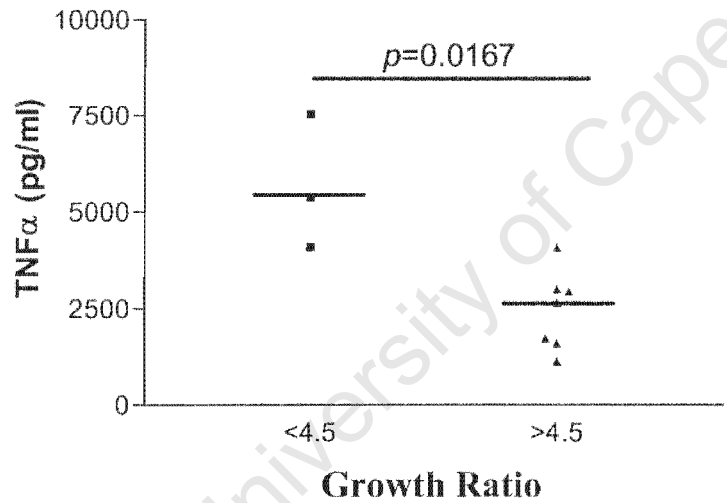
To evaluate the relationship between mycobacterial growth and cytokine production, subjects were separated into those showing restricted growth of BCG *lux* and those who failed to restrict growth of BCG *lux*. These were defined as subjects with a growth ratio below the median of 4.5 (see Table 3) for tuberculin positive individuals and subjects with a growth ratio above the median of 4.5. It was shown that only three of the tuberculin positive individuals had growth ratios below the median of 4.5. The remaining 3 tuberculin positive individuals and all four of the tuberculin negative individuals had growth ratios above the median of 4.5. Those subjects showing restricted growth produced significantly more IFNγ (median: 2111 pg/ml, mean: 2183, p=0.0167) than those who failed to restrict growth (median: 858 pg/ml, mean: 918 pg/ml), **figure 11a**. TNFα production was also significantly higher in the group showing restricted growth (median: 5381 pg/ml, mean: 5671 pg/ml, p=0.0167) than those who failed to restrict growth (median: 2647 pg/ml, mean: 2440 pg/ml), **figure 11b**.

University of Cape Town

a) IFNγ measured after a 96-hour incubation period



b) TNFα measured after a 72-hour incubation period



University of Cape Town

Figure 11. (a) IFNγ and (b) TNFα production from individuals showing restricted mycobacterial growth (■ <4.5) and individuals that failed to restrict mycobacterial growth (▲ >4.5), see appendix 3, **Table 9** for individual test values. TNFα and IFNγ levels were measured by ELISA at 72 and 96 hours respectively. Horizontal lines show the median for the group. Mann-Whitney test analysis (unpaired groups) was carried out to determine the significance of the differences between the two groups. Subjects showing restricted growth produced significantly more IFNγ (median: 2111 pg/ml, p=0.0167) than those who failed to restrict growth (median: 858 pg/ml). TNFα production was also significantly higher in the group showing restricted growth (median: 5381 pg/ml, p=0.0167) than those who failed to restrict growth (median: 2647 pg/ml)

4.4 Discussion

In this chapter we sought to validate the use of a luminescent mycobacterial whole blood assay to assess immune status and immune response cytokine profiles to mycobacterial antigen. We have attempted to identify markers that would best discriminate between individuals who are susceptible or resistant to *M. tuberculosis*. Our work was in agreement with previous work by Kampmann *et al.*, [71] in that tuberculin positive individuals were better able to control mycobacterial growth in whole blood than tuberculin negative individuals. This is consistent with the presence of a cell mediated antigen-specific adaptive (acquired) immune response. Individuals who have had previous exposure to mycobacterial antigens, but remain completely well (protected) are more likely to control *M. tuberculosis* after exposure and infection than individuals who have not been exposed to mycobacterial antigens (susceptible). Although the whole blood assay was able to differentiate between tuberculin positive and tuberculin negative individuals it did not allow us to correlate mycobacterial viability to susceptibility or resistance to tuberculosis. This would require us to follow-up these individuals for a prolonged period (5 to 10 years) in order to be sure that those individuals who were tuberculin positive remained well and those who were tuberculin negative developed disease.

Several attempts by researchers to elucidate the immune response to *M. tuberculosis* have focused on the use of isolated cell fractions, rather than whole blood, as a defined amount of cells can be stimulated. The ability to use small amounts of blood with this assay makes it suitable for its application to paediatric populations. Our results support the importance of cytokines such as IFNγ and TNFα in mycobacterial infections as significantly more IFNγ and TNFα were produced by individuals who were better able to control BCG *lux* growth. The increased production of TNFα by individuals who were better able to control mycobacterial growth is consistent with the presence of activated TNFα producing macrophages. This is also consistent with the increased IFNγ production by these individuals.

University of Cape Town

Chapter 5: Growth of Mycobacteria in Whole Blood of Healthy and HIV-Infected Children

5.1 Introduction

Tuberculosis represents a major health problem in childhood and age-related differences in susceptibility to the disease are well described. The immunological basis for the increased susceptibility in young children is not known in detail. Although the resurgence of TB in both developed and developing countries, and the increasing incidence of the disorder as a result of the HIV pandemic has led to increased research, most of this has focused on the problem of TB in adults. Research specifically focused on TB in childhood is urgently needed for a number of reasons. Failure of initial containment of the bacteria in children lacking an established immune response underlies progressive disease in childhood, whereas breakdown of well-established immune mechanisms may underlie re-activation in adult life. Whereas re-activation of TB in adult life results predominantly in pulmonary disorder, progressive primary TB in childhood can be associated with the devastating problems of TB meningitis, miliary TB and bone and joint destruction. The diagnosis of childhood TB is difficult, due to the low numbers of bacteria, lack of sputum positivity and frequently negative Mantoux. Improved diagnostic methods are thus urgently required for childhood disease. Any successful vaccine against TB will need to induce a protective immune response when administered to young children in the first few years of life.

University of Cape Town

An understanding of the immune responses to mycobacteria in early childhood is thus an essential first step in developing a vaccine that is more effective than BCG. Immune responses in children can vary according to age group and this might have implications for vaccine efficacy. The results of some major BCG trials are shown in **Table 4**. This chapter, compares mycobacterial growth in whole blood obtained from healthy children of different age groups and HIV-infected children in order to study the influence of age and immunocompetence on growth-restricting properties of whole blood. Supernatants obtained from incubation of whole blood with BCG *lux*

were analysed for IFNγ and TNFα. Children are particularly susceptible to TB under the age of 5 years and during puberty [95]. Between the ages of 5 and 10 years children are not as susceptible to TB. Therefore the first aim of this chapter was to determine the effect of age on the immunological response to TB as determined by the ability to restrict the growth of BCG in the whole blood assay. This study may help us understand why those at the paediatric age extremes are particularly susceptible to TB. The second aim was to determine the effect of HIV infection and the resultant immunocompromisation on the ability of whole blood to restrict growth of BCG. This was predicated on the fact that HIV-infected children are particularly susceptible to TB because of their low $CD4^+$ lymphocyte counts.

Table 4. The efficacy of BCG as determined in nine major trials

Region	Year of commencement	Age range	Protection afforded
North America	1935	0-20 years	80
Chicago, USA	1937	3 months	75
Georgia, USA	1947	6-17 years	0
Illinois, USA	1948	Young adults	0
Puerto Rico	1949	1-18 years	31
Georgia, USA	1950	5 years	14
Great Britain	1950	14-15 years	78
South India	1950	All ages	31
South India	1968	All ages	0**

**A 15-year follow up study revealed some protection (17%) in those vaccinated in infancy.
This table was adapted from Tuberculosis in childhood [96].

University of Cape Town

5.2 Methods

Peripheral blood was collected from 88 healthy children of different age groups and 22 HIV-infected children. Briefly, aliquots of the diluted (1:2) whole blood were inoculated with 1.5 x 10^8 RLU/ml BCG-*lux* and luminescence was measured at 0 and 96-hour time points. The growth ratio was calculated using the formula ($RLU_{96hours}$ / RLU_{0hours}) described previously in section 2.6.2. Supernatants were also collected at each time point for cytokine measurements.

5.3 Results

5.3.1 Growth of mycobacteria in the whole blood of healthy children

In order to determine the effect of age on the ability of whole blood to restrict the growth of BCG, 88 healthy children were recruited from two local communities. This cohort gave adequate numbers of children in the age categories of (i.) <1 year, (ii.) 1-4 years, (iii.) 5-9 years and (iv.) 10-15 years. All children were screened with a Mantoux skin test for evidence of prior exposure to mycobacterial antigen. Of the 88 children screened with a Mantoux skin test, only 20 had a positive skin reaction (induration >10 mm) and 15 of those had an induration >15 mm. There was no significant association between the growth of mycobacteria in the whole blood of healthy children in the different age groups as shown in **Table 5**. However, the highest growth ratios were measured in the very young children and those around puberty. These are the periods that epidemiologically coincide with the highest incidence of tuberculosis in children.

