Identification of Immune Correlates of Natural Protection against Tuberculosis in a population with a high incidence of Latent Infection

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: …… Date: ……..

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Summary

Setting

This study was conducted in the Tygerberg area of Cape Town in South Africa.

Background

A third of the world's population is latently infected with *Mycobacterium tuberculosis*, and correlates of protection against progression to active disease urgently need to be identified to facilitate the development of an effective vaccine against the disease. The production of IFN-γ is recognised as an immune correlate of protection from tuberculosis, but other immune regulators have been implicated in playing a significant role in protective immunity. The aims of this project were three-fold: (i) to identify promising TB vaccine candidates by screening a panel of novel MTB antigens, by stimulating whole blood cultures *in vitro* with the novel proteins and quantifying the level of IFN-γ production, (ii) to identify other cytokines and chemokines that may be immune correlates of protection using the Luminex fluorescent bead-based technique and (iii) to compare the performance of the two techniques.

Methods

Antigen Screening study

Whole blood of 57 adult and adolescent participants defined as latently infected individuals was stimulated with a panel of 78 novel TB-specific, DosR- or RD1-encoded antigens. The 7-day culture supernatants were used in IFN-γ ELISA to quantify the level of IFN-γ production.

Luminex Assay study

Whole blood culture supernatants of 15 HIV negative, TST positive adults were used in the Luminex LINCO 21-plex cytokine assay. This was done to determine which of 21 cytokines, that may be LTBI-associated cytokines, were produced after stimulation with 9 TB-specific recombinant antigens, and to quantify their level of expression.

Results

In the antigen screening study, it was found the majority of the 78 proteins tested were able to induce a positive IFN-γ response. The classic TB antigens were used as controls, and the frequency of responses was highest after stimulation with ESAT-6 and TesatCFP10 (80 – 85% of responders). Ten latency antigens elicited an IFN-γ response in 19 – 45% of participants, and five reactivation antigens stimulated a positive reaction in 15 – 48% of responders. The category of antigens that elicited the most frequent and highest responses overall was the resuscitation-promoting factors (Rpf). Over 30% of participants responded to all 5 Rpfs, and the level of responses were equally divided in the low and moderate-to-high levels, with an additional 5% of responses in the high (>1000pg/ml) range.

In the Luminex study, the positive stimulant TesatCFP10 consistently induced expression of most cytokines. In addition latency antigens Rv1733c, Rv0569 and Rv2029c also induced moderate-to-high level cytokine expression. A Th1-biased cytokine profile was observed, with the preferential expression of pro-inflammatory and cell-mediated cytokines like IFN-γ, TNF-α, IP-10, MIP1-α and G-CSF being produced. Th2 cytokines IL-4, IL-5, IL-13 and eotaxin were very poorly expressed or were not expressed at detectable levels. A very strong induction of IL-6, IL-8 and MCP-1 was observed, but this

cytokine/chemokine association suggested contamination of the recombinant antigens with bacterial endotoxins.

Conclusion

In this study of latently infected individuals, the pattern of response observed for both assays is largely a Th1-biased expression profile. The whole blood ELISA method is a well-established assay for quantifying IFN-γ in culture supernatants, and has proven to be effective here. This study has demonstrated, in humans with LTBI, immune recognition of these novel MTB-specific antigens as illustrated by the positive IFN-γ levels induced after stimulation. The multiplex technology is also a very versatile and sensitive assay, capable of detecting multiple analytes simultaneously in one sample. The multiplex has been valuable here in identifying some antigens as potential vaccine candidates, and a subset of cytokines as potential immune mediators and prognostic indicators in TB infection.

Opsomming

Studie-area

Hierdie studie was gedoen in die Tygerberg area van Kaapstad in Suid-Afrika.

Agtergrond

'n Derde van die wêreld se bevolking is latent geïnfekteer met *Mycobacterium tuberculosis* en korrelate van beskerming teen die siekte moet geïdentifiseer word om die ontwikkeling van 'n effektiewe enstof te fasiliteer. Die produksie van IFN-γ is welbekend as 'n immuunkorrelaat van beskerming teen tuberkulose (TB), maar ander immuunreguleerders speel ook 'n belangrike rol in beskermende immuniteit. Die doelwitte van hierdie projek was drievoudig: (i) om belowende TB-entstof kandidate te identifiseer deur die sifting van 'n paneel van nuwe MTB antigene mbv die *in vitro* stimulasie van volbloed kulture, ii) om ander sitokiene en chemokiene as immuunkorrelate van beskerming te identifiseer deur van die *Luminex fluorescent bead-based* tegniek gebruik te maak, en (iii) om die twee tegnieke te vergelyk op grond van hul prestasie as prognostiese of siftings metodes in latente infeksie.

Metodes

Antigeen siftings studie

Volbloed van 57 volwasse en adolessente deelnemers, geïdentifiseer as latent geïnfekteerde individue, was gestimuleer met 'n paneel van 78 nuwe TB-spesifieke DosR- or R-gekodeerde antigene. Die 7-dae kultuur supernatante was gebruik in 'n IFN-γ ELISA om die hoeveelheid IFN-γ produksie the kwantifiseer.

Luminex assay studie

Volbloed kultuur supernatante van 15 HIV negatiewe, TST positiewe volwassenes was gebruik in die *Luminex LINCO 21-plex cytokine assay*. Dit was gedoen om die tipes en hoeveelheid ander LTBI-geassosieerde sitokienes te identifiseer wat geproduseer word na stimulasie met 9 TB-spesifieke rekombinante antigene.