University of Cape Town

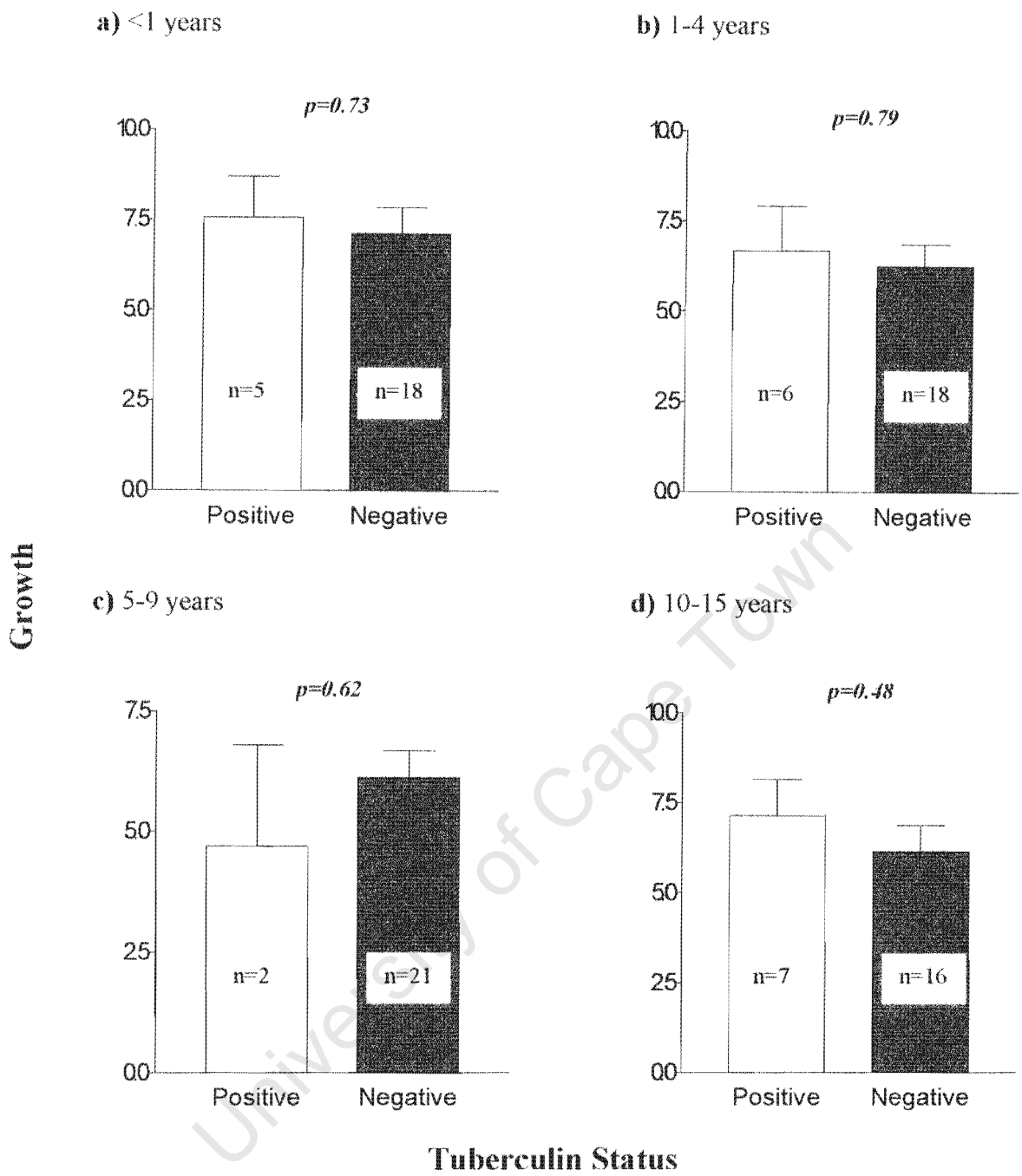
Table 5. Comparison of growth ratios obtained after culture of mycobacteria in whole blood of healthy children between the ages of 0-15

Age	n	Growth Ratio	*p*-value
<1	18	7.5	*NS*
1-4	24	6.3	*NS*
5-9	23	5.8	*NS*
10-15	23	7.0	*NS*

Growth expressed as median growth ratio for each age group
p-value, Kruskall-Wallis test, *NS* = not significant

The 88 healthy children were then grouped according to their immune status (tuberculin positive or tuberculin negative) in the different age groups. We investigated whether prior exposure to mycobacterial antigen as depicted by a positive Mantoux skin test in the healthy children would limit the ability of BCG *lux* to grow in whole blood of the healthy children. There was no significant difference in growth of BCG *lux* in whole blood of tuberculin positive and tuberculin negative healthy children, **figure 12**.

University of Cape Town



University of Cape Town

Figure 12. Growth of mycobacteria in whole blood of tuberculin positive and tuberculin negative healthy children (a) <1 year, (b) 1-4 years, (c) 5-9 years and (d) 10-15 years. Growth of BCG *lux* was measured at 96 hours and expressed as a growth ratio. Open columns represent tuberculin positive (>15 mm) healthy children and shaded columns represent tuberculin negative (<5 mm) healthy children. Error bars represent the standard error of the mean (SEM). The *p* values given were derived from a Mann-Whitney test analysis.

5.3.2 Associated cytokine production in whole blood of healthy children

TNFα and IFNγ production was measured at 72 and 96 hours respectively in supernatants harvested from whole blood cultures inoculated with BCG *lux*. There was a significant difference in IFNγ production between age groups, p=0.006 (Kruskal Wallis test) as shown in **Table 6**. Although there was a trend for IFNγ levels to increase with age there was no correlation between growth ratio and IFNγ levels, r=-0.043 (Spearman), **figure 13a**. In contrast, our data was able to show a correlation between IFNγ levels and age, **figure 13b**. TNFα levels did not reach statistical significance at the different age groups, p=0.681 (Kruskal Wallis test).

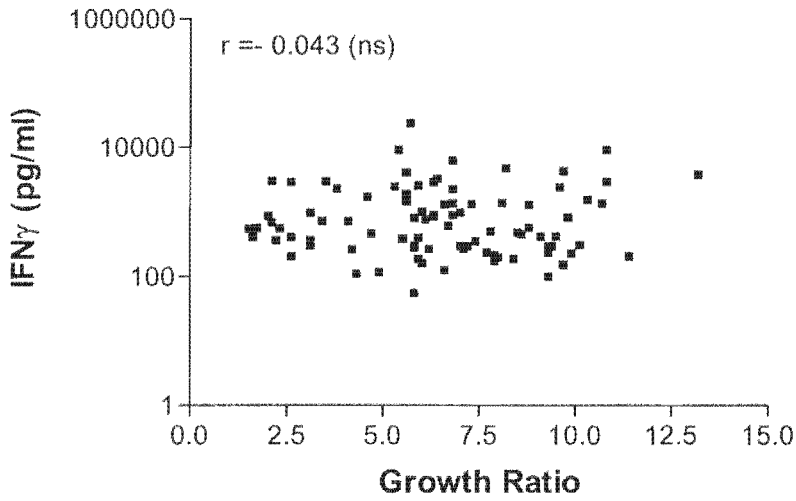
Table 6. Production of IFNγ and TNFα in whole blood from healthy children during *in vitro* infection with BCG *lux*

Age	*IFNγ (pg/ml)	Range (pg/ml)	TNFα (pg/ml)	Range (pg/ml)
<1	278	100-9322	2523	59-6608
1-4	518	56-3000	3107	1032-8553
5-9	878	270-24232	2888	967-8724
10-15	978	190-9238	2711	625-7305

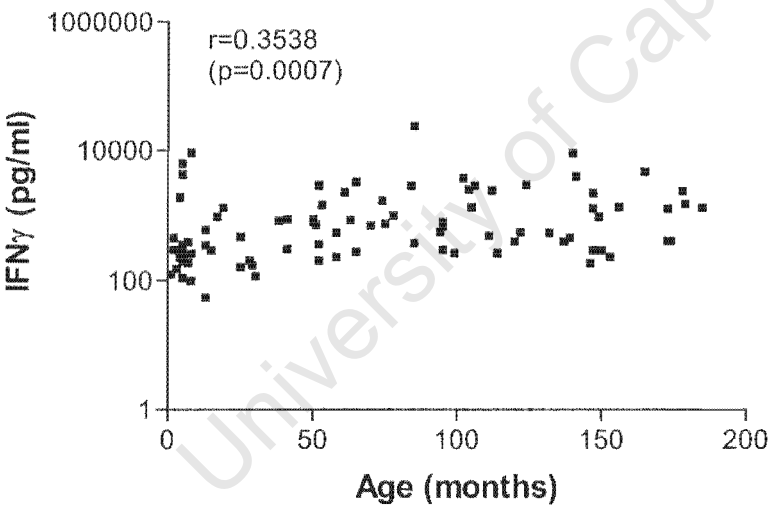
IFNγ and TNFα expressed as medians, *p=0.006, Kruskal Wallis test
See appendix 3, **Table 10** for individual test values.

University of Cape Town

a)



b)



University of Cape Town

Figure 13. Correlation between (a) IFNγ levels and growth ratio and (b) IFNγ levels and age

5.3.3 Comparison of mycobacterial growth in whole blood of healthy and HIV-infected children

To study the influence of compromised immunity on growth restricting properties of whole blood, a cohort of 22 HIV-infected children were recruited from the Infectious Diseases Clinic at Red Cross Children's Hospital in Cape Town. A Mantoux skin test was done on all children to determine prior exposure to mycobacterial antigen. Peripheral blood was collected to study mycobacterial growth restriction, to measure cytokine production and for $CD4^+$ T lymphocyte enumeration. The immunologic and clinical status of the 19 of the HIV-infected children was categorized according to the CDC staging system [92] as illustrated in **Table 7.**

Table 7. Clinical and immunological characteristics of HIV-infected children following the 1994 CDC classification system

Mean Age	**Sex**	**Classification of HIV-infection**			
		Clinical	Immunological category[b]		
59 months	13:9	category[a]	**1**	**2**	**3**
(range: 9-119 months)	Male:female	**A**	1	1	1
		B	0	9	3
		C	0	2	2

[a]A, mild signs/symptoms; B, moderate signs/symptoms; C, severe signs/symptoms
[b]1, no evidence of suppression; 2, evidence of moderate suppression; 3, severe suppression
($CD4^+$ T lymphocyte counts were obtained from 19 of the 22 children)

University of Cape Town

Out of the 19 HIV-infected children, 9 children were moderately immunocompromised with moderate symptoms **(B2)**, 3 children were severely immunocompromised with moderate symptoms **(B3)**, 2 children were moderately immunocompromised with severe symptoms and 2 children were severely immunocompromised with severe symptoms. There were 3 children who were not symptomatic with one showing no evidence of immunosuppresion **(A1)** but another showed moderate evidence of immunosuppression **(A2)** and the last one showed severe immunosupression **(A3)**. The mean age of the HIV-infected group (59 months,

range: 9-119 months) fell within the 1-4 year age group of the healthy control children, who had a slightly lower mean age of 38 months (range: 15-172 months). None of the children had clinical signs of tuberculosis, though all were recruited from an area of Cape Town with a high incidence of disease. BCG *lux* growth was compared in whole blood of HIV-infected children and healthy children, **figure 14**.

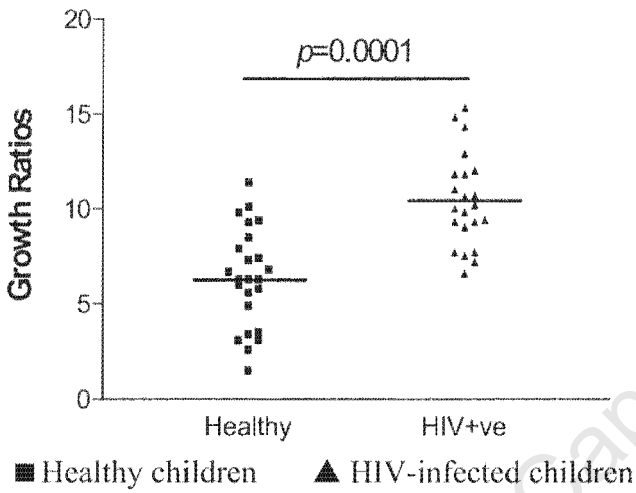


Figure 14. Comparison of mycobacterial growth in whole blood of (▲) HIV-infected children (n=22) and an age-matched (■) control group (n=24), see appendix 3, **Table 11** for individual test values. Growth of BCG *lux* was measured at 96 hours and expressed as a growth ratio. The median growth ratio was 10.1 for the HIV-infected group (range: 6.6-15.3) and 6.5 for the age-matched control group (range: 1.5-11.4). Horizontal lines show the median for the group. The *p* value given was derived from a Mann-Whitney test analysis.

University of Cape Town

The HIV-infected children demonstrated a significantly decreased ability to inhibit mycobacterial growth when compared with an age-matched control group, $p<0.0001$ (Mann-Whitney). The median growth ratio was 10.1 for the HIV-infected group (range: 6.6-15.3) and 6.5 for the age-matched control group (range: 1.5-11.4). $CD4^+$ T lymphocyte counts were determined in the HIV-infected children and were shown to be lower (mean: 460 cells/μl, range: 152-1725 cells/μl) than the reference $CD4^+$ T lymphocyte counts of an age-matched uninfected group (mean: 1606 cells/μl, range: 1310-2201cells/μl) [97]. The Kenyan CD4 results were used due the lack of normal $CD4^+$ T lymphocyte subset values for South African children of different ages. The

$CD4^+$ T cell counts of the HIV-infected children showed no correlation with the growth ratios, r=0.285 (Spearman), **figure 15.**

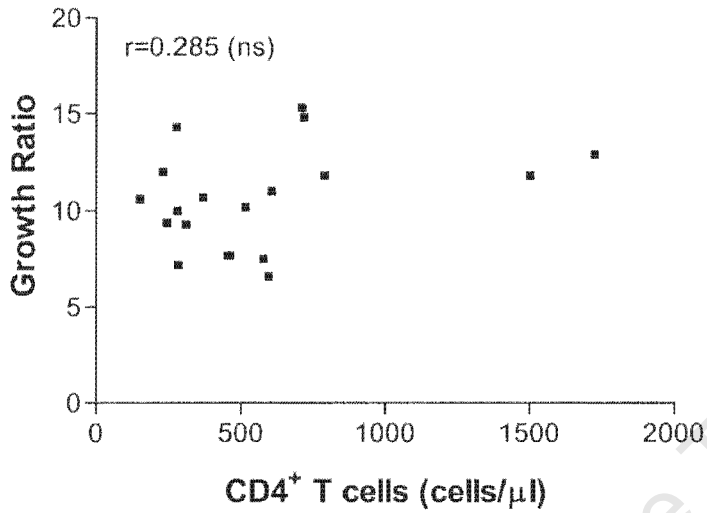


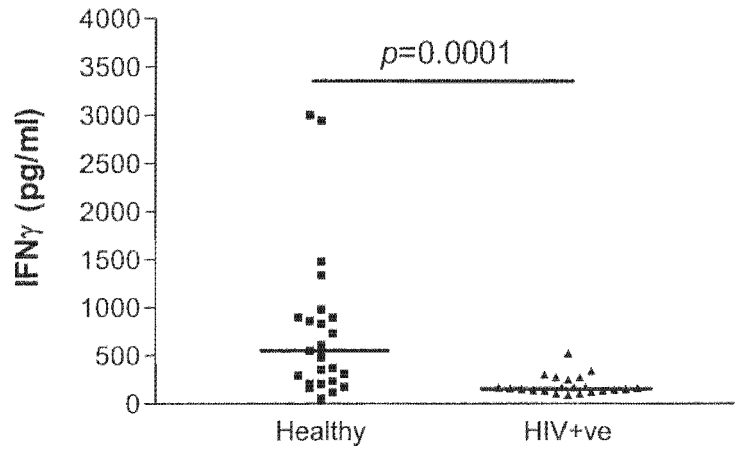
Figure 15. Correlation between growth ratio and $CD4^+$ T cells

5.3.4 Comparison of cytokine production in whole blood of healthy and HIV-infected children

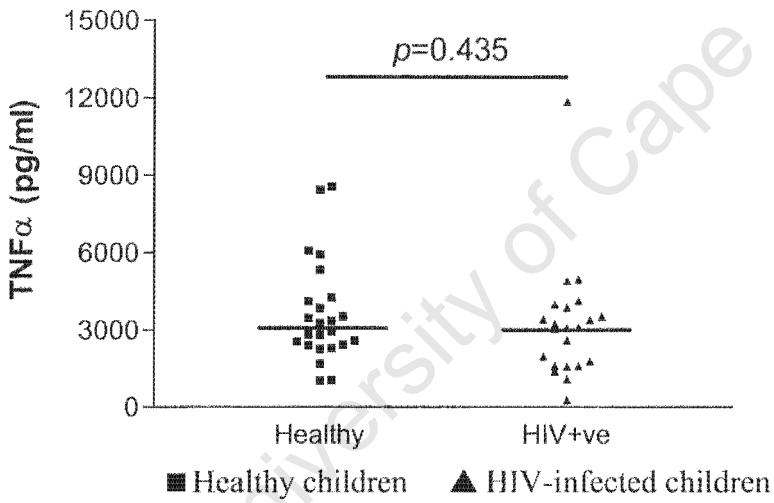
IFNγ and TNFα production was measured in the supernatants harvested from the whole blood assay in the HIV-infected children (n=22) and compared with the IFNγ and TNFα levels measured for the healthy children (n=24) in the 1-4 years age group. There was a significant difference in IFNγ production, p=0.0001 **figure 16a** between the healthy children and the HIV-infected children. The median level of IFNγ in the HIV-infected children was 156 pg/ml (range: 89-523 pg/ml), as compared to 518 pg/ml in the age-matched uninfected group (range: 56-3000 pg/ml). There was no significant difference in TNFα levels measured at the same time, p=0.435 **figure 16b**. The median TNFα levels were 3100 pg/ml (range: 1032-8553 pg/ml) for healthy children and 3080 pg/ml (range: 274-11834 pg/ml) for HIV-infected children.