Resultate

In die antigeen siftings studie is gevind dat die meerderheid van die 78 getoetste proteïene ‘n positiewe IFN-γ reaksie kon induseer. Vir die kontroles was die frekwensie van reaksies die hoogste na stimulasie met ESAT-6 en TesatCFP-10 (80 – 85% van reageerders). Tien latensie antigene was gereeld herken deur 19 – 45% van deelnemers en vyf reaktiverings-antigene het ‘n positiewe reaksie in 15 – 48% van reageerders gestimuleer. Die kategorie van antigene wat die meeste en hoogste response veroorsaak het, was die resusitasie-promoterende faktors (Rpf). Meer as 30% van deelnemers het op al 5 Rpfs gereageer en die vlak van reaksies was gelyk verdeel in die lae en matig-tot-hoog vlakke, met ‘n addisionele 5% van reaksies in die hoë (>1000pg/ml) reeks.

In die *Luminex* studie het die positiewe stimulant TesatCFP-10 konsekwent die positiewe uitdrukking van die meeste sitokiene geïnduseer. Saam met dit het die latente antigene Rv1733c, Rv0569 en Rv2029c ook matige-toe-hoë vlakke van sitokien uitdrukking geïnduseer. ‘n Th1-gebaseerde sitokien profiel was waargeneem, met die begunstigde uitdrukking van pro-inflammatoriese en sel-gemedieerde sitokiene soos IFN-γ, TNF-α, IP-10, MIP1-α en G-CSF. Th2 sitokiene IL-4, IL-5, IL-13 en eotaksien was of baie sleg uitgedruk of onder naspeurbare vlakke uitgedruk. ‘n

Baie sterk induksie van IL-6, IL-8 en MCP-1 was waargeneem, maar hierdie sitokiene/chemokiene assosiasie stel moontlik kontaminasie van die rekombinante antigene met bakteriële endotoksiene voor.

Samevatting

Die reaksiepatroon wat in hierdie studie tussen die twee toetse waargeneem is, was grootliks ‘n Th1-gebaseerde uitdrukkingsprofiel vir latente infeksie met TB. Die volbloed ELISA metode is a betroubare gevestigde toets vir die kwantifisering van IFN-γ in kultuur supernatante, wat ook in hierdie studie bewys is om effektief te wees. Hierdie studie het gedemonstreer dat die nuwe TB-spesifieke antigene effektief positiewe IFN-γ response in mense met LTBI induseer. Die multipleks tegnologie is ook ‘n baie veelsydige en sensitiewe toets, wat in staat is om veelvoudige analite gelyktydig in een monster te kan opspoor. In hierdie studie was dit veral waardevol in die identifisering van ander moontlike antigene as prognostiese kandidate en sitokiene as immuunbemiddelaars in TB-infeksie.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

Ag85A	Antigen 85 A
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guerin
CD	Cluster of differentiation (antigens)
CFP-10	Culture filtrate protein 10
CMI	Cell mediated immunity/ immune (response)
DC	Dendritic cell
DosR	Dormancy regulon
ELISA	Enzyme linked immunosorbent assay
ESAT-6	Early secretory antigenic target 6
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
HHC	Household contacts
HI AB serum	Heat-inactivated AB serum
HI FCS	Heat-inactivated foetal calf serum
HIV	Human immunodeficiency virus
HSP65	Heat shock protein 65
IFN-γ	Interferon gamma
Lab-MAP	Luminex multi-analyte profiling system
LAM	Lipoarabinomannan
LTBI	Latent tuberculosis infection
MHC	Major histocompatibility complex

MIP1-α	Macrophage inflammatory protein-1 alpha
mRNA	messenger RNA
MTB	Mycobacterium tuberculosis (pathogen)
NC	Non-culturable (*in vitro*)
NK	Natural killer cell
NO	Nitric oxide
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PGL-I	Phenolicglycolipid-I
PHA	Phytohaemaglutinin
PPD	Purified protein derivative (of *M. tuberculosis*)
R	Receptor
RD	Region of difference
Rpf	Resuscitation-promoting factor
RPMI-1640	Roswell Park Memorial Institute medium 1640
ROI	Reactive oxygen intermediates
RNI	Reactive nitrogen intermediates
SEB	Staphylococcal enterotoxin B
TB	Tuberculosis
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TST	Tuberculin skin test
TU	Tuberculin units

WBA	Whole blood assay
WHO	World Health Organisation
ZN	Zhiel-Nielson sputum stain

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CHAPTER 1: Introduction

1.1 Epidemiology of Tuberculosis

Tuberculosis (TB) has long been and remains a serious health problem globally, especially in the resource-deficient developing world. A recent resurgence of the disease resulted in TB being declared a global health emergency by the World Health Organisation (WHO) in 1993. The convergence of the epidemic with the increasingly high incidence of HIV-1 infection worldwide has further compounded the problem, making the treatment and control of disease more difficult.