University of Cape Town

a) IFNγ measured after a 96-hour incubation period



b) TNFα measured after a 72-hour incubation period



University of Cape Town

Figure 16. Comparison of (a) IFNγ and (b) TNFα production in whole blood of (■) healthy (n=24) and (▲) HIV-infected children (n=22), see appendix 3, **Table 12** for individual test values. IFNγ and TNFα production was measured at 96 and 72 hours respectively. The median level of IFNγ in the HIV-infected children was 156 pg/ml (range: 89-523 pg/ml), as compared to 518 pg/ml in the age-matched uninfected group (range: 56-3000 pg/ml). There was no significant difference in TNFα levels measured at the same time, p=0.435. The median TNFα levels were 3107 pg/ml (range: 1032-8553 pg/ml) for healthy children and 3080 pg/ml (range: 274-11834 pg/ml) for HIV-infected children. Horizontal lines show the median for the group. The p value given was derived from a Mann-Whitney test analysis.

5.4 Discussion

This assay combines the advantage of rapid enumeration of mycobacteria with the ability to study immune responses in small volumes of whole blood. It is therefore well suited for studies in paediatric populations. In this chapter we investigated the effect of age on the ability of whole blood from healthy children to restrict growth of mycobacteria and explored the differences in restriction between healthy and HIV-infected children. Although a trend could be demonstrated, there was no significant effect of age on the ability to restrict growth of mycobacteria in whole blood of healthy children using this model. TNFα levels showed no significant difference between the varying age groups. This is in contrast to the IFNγ levels, which showed a significant increase with age. Neither of the cytokines was found to correlate with growth ratios.

This model did however, demonstrate that HIV-infected children have an impaired ability to control the growth of mycobacteria in their blood *in vitro*. Their increased susceptibility to tuberculosis might be a clinical reflection of these findings. While we did not observe a precise correlation between the $CD4^+$ count and the ability of an individual to control mycobacterial growth, children with impaired $CD4^+$ T lymphocyte function produced significantly less IFNγ than their age-matched healthy controls. It can be postulated that the lack of IFNγ contributes to the increased growth of mycobacteria in the whole blood observed in these children. However, since the correlation between IFNγ and growth ratio did not reach significance in healthy children and no statistically significant alteration in growth ratios was observed in the assay by the addition of exogenous IFNγ [98], additional factors must also play a role in growth inhibition *in vitro*. Exploring the effect of adding alternative cytokine combinations could elucidate a possible explanation. "While long-term infection with HIV is characterised by a reduction in the absolute number of $CD4^+$ cells, functional abnormalities of viable and normal-appearing cells have been noted at all stages of HIV infection" [99]. Thus the lack of mycobacterial growth inhibition in the HIV-infected children could be due to dysfunction of their viable circulating $CD4^+$ T lymphocytes, resulting in impaired lymphocyte activation [100].

University of Cape Town

Chapter 6: Concluding Discussion and Future Prospects

Bioluminescent mycobacteria lend themselves to the rapid assessment of differences in human immune responses to mycobacterial infection. The ability to provide a readout of the ultimate effector function, mycobacterial survival, make them an attractive vehicle for studying some of the molecular aspects involved in the escalating problem of childhood tuberculosis. The advantage of being able to use small volumes of blood with this assay strengthens our support for its use in paediatric populations. The limitations of working with mycobacteria such as bacterial clumping has allowed for improved standardisation of the assay.

This work is consistent with and extends the observations of Kampmann and colleagues, who studied human immune responses to mycobacterial infection more specifically in adults. Kampmann *et al.*, [71] have shown that tuberculin positive individuals can significantly control growth of mycobacteria in whole blood compared to tuberculin negative individuals. The whole blood assay was also able to differentiate between tuberculin positive and tuberculin negative individuals in the published paper [98]. However, it did not allow for the correlation of mycobacterial viability to susceptibility or resistance to tuberculosis. This would require a long-term follow-up study to establish that the individuals who showed decreased mycobacterial growth stayed free of disease. It will be of interest to carry out more extensive comparisons between mycobacterial growth and the tuberculin response. For example, study of individuals who are tuberculin negative, yet show impaired growth of mycobacteria (such as heath care workers) in whole blood may provide a clue to (possibly innate) immune responses to TB that do not rely on T cell mediated DTH. Our results support the importance of cytokines such as IFNγ and TNFα in mycobacterial infections as individuals who were better able to control the growth of BCG *lux* produced significantly more IFNγ and TNFα. The increased production of these cytokines is consistent with the presence of activated macrophages.

Due to the difficulties in isolating *M. tuberculosis* in children, a definitive diagnosis of paediatric TB is problematic. Consequently, there are few studies of TB in children, particularly those examining the impact of HIV on TB infection. The

University of Cape Town

simplicity of the whole blood assay makes it suitable for application to population-based studies in TB endemic areas. This thesis failed to show age-related changes in mycobacterial growth *in vitro* in healthy children. This might be due to the high proportion of tuberculin negative children in the various age groups. However, it was able to show differences in mycobacterial growth between healthy and HIV-infected children. The two groups of children share similar environments and all had received BCG vaccination at birth. Zhang and colleagues [101] have previously shown the absence of a precise correlation between $CD4^+$ T lymphocyte count and the ability of an individual to control mycobacterial growth for the onset of clinical tuberculosis in HIV-infected individuals. It is likely that the difference between the two groups reflects HIV-mediated impairment rather than a difference in exposure to mycobacterial antigens.

We demonstrated in this thesis, that the reduced ability to control mycobacterial growth in peripheral blood is consistent with the known susceptibility to clinical tuberculosis in HIV-infected populations. The synergy between the two diseases represents one of the most important threats to global health [102]. The children involved in the present study did not have access to highly active antiretroviral therapy (HAART), but it would be of considerable interest to use this assay in analogous cohorts to determine whether HAART is able to restore HIV-impaired antimycobacterial immunity.

Individuals with mutations in the genes encoding IFNγ or IFNγ-receptor often succumb to mycobacterial infection [103]. This emphasises the importance of IFNγ in protection against human infection with mycobacteria. However, *in vitro*, it has been difficult to demonstrate IFNγ-induced killing of *M. tuberculosis* within human macrophages [104]. Our work was able to show a correlation between the production of IFNγ and the ability to control infection, but we have been unable to substitute the T cell contribution in the assay by addition of exogenous IFNγ [98]. A possible explanation would be that the IFNγ signal on its own is insufficient to trigger the appropriate activation pathway, but has to occur in combination with some second signal delivered by an additional cytokine or by cell-cell interaction. Alternatively, it can be proposed that some additional impairment in immune regulation counteracts

University of Cape Town

the effect of IFNγ in the HIV-infected blood [105]. It would be of interest to examine the effect of depletion or addition of various cytokine combinations or lymphocyte subsets in order to dissect the role of different immune mechanisms in the control of tuberculosis. This model is therefore applicable to further studies of antimycobacterial responses such as in TB infection and disease. It will be of interest to define the response of patients with different forms of TB and at different stages of the illness.

Availability of the genomic information and the development of molecular genetic tools has stimulated the development of novel TB vaccine candidates, some of which show encouraging efficacy in experimental models of infection [106], [107]. But, translation of progress in the laboratory into clinical vaccine trials in humans will depend on the development of improved assays for monitoring functional immune responses in vaccinees. Our data suggest that the whole blood luminescent assay might be a useful tool to assess the immunogenicity of promising new vaccine candidates, prior to large clinical trials assessing efficacy.