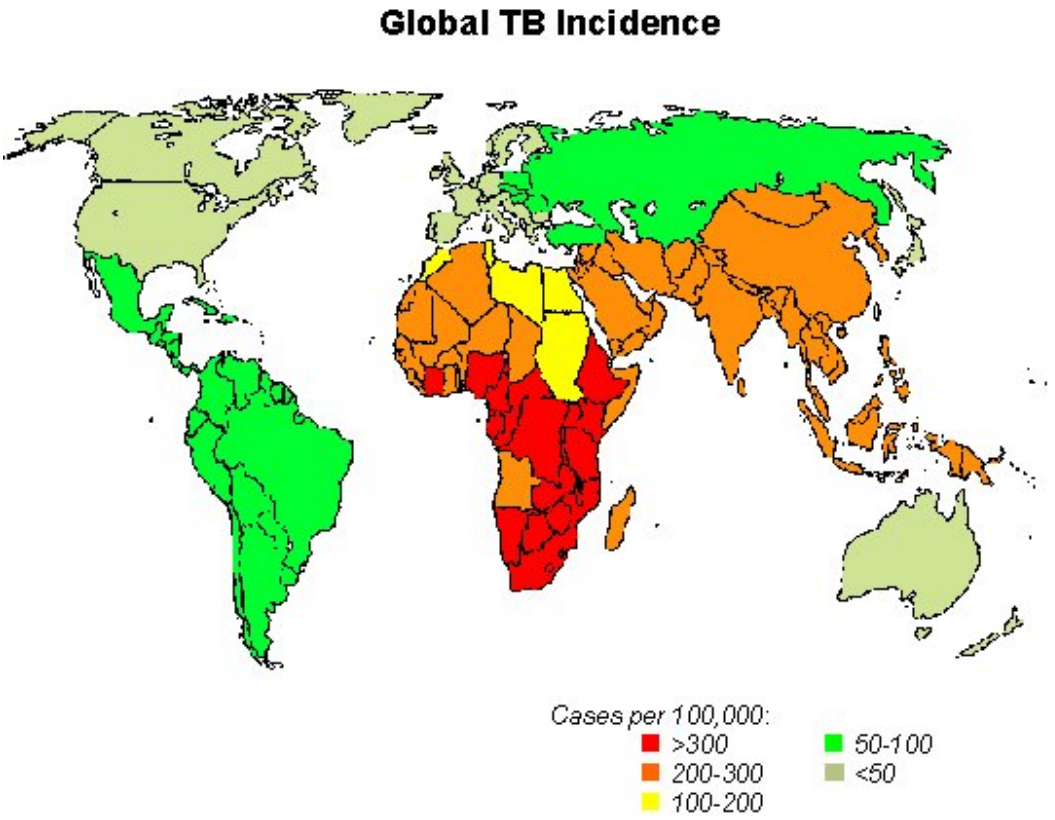


Figure 1: Incidence Rates of Tuberculosis worldwide, as estimated by the WHO in 2005.

Death from dual TB-HIV infection is currently the leading cause of mortality worldwide, most strikingly in Sub-Saharan Africa. An estimated one-third of the world's population is currently infected with the causative pathogen *Mycobacterium tuberculosis* (MTB), resulting in about 2 million deaths and a reported 9 to 10 million new cases annually (Dye C. et al., 2005). A recent projection by The Stop TB Department of the WHO has predicted that a reduction in the spread, prevalence and death rate can be achieved in most parts of the world by 2015, though the task will be most daunting in the worst-affected areas of Africa and Eastern Europe (Dye C. et al., 2005). Discovery of a vaccine for the disease, the attenuated form of *Mycobacterium bovis* called Bacillus Calmette Guerin (BCG), provided hope of positive prospects for treatment and cure. In most countries worldwide, BCG vaccination at birth is and has been routinely practised, and an estimated 3 billion doses has been administered worldwide during the past four decades (Gupta U.D et al., 2007). The positive outlook in its effects was short-lived however, as the vaccine went to demonstrate highly variable efficacy in protecting adult subjects from pulmonary TB, the most common variety (Fine P.E, 1995). In addition, BCG has been particularly disappointing in providing reliable protection in the developing world, where unfortunately, TB is highly endemic.

One of the most challenging aspects of controlling TB however, lies not only in effective treatment of active disease but in understanding latent infection. Of the 2 billion people currently infected, only 5-10% ever present with clinically active disease (Styblo K., 1991). Most healthy persons are able to contain the pathogen for extended periods without actually eliminating the initial infection, a state of dormancy known as clinical

latency or latent infection. This population of asymptomatic individuals represents a massive reservoir of the bacillus that keeps perpetuating the spread of the disease. Ironically, they also clearly illustrate the importance of a potent immune response that is obviously required to prevent the disease from ever gaining headway. Beginning to understand why only a relatively small proportion of people progress to disease would provide insight into how the host immune system works to control TB. Elucidating the immune profile post-infection and defining what constitutes protective immunity would ultimately give rise to valuable tools in drug and vaccine therapy.

1.2 MTB Infection and Immunity

1.2.1 Early Phase Infection

TB is transmitted via the aerosol route, when expelled droplets harbouring *Mycobacterium tuberculosis* bacilli are inhaled and begin the initial interaction in the lungs with alveolar macrophages. *M. tuberculosis* infects and replicates rapidly in the macrophages of the airways, quickly migrating to those of the lung parenchyma and also to differentiated monocytes that are recruited to the site (Algood H.M.S et al., 2003). The result is an activation of a cascade of inflammatory molecules that include the all-important cytokine and chemokine mediators that regulate the course of infection. Circulating dendritic cells in the vicinity are also infected by MTB and consequently migrate to the lymph nodes to prime naïve T cells (Gonzalez-Juarrero M. et al., 2001). Subsequent lung inflammation is the signal that recruits effector T lymphocytes back to the region of infection, where a conglomeration of these cells forms a granuloma.

1.2.2 Granulomas: Composition and Function

Granulomas typify MTB infection, and represent an attempt on the part of the host's immune system to contain and repress the effects of the bacillus. Their formation is resultant of a highly activated cell mediated response, as they are composed of macrophages, CD4, CD8 and γδ T lymphocytes, as well as B lymphocytes. Granulomas function to physically contain infectious bacilli at the primary site of infection, thereby depriving them of oxygen and nutrients and limiting their spread to other regions. They also function to create a microenvironment for immune cross-talk between host cells, leading to continual macrophage activation, cytokine production and effective killing of bacteria by CD8 T lymphocytes (Algood H.M.S et al., 2003; Andersen P., 2007). This effectively limits bacterial replication and localises inflammation to the infectious site. The structure of the granuloma comprises a macrophage-rich centre with surrounding lymphocytes that penetrate the core. This macrophage-lymphocyte aggregate begins to form 2-3 weeks post-infection, maturing into larger, more distinct bodies within 4-5 weeks (Algood H.M.S et al., 2003).

Granulomatous cavities containing live but dormant MTB can persist for decades, as significant numbers of bacteria can escape eradication by the cell-mediated response, giving rise to the state of latent infection. Therefore whilst initially beneficial, this attempt to contain the infection can result in subsequent immunopathology that is detrimental to the host.

1.2.3 Cell-mediated Immune (CMI) Response

1.2.3.1 CD4 T Cells

Components of the T-cell compartment form a highly essential part of protective immunity against an intracellular pathogen like *M. tuberculosis*. Since the primary residence of the bacteria is inside the phagosome of macrophages, their secreted peptide products are readily presented to CD4 T cells via the MHC II pathway. CD4 T cells are of the T-helper 1 type and are prime mediators of protection against TB. They typically produce IFN-γ, which, along with an array of other cytokines like TNF-α and interleukin-2 (IL-2) that characterise the Th1 profile, activate macrophages (Flynn J.L and Chan J., 2001). Activated macrophages initiate the production of effector molecules such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), and begin the development of granulomas to isolate and control bacterial replication (Walker L. and Lowrie D.B, 1981; MacMicking J. et al., 1997). In addition to possessing antimycobacterial activity, macrophages can also kill intracellular invaders in some cases (Flesch I. et al., 1987; Chan J. et al, 1992). In murine studies, methods resulting in antibody depletion of CD4 cells (Muller I. et al., 1987), adoptive transfer (Orne I. and Collins F., 1984), or the use of knock-out mice (Caruso A.M et al., 1999) have provided results that show the $CD4^+$ T cell subset to be vital for controlling infection. The production of IFN-γ and its synergistic effects with TNF-α, whilst important, is thought to be only one of the roles of CD4 T cells in tuberculosis infection, others remaining unclear or less prominent. In MHC II- and CD4-knockout mice where IFN-γ was severely depleted in early infection, the mice were still not rescued by a later surge in IFN-γ production within the $CD8^+$ T cell subset, and succumbed to infection nevertheless

(Caruso A.M et al., 1999). Other studies have shown that the protective response against tuberculosis does not wholly rely on the associated induction of nitric oxide synthase (NOS2) after IFN-γ production, and there may be more functions for $CD4^+$ T cells (Scanga C.A et al., 2000). Speculation as to how these T cells contribute to the immune response include helping to activate or mature antigen-presenting cells (APCs) (Campos-Neto A. et al., 1998), priming and maintaining $CD8^+$ effector and memory function (Kalams S.A et al, 1998), affecting the role of B cells in infection (Bosio C.M et al, 2000), and inducing apoptosis or cell lysis (Keane J. et al, 1997). Clearly, CD4 T cell responses are an important facet of the host immune response after MTB infection.

1.2.3.2 CD8 T cells

The $CD8^+$ T cell subset is also an important component during the course of infection with TB. In addition to also being potent producers of IFN-γ like CD4 cells, CD8 T cells are effective killer cells that are key mediators in acquired resistance against disease (Kaufmann S.H.E et al, 2006). They secrete perforin and granulysin that target and kill infected cells. Other roles for CD8 T cells have also been studied. Since *M. tuberculosis* classically resides in vacuoles and there are very few bacilli free in the cytosol, the mechanism of antigen presentation via the cytoplasmic MHC I route, which involves $CD8^+$ T cells, was largely neglected for many years. More recently though, evidence for how mycobacterial antigens can be loaded and recognised via the MHC I route have been found. *M. tuberculosis* is a pathogen that induces apoptosis, resulting in the formation of vesicles containing proteins and glycolipids that get taken up by dendritic cells (DC). As the most efficient APCs of the immune system, dendritic cells then facilitate loading of

the vesicular contents, allowing stimulation of the associated MHC I and II T cell populations (Geijtenbeek T.B.H et al., 2003; Schaible U.E et al., 2003). CD1 loading, which represents an unconventional or MHC I-unrestricted antigen presenting pathway, also occurs via dendritic cells (Henderson R.A et al., 1997; Stenger S. et al, 1998). It has been noted that the high glycolipid content of the vesicles increases their antigen-presenting ability because it enables them to stimulate DC using toll-like receptors (TLR) (Beatty W.L et al., 2000; Kaufmann S.H.E et al, 2006).

1.3 Mtb Immune Evasion Techniques and Persistence in Macrophages

1.3.1 Adaptation to the Intracellular Microenvironment

The triumph of tuberculosis as a chronic infection hinges on its ability to establish a dormant state inside the host for many years, making it difficult to achieve sterile cure of disease even with multi-drug chemotherapy. The bacillus is able to survive and replicate for long periods in phagosomes by altering their normal state in a number of ways. Phagosomes are vacuoles inside phagocytes, which are macrophages with the endocytotic ability to engulf invading microorganisms, and are the direct product of the endocytic pathway. After being phagocytosed, *M. tuberculosis* firstly disturbs the normal maturation pathway of the phagosome. This prevents it from fusing with lysosomes to form a body called a phagolysosome, where invading microorganisms are degraded by hydrolytic enzymes (Hart P.D et al., 1972; Mwandumba H.C et al., 2004). This allows the bacteria to survive in immature phagosomes, where the iron-rich conditions favour growth and replication (Kaufmann S.H.E et al, 2006), and there is limited acidification because the vesicle compartment is deficient in the hydrolases present in lysosomes

(Russell D.G et al., 2002; Mwandumba H.C et al., 2004). Studies have shown that aborted fusion with lysosomes was commonly observed only with phagosomes that contained viable bacilli, demonstrating how an integral part of the ability of *M. tuberculosis* to survive inside phagocytes relies on its interference of phagosome-lysosome fusion (Armstrong J. and D'arcy Hart P., 1971). Studies have also shown that *M. tuberculosis* is capable of adversely affecting the antigen-processing and -presenting capacity of macrophages by producing MHC I and II receptor inhibitors (Boom W.H et al., 2003). These are ways employed by the bacteria to evade destruction by the host's immune system.