University of Cape Town

Appendix 1

Consent form: Ex-vivo models of killing of mycobacteria: cellular and molecular mechanisms and relevance to protective immunity in humans (Healthy children)

Researchers at Red Cross War Memorial Children's Hospital in Cape Town, St Mary's Hospital in London and Stanford University in the USA are investigating aspects of the immunological response that develops in children with active TB. Because there is a high prevalence of TB in the Western Cape, including Cape Town, the research is being conducted on blood samples obtained in this region. The immunological responses to TB in children who have received the BCG vaccine, children who have had active TB and children who are immuno-suppressed will be investigated. Healthy children are particularly susceptible to TB under the age of 5 years and during puberty. Between the ages of 5 and 10 years children are not as susceptible to TB. Therefore the aim of this project is to determine the immunological responses to TB at different ages. This study may help us understand why those at the paediatric age extremes are particularly susceptible to TB. One hundred healthy children at different ages (25 less than 1 year, 25 between 1 and 5 years, 25 between 6 and 10 years and 25 between 11 and 15 years) will be enrolled.

A single blood sample will be obtained for your child. A TB skin test will also be done to determine whether they have been exposed to TB in the past. This test will be read after 48 hours. The blood sample will be used to determine whether healthy children are able to kill the TB bug. Some of the blood will be used for other immunological tests (measurement of cytokines, chemokines, and effector molecules). All the research work will take place in the laboratory. The results will help doctors treating children with TB to understand how the body defends itself against TB.Children with a highly reactive mantoux (> 15 mm) will be referred for radiological evaluation and anti-tuberculous prophylaxis or treatment.

Your child will not be treated any differently if you decide not to permit him or her to participate in this study. He / she will receive the best treatment that the health system can provide.

Consent

I understand the above information as explained to me by _____
and am prepared to have my child NAME: _____
participate in this study.
NAME: _____

RELATION TO CHILD: _____

SIGNATURE: _____

DATE: _____

WITNESSES: _____

_____

University of Cape Town

Appendix 2

Consent form: Ex-vivo models of killing of mycobacteria: cellular and molecular mechanisms and relevance to protective immunity in humans (paediatric HIV infection)

Researchers at Red Cross War Memorial Children's Hospital in Cape Town, St Mary's Hospital in London and Stanford University in the USA are investigating aspects of the immunological response that develops in children with active TB. Because there is a high prevalence of TB in the Western Cape the research is being conducted on blood samples obtained in this region. HIV-infected children are particularly susceptible to TB because of their low CD counts. This project aims to determine the extent to which HIV-infected children are able to mount a response to TB. To complete the project successfully we require blood samples from 50 HIV-infected children.

Children will be recruited at the Infectious Diseases Clinic at Red Cross Children's Hospital. A single sample of blood will be obtained. The blood will be used to determine whether children with HIV infection are able to kill the TB bug. Some of the blood will be used for other immunological tests (measurement of cytokines, chemokines, effector molecules), determination of CD4 count and to study the pattern of gene expression in response to the TB bug. A TB skin test will also be done to determine whether the children were exposed to TB in the past. This test will be read after 48 hours. The results will help us to understand how the body defends itself against TB. Children with a reactive mantoux (> 5 mm) will be referred for radiological evaluation and anti-tuberculous treatment.

Your child will not be treated any differently if you decide not to permit him or her to participate in this study. He / she will receive the best treatment that the health system can provide.

Consent

I understand the above information as explained to me by _____
and am prepared to have my child NAME: _____
participate in this study.
NAME: _____

RELATION TO CHILD: _____

SIGNATURE: _____

DATE: _____

WITNESSES: _____

_____

University of Cape Town

Appendix 3

Tables of individual test values

Table 8. IFNγ and TNFα produced during incubation of whole blood obtained from tuberculin positive and negative individuals with BCG *lux*

Adult Control	Tuberculin Status	IFNγ (pg/ml)	TNFα (pg/ml)
1	positive	1554	2999
2	positive	2454	2647
3	positive	1009	7534
4	positive	1576	5381
5	positive	2111	4098
6	positive	1983	4061
7	negative	389	2934
8	negative	858	1724
9	negative	568	1122
10	negative	474	1593

NOTE. IFNγ: Tuberculin positive individuals (median: 1780 pg/ml, range: 1009-2454 pg/ml), tuberculin negative individuals (median: 521 pg/ml, range: 389-858 pg/ml), p=0.0095, Mann-Whitney test. TNFα: Tuberculin positive individuals (median: 4080 pg/ml, range: 2647-7534 pg/ml), tuberculin negative individuals (median: 1659 pg/ml, range: 1122-2934 pg/ml), p=0.0190, Mann-Whitney test.

University of Cape Town

Table 9. IFNγ and TNFα production from individuals showing restricted mycobacterial growth (growth ratio <4.5) and individuals that failed to restrict mycobacterial growth (growth ratio >4.5)

Growth Ratio	IFNγ (pg/ml)	TNFα (pg/ml)
<4.5	2111	7534
<4.5	2454	5381
<4.5	1983	4098
>4.5	1554	2999
>4.5	1009	2647
>4.5	1576	4061
>4.5	389	2934
>4.5	858	1724
>4.5	568	1122
>4.5	474	1593

NOTE. IFNγ: Growth ratio <4.5 (median: 2111 pg/ml, range: 1983-2454 pg/ml), growth ratio >4.5 (median: 858 pg/ml, range: 389-1576 pg/ml), p=0.0167, Mann-Whitney test. TNFα: Growth ratio <4.5 (median: 5381 pg/ml, range: 4098-7534 pg/ml), growth ratio >4.5 (median: 2647 pg/ml, range: 1122-4061 pg/ml), p=0.0167, Mann-Whitney test.

Table 10. IFNγ and TNFα produced during incubation of whole blood obtained from healthy children of different ages with BCG *lux*

IFNγ (pg/ml)				TNFα (pg/ml)			
<1 yr	**1-4 yrs**	**5-9 yrs**	**10-15 yrs**	**<1 yr**	**1-4 yrs**	**5-9 yrs**	**10-15 yrs**
4332	174	1730	410	192	1051	4726	3155
6376	860	302	410	59	1694	1825	3658
9322	898	572	1316	398	2424	2888	1148
200	610	270	1360	5882	8553	996	1573
218	208	502	564	4225	2808	6149	1698
302	554	816	1396	5418	2591	1794	1735
366	730	2328	2434	1584	2944	5318	2736
112	296	774	4100	6608	2426	2898	2573
402	354	282	3034	3534	3270	2743	4680
126	1338	1018	2276	4631	2821	1924	4252
462	164	24232	420	2018	3537	8724	4740
295	56	3346	300	3339	3858	3577	7305
100	370	2954	978	2653	3353	6473	1310
230	118	3814	470	2296	2308	3271	6259
1938	2946	698	296	2337	8430	4512	625
154	208	270	190	2111	4253	1389	2048
190	830	2496	4824	3243	3470	2495	2711
264	896	878	420	2393	1032	1945	1993
	236	2590	1330		2556	967	2476
	980	386	9238		6063	4065	4807
	3000	1374	238		4124	1012	1049
	1480	2924	1550		5922	1535	4170
	312	724	558		2270	3157	3986
	482				5347		

NOTE. IFNγ: <1 yrs, median: 278 pg/ml, range: 100-9322 pg/ml; 1-4 yrs, median: 518 pg/ml, range: 56-3000 pg/ml; 5-9 yrs, median: 878 pg/ml, range: 270-24232 pg/ml; 10-15 yrs, median: 978 pg/ml, range: 190-9238 pg/ml, p=0.006, Kruskal Wallis test. TNFα: <1 yrs, median: 2523 pg/ml, range: 59-6608 pg/ml; 1-4 yrs, median:3107 pg/ml, range: 1032-8553 pg/ml; 5-9 yrs, median: 2888 pg/ml, range: 967-8724 pg/ml; 10-15 yrs, median: 2711, range: 625-7305 pg/ml.

University of Cape Town

Table 11. Growth ratios of HIV-infected children and an age-matched control group (1-4 years)

Healthy Children 1-4 years	HIV-infected children
7.9	7.7
6.3	10.0
6.8	11.8
6.7	9.0
2.6	9.3
1.5	11.0
3.4	15.3
9.4	12.9
7.4	14.8
7.3	10.2
6.0	7.2
5.8	9.4
3.1	9.3
4.9	6.6
6.3	7.7
11.4	12.0
9.8	10.7
6.3	9.8
9.3	7.5
3.1	11.8
3.5	14.3
5.6	10.6
10.1	
8.5	

NOTE. Growth ratio: Healthy children (n=24), median: 6.3, range: 1.5-11.4, HIV-infected children (n=22), median: 10.1, range: 6.6-15.3, p=0.0001, Mann-Whitney.