1.3.2 Persistence in Macrophages

After the immune system's primary encounter with *M. tuberculosis*, the cell-mediated response results in the formation of granulomas, conglomerates of macrophages and lyphocytes, in the lungs. A granuloma effectively represents an attempt to contain the infectious agent whilst also providing a haven for their replication and survival, highlighting a paradox of tuberculosis infection. The cellular environment of granulomas is characterised by low pH, nutrient deficiency, hypoxia and the production of other inhibitory organic acids (Warner D.F and Mizrahi V., 2006). Furthermore, the bacillus is exposed to other immune effectors like ROI and RNI, which also act to drive it from the active replicating to the dormant state.

1.3.2.1 Low pH

As previously described, phagosome maturation and subsequent phagosome-lysosome fusion is vital for bacterial killing, as the microbes are bombarded with bacteriocidal pore-forming peptides and lysosomal hydrolases (Flynn J.L and Chan J., 2001, Kaufmann S.H.E et al, 2006). Effective maturation of the phagosome is accompanied by a drop in the pH from neutral to acidic, which is the optimal environment required for the acidic hydrolases of the lysosomes to work after fusion has occurred (Hingley-Wilson S.M et al., 2003). *M. tuberculosis* expresses urease, which catalyses the production of ammonia (NH_4^+) and neutralises the pH of the phagosome, which is thought to arrest it in the early stages of development (Gordon A. et al., 1980). It has also been shown that other tuberculosis-associated products and enzymes like sulfatides (Goren M. et al., 1976) and glutamine synthetase (Harth, G. and Horwitz M.A, 1999) may also affect pH via ammonia production, but much speculation still surrounds exactly how this may be so.

1.3.2.2 Hypoxia

Low oxygen concentration and its effects on the control of bacterial replication has been one of the most comprehensively studied aspects of *in vivo* and *in vitro* research in tuberculosis. Oxygen depletion is another characteristic of the microenvironment of the granuloma and gives insight into how the bacillus is driven into a dormant state by hypoxia. In a landmark series of experiments, it was demonstrated how the gradual depletion of oxygen forces bacterial respiration not only to shift to involve nitrate

reduction (Wayne L.G et al., 1998) but also induces chromosomal, metabolic and structural changes in the dormant bacteria (Wayne L.G et al., 1979 and 1982). These studies went a long way to identifying the role of a prominent two component regulator system, DosR-DosS/DosT, of which the DosR regulon has turned out to be significantly important. This section of the MTB genome includes genes associated with multiple stresses known to drive the bacteria into a dormant state, including anaerobic respiration and non-toxic nitric oxide (NO) (Kendall S.L et al., 2004; Schnappinger D. et al., 2003).

1.3.2.3 Nutrient starvation

Nutrient limitation is also thought to occur when *M. tuberculosis* resides inside the phagsome and granuloma, depriving the replicating bacteria of an adequate food source and essential elements that are vital for proper development. Recent *in vitro* starvation models that mimic intracellular conditions as closely as possible have allowed for the identification of metabolites and broad conditions that illustrate how persistence in macrophages can result. Glucose deficiency has been shown to be relevant, as well as over-production of fatty acids (McKinney J. D et al., 2000; Betts, J.C et al., 2002). In general, the up-regulation of genes involved in β-oxidation, gluconeogenesis, the glyoxylate shunt, RNA synthesis and amino acid/amine degradation was observed, whilst those associated with de novo ATP production, carbon degradation, and purine/pyrimidine synthesis were down-regulated (Betts, J.C et al., 2002; Hampshire T. et al., 2004). Depletion of isocitrate lyase, which is an important enzyme in fatty acid metabolism and bacterial replication in late-stage infection in mice, has given strong evidence that fatty acids are critical in times of nutrient starvation. When present in

normal amounts, bacteria were capable of replicating normally, which was still the case when the isocitrate lyase gene was disrupted in IFN-γ knockout mice (McKinney J. D et al., 2000). This meant that IFN-γ-producing macrophages deprive the bacteria of carbon-based nutrients and forced its metabolism to switch to fatty acid breakdown, which demonstrated how the host's immune activation can act to curtail the survival pattern of the bacterium. In murine models, the transcription profile of the MTB genome was also seen to change to produce more siderophores, which facilitated in the uptake of more iron which was necessary for growth (Timm J. et al., 2003).

1.3.2.4 Free Radical-based Intermediates

The most effective effector molecules encountered by *M. tuberculosis* in the cell are reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). These compounds are produced by activated phagocytes that generate nitric oxide (NO) and related RNI via NOS2 using L-arginine as the substrate (Flynn J.L and Chan J., 2001). The role of RNI in controlling acute and chronic MTB infection has been well documented, showing that it not only has a protective role in mice (MacMicking J. et al., 1997; Shiloh M. and Nathan C.F, 2000) but also that it may augment the host defence in humans. Immunohistochemical analysis has detected high expression levels of NOS2 in pulmonary macrophages of people with active tuberculosis, as well as high levels of exhaled NO (Nicholson S. et al., 1998; Wang C-H et al., 1996). In contrast, the role of ROI in infection is still the subject of debate. It has been shown that hydrogen peroxide (H_2O_2) present in macrophages has a mycobacteriocidal effect, but the combined effect of ROI is yet to be confirmed conclusively. On one hand, the mycobacterium appears to

possess mechanisms of evading toxic ROI effects, such as releasing lipoarabinomannan (LAM) and phenolicglycolipid I (PGL-I) which are strong oxygen radical scavengers (Chan J. et al., 1989). Yet there is evidence that mice deficient in the NADPH oxidase complex do show a moderately increased susceptibility to *M. tuberculosis* infection (Adams L. et al., 1997).

1.4 Identification of MTB-specific Antigens

1.4.1 Genomic Profiling and the RD1 Expression Proteins

The genome of *M. tuberculosis* spans about 4000 genes in total, and one of the first studies to recognise the importance of identifying MTB-specific antigens that would be useful in vaccine research and effective drug design was carried out in 1996. In order to qualify exactly how *M. tuberculosis* differs from BCG, which would provide insight into developing a better vaccine, a technique called subtractive genome hybridisation was carried out to identify genetic differences between *M. tuberculosis, M. bovis* and BCG (Mahairas et al., 1996; Mustafa A.S, 2005). Three regions of difference (RD), designated RD1, RD2 and RD3, were discovered. With respect to MTB infection, RD2 and RD3 demonstrated little or no research potential, because they either showed too little homology with *M. tuberculosis* or *M. bovis* to be of any diagnostic use against TB, or because their encoded proteins showed little or no protective effects against TB in murine or human models (Brewer T.F and Colditz G.A, 1995). In contrast, RD1 was highly conserved in all tested laboratory and clinical isolates of *M. tuberculosis*, but absent from all BCG substrains (Mahairas et al., 1996), and interest in this has region prompted new and innovative research into host-pathogen interactions.

1.4.1.1 Latency and the DosR Regulon

Recent experiments using gene expression profiling have studied the bacillus under different stress conditions that mimic *in vivo* characteristics of the bacteria in its dormant state. During latent infection, individuals appear healthy and disease-free, but harbour dormant bacteria that are virtually inactive metabolically and reproductively (Kaufmann S.H.E, 2006). The genes under the control of what has been named the DosR, or dormancy regulon, typify the profile of latent infection, and upregulated latency genes have been linked to nutrient starvation (Wayne L.G and Hayes L.G, 1996; Cole S.T et al., 1998; Wayne L.G and Sohaskey C.D, 2001; Voskuil M.I, 2004), hypoxia (Rosenkrands I. et al., 2002; Leyten et al., 2006) and varying NO levels (Voskuil M.I et al., 2003). It has been shown that not all DosR genes are up-regulated during latency, whilst some non-DosR-regulated genes are (Boshoff H.I and Barry C.E, 2005), and this could provide important clues as to which genes are of interest during the dormant phase of the bacterium. Characterising a set of latency antigens that are important would provide interesting candidates for postexposure vaccines, as they may be potentially protective proteins against progression to active disease.

1.4.1.2 Reactivation

Infected individuals stand a 10% chance that the latent condition may roll over into active disease, a state called reactivation. The clinical factors responsible for reactivation are not well understood but are thought to be the result of a temporary suppression of the immune response (Collins F.M, 1989; Rook G.A.W and Bloom B.R, 1994). It has been

determined that there is a shift from the Th1-biased response during latency to a Th2 one during reactivation, a pattern that was observed in the cytokine production in both CD4 and CD8 T cells (Howard A.D and Zwilling B.S, 1999). During latency, infectious microbes are contained in macrophages which reside in granulomas that do not largely affect the surrounding tissue. However during reactivation, granulomas increase in size, form cavities and large amounts of bacteria ($>10^{10}$ organisms) can escape and replicate extracellularly in the debris surrounding the granuloma (Kaufmann S.H.E, 2006). Gene expression profiling has thus identified proteins associated with reactivation of disease as useful vaccine candidates.

1.4.1.3 Resuscitation

The bacteria's ability to regress to a stable but metabolically inactive state when grown under suboptimal conditions is referred to as 'non-culturable' (NC) *in vitro* (Shleeva M. et al., 2004). It is hypothesized that induction of the NC state may be an adaptive response of the bacillus to their metabolism being compromised by a missing growth factor, possibly the simultaneous action of several factors (Shleeva M. et al., 2004). The change-over from dormancy to a reactivated state can occur when these growth factors (such as oxygen and nutrients) which were removed from the growth medium are replenished. There is tentative evidence that reactivated mycobacteria can resuscitate other neighbouring microbes by secreting resuscitation-promoting factors or Rpf (Cohen-Gonsaud et al., 2005). The Rpf of *Micrococcus luteus* has been shown to restore active growth to *M. luteus* cultures in the stationary growth phase, and five Rpf homologues specific for *M. tuberculosis* have demonstrated similar effects in *M. tuberculosis* cultures

(Mukamolova G.V et al., 2002; Munoz-Elias E.J et al., 2005). Very little is still known about the *in vivo* effects of Rpf proteins.

1.4.1.4 Classic Vaccine or TB Control Antigens

The serodiagnostic potential of various new vaccine candidates have been evaluated by a large number of research groups in the search for an effective vaccine with protein/peptide epitopes that are more MTB-specific and confer better protection than BCG. From the assortment of antigens that have generated particular interest, a group now referred to as classic or typical TB vaccine antigens have been identified. It is generally accepted that these antigens strongly induce cytokine production in the sera and whole blood supernatant of both actively and latently infected individuals, living in high and low endemic regions and across ethnic groups (Lalvani A. et al., 2001; Porsa E. et al., 2006; Hoff S.A et al., 2007). Proteins produced from the culture filtrate of *M. tuberculosis*, such as ESAT-6, Ag85A, Ag85B and CFP10 have been shown to induce a protective immune response against *M. tuberculosis* in animal and human models (Wang J. and Xing Z., 2002; Doherty T.M, 2005). These antigens are currently being used to develop or optimise TB diagnostic assays (Samanich K.M et al., 2000; Porsa E. et al., 2006) or are entering clinical trials subunit vaccines (Langermans J.A.M et al., 2005; Radošević K et al., 2007). TB10.4 is another recently identified protein that appears to be important for *M. tuberculosis* virulence and growth. It is strongly recognised by TB-exposed individuals, and produces a high frequency of antigen-specific $CD4^+$ T cells that correlate to protection against MTB.