University of Cape Town

Table 12. IFNγ and TNFα produced during incubation of whole blood obtained from healthy children (1-4 years) and HIV-infected children with BCG *lux*

IFNγ (pg/ml)		TNFα (pg/ml)	
Healthy	**HIV**	**Healthy**	**HIV**
174	102	1051	11834
860	157	1694	1598
898	138	2424	274
610	168	8553	3049
208	89	2808	4948
554	161	2591	3503
730	299	2944	3503
296	149	2426	3394
354	118	3270	1386
338	148	2821	3233
164	337	3537	3376
56	523	3858	1968
370	523	3353	3092
118	168	2308	4113
2946	100	8430	1575
208	130	4253	2590
830	272	3470	1777
896	130	1032	4888
236	155	2556	3067
980	273	6063	3862
3000	170	4124	1078
1480	142	5922	1594
312		2270	
482		5347	

NOTE. IFNγ: Healthy children (n=24), median: 518 pg/ml, range: 56-3000 pg/ml, HIV-infected children (n=22), median: 156 pg/ml, range: 89-523 pg/ml, p=0.0001, Mann-Whitney. Healthy children (n=24), median: 3107 pg/ml, range: 1032-8553 pg/ml, HIV-infected children (n=22), median: 3080 pg/ml, range: 274-11834 pg/ml, p=0.435, Mann-Whitney.

University of Cape Town

References

1. NIAID, *"Tuberculosis"*. <http://www.niaid.nih.gov/factsheet/tb.htm>, 2002. **(25 Apr 2003)**.
2. Dye, C., Scheele, S., Dolin, P., Pathania, V. and Raviglione, M. C., *Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project.* Jama, 1999. **282**(7): p. 677-86.
3. WHO, *"Tuberculosis"*. <http://www.who.int/factsheets/who104/en/>, 2002.
4. Snider, D.E., and Raviglione, M., *Global Burden of Tuberculosis*, in *Tuberculosis: Pathogenesis, Protection and Control*, B.R. Bloom, Editor. 1994, ASM Press: Wazhington D. C. p. 3-11.
5. Frieden, T.R., Sterling, T. R., Munsiff, S. S., Watt, C. J. and Dye, C., *Tuberculosis.* Lancet, 2003. **362**(9387): p. 887-99.
6. Sung, S.W., Kang, C. H., Kim, Y. T., Han, S. K., Shim, Y. S. and Kim, J. H., *Surgery increased the chance of cure in multi-drug resistant pulmonary tuberculosis.* Eur J Cardiothorac Surg, 1999. **16**(2): p. 187-93.
7. Fourie, B., *"The burden of tuberculosis in South Africa"*. MRC National Tuberculosis Research Programme, South Africa, SA HealthInfo Consortium, 2002(February): p. 1-3.
8. Edginton, M., *Tuberculosis in South Africa.* Health Systems Trust Update, 2000(56): p. 1-16.
9. McMurray, D.N., *Disease model: pulmonary tuberculosis.* Trends Mol Med, 2001. **7**(3): p. 135-7.
10. van Rie, A., Warren, R. M., Beyers, N., Gie, R. P., Classen, C. N., Richardson, M., Sampson, S. L., Victor, T. C. and van Helden, P. D., *Transmission of a multidrug-resistant Mycobacterium tuberculosis strain resembling "strain W" among noninstitutionalized, human immunodeficiency virus-seronegative patients.* J Infect Dis, 1999. **180**(5): p. 1608-15.
11. Koch, R., *Classics in infectious diseases. The etiology of tuberculosis: Robert Koch. Berlin, Germany 1882.* Rev Infect Dis, 1982. **4**(6): p. 1270-4.

University of Cape Town

12. Grange, J.M., *The bacteriology of tuberculosis*, in *Tuberculosis in childhood*, P.R. Donald, P. B. Fourie, and J. M. Grange, Editor. 1999, J. L. van Schaik Publishers: Hatfield, Pretoria. p. 1-11.

13. Goren, M.B., Cernich, M. and Brokl, O., *Some observations of mycobacterial acid-fastness.* Am Rev Respir Dis, 1978. **118**(1): p. 151-4.

14. Fine, P.E., and Rodrigues, L. C., *Modern vaccines. Mycobacterial diseases.* Lancet, 1990. **335**(8696): p. 1016-20.

15. Colditz, G.A., Brewer, T. F., Berkey, C. S., Wilson, M. E., Burdick, E., Fineberg, H. V. and Mosteller, F., *Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature.* Jama, 1994. **271**(9): p. 698-702.

16. Hart, P.D., and Sutherland, I., *BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life.* Br Med J, 1977. **2**(6082): p. 293-5.

17. Fine, P.E., *Variation in protection by BCG: implications of and for heterologous immunity.* Lancet, 1995. **346**(8986): p. 1339-45.

18. Han, T.I., Kim, I. O., Kim, W. S. and Yeon, K. M., *Disseminated BCG infection in a patient with severe combined immunodeficiency.* Korean J Radiol, 2000. **1**(2): p. 114-7.

19. Casanova, J.L., Jouanguy, E., Lamhamedi, S., Blanche, S. and Fischer, A., *Immunological conditions of children with BCG disseminated infection.* Lancet, 1995. **346**(8974): p. 581.

20. Abramowsky, C., Gonzalez, B. and Sorensen, R. U., *Disseminated bacillus Calmette-Guerin infections in patients with primary immunodeficiencies.* Am J Clin Pathol, 1993. **100**(1): p. 52-6.

21. Talbot, E.A., Perkins, M. D., Silva, S. F. and Frothingham, R., *Disseminated bacille Calmette-Guerin disease after vaccination: case report and review.* Clin Infect Dis, 1997. **24**(6): p. 1139-46.

22. Doffinger, R., Jouanguy, E., Dupuis, S., Fondaneche, M. C., Stephan, J. L., Emile, J. F., Lamhamedi-Cherradi, S., Altare, F., Pallier, A., Barcenas-Morales, G., Meinl, E., Krause, C., Pestka, S., Schreiber, R. D., Novelli, F. and Casanova, J. L., *Partial interferon-gamma receptor signaling chain deficiency in a patient with bacille Calmette-Guerin and Mycobacterium abscessus infection.* J Infect Dis, 2000. **181**(1): p. 379-84.

University of Cape Town

23. Baldwin, S.L., D'Souza, C., Roberts, A. D., Kelly, B. P., Frank, A. A., Lui, M. A., Ulmer, J. B., Huygen, K., McMurray, D. M. and Orme, I. M., *Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis.* Infect Immun, 1998. **66**(6): p. 2951-9.

24. Orme, I.M., *Beyond BCG: the potential for a more effective TB vaccine.* Mol Med Today, 1999. **5**: p. 487-492.

25. McMurray, D.N., *A nonhuman primate model for preclinical testing of new tuberculosis vaccines.* Clin Infect Dis, 2000. **30**(Suppl 3): p. S210-2.

26. Toossi, Z., and Ellner, J. J., *Host response to mycobacterium tuberculosis.* Front Biosci, 1998. **3**: p. E133-40.

27. Teitelbaum, R., Glatman-Freedman, A., Chen, B., Robbins, J. B., Unanue, E., Casadevall, A. and Bloom, B. R., *A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15688-93.

28. Schlesinger, L.S., Bellinger-Kawahara, C. G., Payne, N. R. and Horwitz, M. A., *Phagocytosis of Mycobacterium tuberculosis is mediated by human monocyte complement receptors and complement component C3.* J Immunol, 1990. **144**(7): p. 2771-80.

29. Hirsch, C.S., Ellner, J. J., Russell, D. G. and Rich, E. A., *Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of Mycobacterium tuberculosis by human alveolar macrophages.* J Immunol, 1994. **152**(2): p. 743-53.

30. Ratliff, T.L., McGarr, J. A., Abou-Zeid, C., Rook, G. A., Stanford, J. L., Aslanzadeh, J. and Brown, E. J., *Attachment of mycobacteria to fibronectin-coated surfaces.* J Gen Microbiol, 1988. **134**(Pt 5): p. 1307-13.

31. Schlesinger, L.S., *Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors.* J Immunol, 1993. **150**(7): p. 2920-30.

32. Schaible, U.E., Collins, H. L. and Kaufmann, S. H., *Confrontation between intracellular bacteria and the immune system.* Adv Immunol, 1999. **71**: p. 267-377.

33. Rock, F.L., Hardiman, G., Timans, J. C., Kastelein, R. A. and Bazan, J. F., *A family of human receptors structurally related to Drosophila Toll.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-93.

University of Cape Town

34. Chaudhary, P.M., Ferguson, C., Nguyen, V., Nguyen, O., Massa, H. F., Eby, M., Jasmin, A., Trask, B. J., Hood, L. and Nelson, P. S., *Cloning and characterization of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans.* Blood, 1998. **91**(11): p. 4020-7.

35. Takeuchi, O., Kawai, T., Sanjo, H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Takeda, K. and Akira, S., *TLR6: A novel member of an expanding toll-like receptor family.* Gene, 1999. **231**(1-2): p. 59-65.

36. van Crevel, R., Ottenhoff, T. H. and van der Meer, J. W., *Innate immunity to Mycobacterium tuberculosis.* Clin Microbiol Rev, 2002. **15**(2): p. 294-309.

37. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.