1.5 Immune Correlates of Protection against Disease

1.5.1 The Importance of IFN-γ

It has been well documented that in tuberculosis, as in many infectious diseases, the role of IFN-γ is of critical importance as it plays a pivotal role in the protective CMI response. It is produced by both CD4 and CD8 T cells, as well as natural killer cells (NK). As previously stated, IFN-γ, in concert with other cytokines, activates macrophages and begins the cascade of events that lead to granuloma formation and killing of infected cells by cytotoxic T cells. Using IFN-γ -/- mice, it has been shown that with depleted levels of or in the absence of IFN-γ, T cells still migrate to the lungs to form granulomas. However, due to the insufficient levels of the cytokine and poor resultant macrophage activation, these granulomas rapidly became necrotic and failed to contain the infection (Flynn J.L et al., 1993; Algood H.M.S et al., 2003). Genetic studies on patients with faulty IFN-γ receptor (IFN-γ R) have provided the most clear and striking proof that flaws in the pathway of this Th1 cytokine is closely associated with increased susceptibility to mycobacteria (Fletcher H., 2007). Patients with either completely defective IFN-γ R1 or IFN-γ R2 suffered recurrent infections with BCG and other mycobacteria that are typically known to be non-infectious, whilst partial deficiency in the IL-12 receptor (IL-12 R) led to increased susceptibility to mycobacteria (Newport M.J et al., 1996; Altare F. et al., 1998; Lichtenauer-Kalis E.G et al, 2003). Murine studies using gene knock-out mice (GKO) mice have shown that not only is macrophage activation low when IFN-γ is defective, but NOS2 expression is also low, resulting in unimpeded bacillary growth in the lung cavities (Dalton D.K et al., 1993).

Although IFN-γ is essential in controlling mycobacterial infection, it is also generally accepted that it cannot be the only cytokine important in directing the immune response during the course of tuberculosis. Like in many diseases, infection with TB is multifactorial and complex, and other correlates of protection need to be identified. A recent study has shown a poor correlation between the frequencies of IFN-γ-secreting cells and the level of protection against MTB (Majlessi L. et al., 2006). IFN-γ itself may be unreliable as an immune correlate when its effects are taken alone, as levels of production may vary in affected subjects. IFN-γ is produced by both healthy PPD^+ patients and those with active disease, and even though it has been shown that its levels are depressed in actively infected people (Flynn J.L and Chan J., 2001, Raja A., 2004), this may be insufficient to distinguish latent and active disease. One study demonstrated that *M. tuberculosis* may prevent macrophages from adequately responding to IFN-γ, suggesting that the activated cells' response to the cytokine may provide better indications as to disease progression than measuring levels of the cytokine itself (Ting L.M et al., 1999). Also, it appears that the initial surge of IFN-γ production, which indicates an assaulted immune system, usually leads to suppression of the infection by downregulating the cytokine response. However in the case of *M. tuberculosis,* where the bacilli can evade the host's counter-attack mechanisms and continue to replicate, the immune response can remain elevated throughout infection without sterile cure being attained. IFN-γ alone cannot provide protective immunity, and in some scenarios its elevated levels can be detrimental rather than protective. It would be therefore be innovative to profile other cytokines that are associated with bacterial burden in an effort to establish their effect on immunity as well.

1.5.2 Associated Cytokines

1.5.2.1 TNF-α, IL-6 and IL-12

M. tuberculosis induces TNF-α secretion by macrphages, dendritic APCs and T cells, and has been well-recognised to play an important role with IFN-γ as a pro-inflammatory cytokine in tuberculous infection. Its exact role appears to be complex, but it has been implicated in macrophage activation, where it was shown that TNF-α- or TNF receptor-deficient mice died much more rapidly than control mice, with markedly higher bacterial burdens (Flynn J.L et al., 1995; Bean A.G.D et al., 1999). The cytokine appears to affect cell migration to and localisation at affected tissue sites. Its synergistic effects with IFN-γ to induce NOS2 has also been well characterised, showing its downstream importance in granuloma formation (Chan J. et al., 1992; Flynn J.L and Chan J., 1995). It also affects the expression of adhesion chemokines and their receptors, which also plays a role in granuloma formation.

IL-6 is another cytokine with multiple roles in the immune system. It has pro-inflammatory and haematopoietic functions, and also aids in the differentiation of T cells (Raja A., 2004). Its role has also been extended to T cell suppression. Studies provided evidence that IL-6 is vital in the innate response to the MTB pathogen by showing how IL-6 -/- mice had earlier and higher bacterial load in the lungs, in addition to decreased IFN-γ production (VanHeyningen T.K et al., 1997; Saunders B.M et al., 2000). IL-6 has also been shown to be stimulated by other bacterial components like endotoxins, of which lipopolysaccharide (LPS) and lipoarabinomannan (LPS) are examples (Zhang et al., 1994, Christodoulides M. et al., 2002).