38. Chan, J., and Kaufmann, H. E., *Immune mechanisms of protection*, in *Tuberculosis: Pathogenesis, Protection and Control*, B.R. Bloom, Editor. 1994, ASM Press: Wazhington D. C. p. 389-415.

39. Flynn, J.L., and Chan, J., *Immunology of tuberculosis.* Annu Rev Immunol, 2001. **19**: p. 93-129.

40. Bogdan, C., Rollinghoff, M. and Diefenbach, A., *Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity.* Curr Opin Immunol, 2000. **12**(1): p. 64-76.

41. Orme, I.M., Andersen, P. and Boom, W. H., *T cell response to Mycobacterium tuberculosis.* J Infect Dis, 1993. **167**(6): p. 1481-97.

42. Cooper, A.M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G. and Orme, I. M., *Disseminated tuberculosis in interferon gamma gene-disrupted mice.* J Exp Med, 1993. **178**(6): p. 2243-7.

43. Flynn, J.L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A. and Bloom, B. R., *An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection.* J Exp Med, 1993. **178**(6): p. 2249-54.

44. Tsukaguchi, K., Balaji, K. N. and Boom, W. H., *CD4+ alpha beta T cell and gamma delta T cell responses to Mycobacterium tuberculosis. Similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production.* J Immunol, 1995. **154**(4): p. 1786-96.

University of Cape Town

45. Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I. S., Bradley, A. and Stewart, T. A., *Multiple defects of immune cell function in mice with disrupted interferon-gamma genes.* Science, 1993. **259**(5102): p. 1739-42.

46. Newport, M.J., Huxley, C. M., Huston, S., Hawrylowicz, C. M., Oostra, B. A., Williamson, R. and Levin, M., *A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection.* N Engl J Med, 1996. **335**(26): p. 1941-9.

47. Jouanguy, E., Lamhamedi-Cherradi, S., Altare, F., Fondaneche, M. C., Tuerlinckx, D., Blanche, S., Emile, J. F., Gaillard, J. L., Schreiber, R., Levin, M., Fischer, A., Hivroz, C. and Casanova, J. L., *Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis.* J Clin Invest, 1997. **100**(11): p. 2658-64.

48. Chan, J., Xing, Y., Magliozzo, R. S. and Bloom, B. R., *Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages.* J Exp Med, 1992. **175**(4): p. 1111-22.

49. MacMicking, J.D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K. and Nathan, C. F., *Identification of nitric oxide synthase as a protective locus against tuberculosis.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5243-8.

50. Lalvani, A., Nagvenkar, P., Udwadia, Z., Pathan, A. A., Wilkinson, K. A., Shastri, J. S., Ewer, K., Hill, A. V., Mehta, A. and Rodrigues, C., *Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians.* J Infect Dis, 2001. **183**(3): p. 469-77.

51. Lewinsohn, D.M., Briden, A. L., Reed, S. G., Grabstein, K. H. and Alderson, M. R., *Mycobacterium tuberculosis-reactive CD8+ T lymphocytes: the relative contribution of classical versus nonclassical HLA restriction.* J Immunol, 2000. **165**(2): p. 925-30.

52. Porcelli, S.A., and Modlin, R. L., *The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids.* Annu Rev Immunol, 1999. **17**: p. 297-329.

53. Constant, P., Davodeau, F., Peyrat, M. A., Poquet, Y., Puzo, G., Bonneville, M. and Fournie, J. J., *Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands.* Science, 1994. **264**(5156): p. 267-70.

University of Cape Town

54. D'Souza, C.D., Cooper, A. M., Frank, A. A., Mazzaccaro, R. J., Bloom, B. R. and Orme, I. M., *An anti-inflammatory role for gamma delta T lymphocytes in acquired immunity to Mycobacterium tuberculosis.* J Immunol, 1997. **158**(3): p. 1217-21.

55. Boom, W.H., Chervenak, K. A., Mincek, M. A. and Ellner, J. J., *Role of the mononuclear phagocyte as an antigen-presenting cell for human gamma delta T cells activated by live Mycobacterium tuberculosis.* Infect Immun, 1992. **60**(9): p. 3480-8.

56. Constant, P., Poquet, Y., Peyrat, M. A., Davodeau, F., Bonneville, M. and Fournie, J. J., *The antituberculous Mycobacterium bovis BCG vaccine is an attenuated mycobacterial producer of phosphorylated nonpeptidic antigens for human gamma delta T cells.* Infect Immun, 1995. **63**(12): p. 4628-33.

57. Griffin, J.P., Harshan, K. V., Born, W. K. and Orme, I. M., *Kinetics of accumulation of gamma delta receptor-bearing T lymphocytes in mice infected with live mycobacteria.* Infect Immun, 1991. **59**(11): p. 4263-5.

58. Jouanguy, E., Altare, F., Lamhamedi-Cherradi, S. and Casanova, J. L., *Infections in IFNGR-1-deficient children.* J Interferon Cytokine Res, 1997. **17**(10): p. 583-7.

59. Cooper, A.M., Roberts, A. D., Rhoades, E. R., Callahan, J. E., Getzy, D. M. and Orme, I. M., *The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection.* Immunology, 1995. **84**(3): p. 423-32.

60. Cooper, A.M., Magram, J., Ferrante, J. and Orme, I. M., *Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis.* J Exp Med, 1997. **186**(1): p. 39-45.

61. Altare, F., Durandy, A., Lammas, D., Emile, J. F., Lamhamedi, S., Le Deist, F., Drysdale, P., Jouanguy, E., Doffinger, R., Bernaudin, F., Jeppsson, O., Gollob, J. A., Meinl, E., Segal, A. W., Fischer, A., Kumararatne, D. and Casanova, J. L., *Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency.* Science, 1998. **280**(5368): p. 1432-5.

62. de Jong, R., Altare, F., Haagen, I. A., Elferink, D. G., Boer, T., van Breda Vriesman, P. J., Kabel, P. J., Draaisma, J. M., van Dissel, J. T., Kroon, F. P., Casanova, J. L. and Ottenhoff, T. H., *Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients.* Science, 1998. **280**(5368): p. 1435-8.

University of Cape Town

63. Altare, F., Lammas, D., Revy, P., Jouanguy, E., Doffinger, R., Lamhamedi, S., Drysdale, P., Scheel-Toellner, D., Girdlestone, J., Darbyshire, P., Wadhwa, M., Dockrell, H., Salmon, M., Fischer, A., Durandy, A., Casanova, J. L. and Kumararatne, D. S., *Inherited interleukin 12 deficiency in a child with bacille Calmette-Guerin and Salmonella enteritidis disseminated infection.* J Clin Invest, 1998. **102**(12): p. 2035-40.

64. Flynn, J.L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., Schreiber, R., Mak, T. W. and Bloom, B. R., *Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice.* Immunity, 1995. **2**(6): p. 561-72.

65. Beyers, N., Gie, R. P., Schaaf, H. S., Van Zyl, S., Talent, J. M., Nel, E. D. and Donald, P. R., *A prospective evaluation of children under the age of 5 years living in the same household as adults with recently diagnosed pulmonary tuberculosis.* Int J Tuberc Lung Dis, 1997. **1**(1): p. 38-43.

66. van Rie, A., Beyers, N., Gie, R. P., Kunneke, M., Zietsman, L. and Donald, P. R., *Childhood tuberculosis in an urban population in South Africa: burden and risk factor.* Arch Dis Child, 1999. **80**(5): p. 433-7.

67. Smith, S., Jacobs, R. F. and Wilson, C. B., *Immunobiology of childhood tuberculosis: a window on the ontogeny of cellular immunity.* J Pediatr, 1997. **131**(1 Pt 1): p. 16-26.

68. Clark, J.E., and Cant, A. J., *Pitfalls in contact tracing and early diagnosis of childhood tuberculosis.* Bmj, 1996. **313**(7051): p. 221-2.

69. Weismuller, M.M., Graham, S. M., Claessens, N. J., Meijnen, S., Salaniponi, F. M. and Harries, A. D., *Diagnosis of childhood tuberculosis in Malawi: an audit of hospital practice.* Int J Tuberc Lung Dis, 2002. **6**(5): p. 432-8.

70. Schaaf, H.S., Cotton, M. F., de Villiers, G. S. and Donald, P. R., *Clinical insights into the interaction of childhood tuberculosis and HIV in the Western Cape.* The SAJ HIV Medicine, 2000: p. 33-35.

71. Kampmann, B., Gaora, P. O., Snewin, V. A., Gares, M. P., Young, D. B. and Levin, M., *Evaluation of human antimycobacterial immunity using recombinant reporter mycobacteria.* J Infect Dis, 2000. **182**(3): p. 895-901.

72. Meighen, E.A., *Bacterial bioluminescence: organization, regulation, and application of the lux genes.* Faseb J, 1993. **7**(11): p. 1016-22.

University of Cape Town

73. Meighen, E.A., *Enzymes and genes from the lux operons of bioluminescent bacteria.* Ann Rev Microbiol, 1988. **42**: p. 151-176.

74. Meighen, E.A., *Molecular biology of bacterial bioluminescence.* Microbiol Rev, 1991. **55**(1): p. 123-42.

75. Andrew, P.W., and Roberts, I. S., *Construction of a bioluminescent mycobacterium and its use for assay of antimycobacterial agents.* J Clin Microbiol, 1993. **31**(9): p. 2251-4.

76. Rauzier, J., Moniz-Pereira, J. and Gicquel-Sanzey, B., *Complete nucleotide sequence of pAL5000, a plasmid from Mycobacterium fortuitum.* Gene, 1988. **71**(2): p. 315-21.

77. Lee, M.H., Pascopella, L., Jacobs, W. R., Jr. and Hatfull, G. F., *Site-specific integration of mycobacteriophage L5: integration-proficient vectors for Mycobacterium smegmatis, Mycobacterium tuberculosis, and bacille Calmette-Guerin.* Proc Natl Acad Sci U S A, 1991. **88**(8): p. 3111-5.

78. Garbe, T.R., Barathi, J., Barnini, S., Zhang, Y., Abou-Zeid, C., Tang, D., Mukherjee, R. and Young, D. B., *Transformation of mycobacterial species using hygromycin resistance as selectable marker.* Microbiology, 1994. **140**(Pt 1): p. 133-8.

79. Hill, P.J., and Stewart, G. S., *Use of lux genes in applied biochemistry.* J Biolumin Chemilumin, 1994. **9**(3): p. 211-5.

80. Gaora, P.O., *Expression of genes in mycobacteria.* Methods Mol Biol., 1998. **101**: p. 261-73.

81. Arain, T.M., Resconi, A. E., Singh, D. C. and Stover, C. K., *Reporter gene technology to assess activity of antimycobacterial agents in macrophages.* Antimicrob Agents Chemother, 1996. **40**(6): p. 1542-4.

82. Carriere, C., Riska, P. F., Zimhony, O., Kriakov, J., Bardarov, S., Burns, J., Chan, J. and Jacobs, W. R., Jr., *Conditionally replicating luciferase reporter phages: improved sensitivity for rapid detection and assessment of drug susceptibility of Mycobacterium tuberculosis.* J Clin Microbiol, 1997. **35**(12): p. 3232-9.

83. Snewin, V.A., Gares, M. P., Gaora, P. O., Hasan, Z., Brown, I. N. and Young, D. B., *Assessment of immunity to mycobacterial infection with luciferase reporter constructs.* Infect Immun, 1999. **67**(9): p. 4586-93.

University of Cape Town

84. Bonay, M., Bouchonnet, F., Pelicic, V., Lagier, B., Grandsaigne, M., Lecossier, D., Grodet, A., Vokurka, M., Gicquel, B. and Hance, A. J., *Effect of stimulation of human macrophages on intracellular survival of Mycobacterium bovis Bacillus Calmette-Guerin. Evaluation with a mycobacterial reporter strain.* Am J Respir Crit Care Med, 1999. **159**(5 Pt 1): p. 1629-37.

85. Silver, R.F., Li, Q., Boom, W. H. and Ellner, J. J., *Lymphocyte-dependent inhibition of growth of virulent Mycobacterium tuberculosis H37Rv within human monocytes: requirement for CD4+ T cells in purified protein derivative-positive, but not in purified protein derivative-negative subjects.* J Immunol, 1998. **160**(5): p. 2408-17.

86. Orme, I.M., Roberts, A. D., Furney, S. K. and Skinner, P. S., *Animal and cell-culture models for the study of mycobacterial infections and treatment.* Eur J Clin Microbiol Infect Dis, 1994. **13**(11): p. 994-9.

87. Bouza, E., Diaz-Lopez, M. D., Moreno, S., Bernaldo de Quiros, J. C., Vicente, T. and Berenguer, J., *Mycobacterium tuberculosis bacteremia in patients with and without human immunodeficiency virus infection.* Arch Intern Med, 1993. **153**(4): p. 496-500.

88. McDonald, L.C., Archibald, L. K., Rheanpumikankit, S., Tansuphaswadikul, S., Eampokalap, B., Nwanyanawu, O., Kazembe, P., Dobbie, H., Reller, L. B. and Jarvis, W. R., *Unrecognised Mycobacterium tuberculosis bacteraemia among hospital inpatients in less developed countries.* Lancet, 1999. **354**(9185): p. 1159-63.

89. Starke, J.R., *Childhood tuberculosis: treatment strategies and recent advances.* Paediatr Respir Rev, 2001. **2**(2): p. 103-12.

90. Weir, R.E., Morgan, A. R., Britton, W. J., Butlin, C. R. and Dockrell, H. M., *Development of a whole blood assay to measure T cell responses to leprosy: a new tool for immuno-epidemiological field studies of leprosy immunity.* J Immunol Methods, 1994. **176**(1): p. 93-101.

91. Weir, R.E., Brennan, P. J., Butlin, C. R. and Dockrell, H. M., *Use of a whole blood assay to evaluate in vitro T cell responses to new leprosy skin test antigens in leprosy patients and healthy subjects.* Clin Exp Immunol, 1999. **116**(2): p. 263-9.

University of Cape Town

92. CDC, *1994 revised guidelines for the performance of CD4+ T-cell determinations in persons with human immunodeficiency virus (HIV) infections. Centers for Disease Control and Prevention.* MMWR Recomm Rep, 1994. **43**(RR-3): p. 1-21.

93. CDC, *1994 revised classification system for human immunodeficiency virus infection in children less than 13 years of age. Centers for Disease Control and Prevention.* MMWR Recomm Rep, 1994. **43**(RR-12): p. 1-20.

94. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular cloning: a laboratory manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989.

95. Milburn, H.J., *Primary tuberculosis.* Curr Opin Pulm Med, 2001. **7**(3): p. 133-41.

96. Grange, J.M., *The immunology of tuberculosis*, in *Tuberculosis in childhood*, P.R. Donald, P. B. Fourie, and J. M. Grange, Editor. 1999, J. L. van Schaik Pulishers: Hatfield, Pretoria.

97. Embree, J., Bwayo, J., Nagelkerke, N., Njenga, S., Nyange, P., Ndinya-Achola, J., Pamba, H. and Plummer, F., *Lymphocyte subsets in human immunodeficiency virus type 1-infected and uninfected children in Nairobi.* Pediatr Infect Dis J, 2001. **20**(4): p. 397-403.

98. Tena, G.N., Young, D. B., Eley, B., Henderson, HH., Nicol, M. P., Levin, M. and Kampmann, B., *Failure to control growth of mycobacteria in blood from children infected with human immunodeficiency virus and its relationship to T cell function.* J Infect Dis, 2003. **187**(10): p. 1544-51.

99. Rosenberg, Z.F., and Fauci, A. S., *The immunopathogenesis of HIV infection.* Adv Immunol, 1989. **47**: p. 377-431.

100. Rosenberg, Z.F., and Fauci, A. S., *Immunopathogenesis of HIV infection.* Faseb J, 1991. **5**(10): p. 2382-90.

101. Zhang, M., Gong, J., Iyer, D. V., Jones, B. E., Modlin, R. L. and Barnes, P. F., *T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection.* J Clin Invest, 1994. **94**(6): p. 2435-42.

102. Lienhardt, C., and Rodrigues, L. C., *Estimation of the impact of the human immunodeficiency virus infection on tuberculosis: tuberculosis risks re-visited?* Int J Tuberc Lung Dis, 1997. **1**(3): p. 196-204.

University of Cape Town

103. Dupuis, S., Doffinger, R., Picard, C., Fieschi, C., Altare, F., Jouanguy, E., Abel, L. and Casanova, J. L., *Human interferon-gamma-mediated immunity is a genetically controlled continuous trait that determines the outcome of mycobacterial invasion.* Immunol Rev, 2000. **178**: p. 129-37.

104. Rook, G.A., Steele, J., Ainsworth, M. and Champion, B. R., *Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages.* Immunology, 1986. **59**(3): p. 333-8.

105. Hertoghe, T., Wajja, A., Ntambi, L., Okwera, A., Aziz, M. A., Hirsch, C., Johnson, J., Toossi, Z., Mugerwa, R., Mugyenyi, P., Colebunders, R., Ellner, J. and Vanham, G., *T cell activation, apoptosis and cytokine dysregulation in the (co)pathogenesis of HIV and pulmonary tuberculosis (TB).* Clin Exp Immunol, 2000. **122**(3): p. 350-7.

106. Mustafa, A.S., *Development of new vaccines and diagnostic reagents against tuberculosis.* Mol Immunol, 2002. **39**(1-2): p. 113-9.

107. Ginsberg, A.M., *What's new in tuberculosis vaccines?* Bull World Health Organ, 2002. **80**(6): p. 483-8.

University of Cape Town