IL-12 is also closely associated with the development of a pro-inflammatory, Th1 response. Its production follows the cell's first encounter with *M. tuberculosis*, after phagocytosis of the bacillus by macrophages and dendritic cells (Ladel C.H et al., 1997), and its induction is strongly tied to enhanced mycobacterial resistance. Mice deficient in IL-12p40, its homodimer, showed a remarkably increased infection susceptibility and bacterial burden, and decreased survival time, which related to a reduction in IFN-γ production (Cooper A.M et al., 1997).

1.5.2.2 IL-2

This cytokine plays a critical part in the clonal expansion of lymphocytes that are specific to bacterial antigens, which is important for an immune response against mycobacteria (Raja A., 2004). IL-2 secretion also constitutes an important protective facet of the Th1 response as it is produced by CD4 cells, and several studies have demonstrated that IL-2 levels, alone or associated with that of other cytokines, can influence the outcome of mycobacterial infections (Blanchard D.K et al., 1989).

1.5.2.3 IL-10

IL-10 is an anti-inflammatory cytokine and has an immune inhibitory function. It is produced by macrophages and T cells and has macrophage-deactivating properties, which includes the down-regulation of IL-12 production, which subsequently leads to the decrease in IFN-γ production (Raja A., 2004). The cultured macrophages of patients with active TB were T cell-suppressive and inhibition of IL-10 partially reversed this pattern

(Gong J-H et al., 1996). IL-10 directly inhibits CD4+ T cell and APC function during infection (Rojas M. et al., 1999). Transgenic mice constitutively expressing IL-10 lacked an adequate ability to clear a chronic BCG infection, although T cell responses and IFN-γ production were unaffected. These results suggested that IL-10 may counteract the macrophage-activating properties of IFN-γ (Murray P.J et al., 1997).

1.5.2.4 IL-4

IL-4 typifies the profile of a Th2-skewed response, but its effects during MTB infection have been variable and controversial. In human studies, it was observed that a lowered Th1 but not a heightened Th2 response characterised the PBMC of tuberculosis patients (Zhang M. et al., 1995; Lin Y. et al., 1996). In the granulomatous lymph nodes of patients with tuberculous lymphadenitis, elevated levels of IFN-γ were found but IL-4 mRNA was difficult to detect (Lin Y. et al., 1996). This shows that in humans, a strong Th2 response is not associated with tuberculosis. Murine infection models using knockout mice showed that in the absence of a reliable Th1 response, a Th2-directed outlook is not necessarily promoted, and it is an IFN-γ deficiency rather than the presence of IL-4 and other Th2 cytokines that prevents control of infection (Flynn J.L et al., 1993; Cooper A.M et al., 1997). How the presence of IL-4 affects clinical outcome still needs to be further elucidated.

1.5.2.5 TGF-β

This anti-inflammatory cytokine has its functions in inhibition of T cell proliferation (Rojas R.E et al., 1999), interference with NK and CTL function and downregulation of

IFN-γ, deactivation of the production of RNI and ROI by macrophages (Ding A. et al., 1990), and TNF-α and IL-1 release (Ruscetti F. et al., 1993). It has been demonstrated that when TGF-β is added to co-cultures of mononuclear phagocytes and *M. tuberculosis*, both growth inhibition and phagocytosis were reduced in a dose-dependent manner (Toosi et al., 1995). The ability of macrophages to inhibit mycobacterial growth may depend on the relative influence of TGF-β and IFN-γ in infection (Raja A., 2004).

1.5.3 Diagnosing Latent Tuberculosis Infection: Old and New Techniques

Currently, diagnosing LTBI is still heavily reliant on the tuberculin skin test (TST), which is an intradermal inoculation of a mixture of over 200 *M. tuberculosis* proteins called a purified protein derivative (PPD). The underlying problem with this test is that is has poor TB-specificity in BCG-vaccinated people and low sensitivity in those with a suppressed cellular immunity, for example HIV-infected persons (Andersen P. et al., 2000; Lalvani A., 2007). Two more specific methods that now exist for measuring TB antigen-specific T cell responses are the now-conventional ELISA method and the more recent multiplex technology.

The whole-blood ELISA relies on measuring the release of IFN-γ within the T lymphocyte compartment, meaning production of the cytokine by antigen-specific T cells follows stimulation with mycobacterial antigens. The ELISA can be a commercially available assay that measures cytokine release after a 16 – 24hour incubation period (Desem N. and Jones S.L, 1998) or an in-house method designed to detect response levels after longer (2 – 7 day) periods. One fundamental drawback of whole blood ELISA

is that it can usually measure only one to a few cytokines. This is in stark contrast to the multiplex technique that has the potential to measure up to 100 different cytokines in a 50µl sample. This fluorescent bead-based technology is becoming a very popular method of detecting a mixture of cytokines in serum, supernatant and PBMC of humans and mice (Oliver K.G et al., 1998; Carson R.T and Vignali D.A, 1999; Kellar K.L et al., 2001). Both these assays are very important in improving and expanding current knowledge on the cytokines produced and detectable during LTBI in humans.

1.6 Summary

The exact correlates of protection against MTB during latent tuberculosis infection are not yet known or fully understood. The protective efficacy of BCG has proven to be highly variable in different populations and geographic regions, not to mention its protective effects in children wanes significantly with age and is affected by coincident exposure to environmental mycobacetria (De Groot A.S et al., 2005; Gupta U.D et al., 2007). In order to find a new vaccine that effectively controls TB infection, these issues must be tackled. Finding a new vaccine against TB would entail further elucidation of the infectious profile, indentifying new antigen components that could provide better protection and investigating novel vaccine delivery systems. There is urgent need for a well-defined profile of immunity with respect to the cytokines and chemokines produced during LTBI, as well as for the development and optimisation of reliable assays that can competently measure the response itself. This project, to my knowledge, describes the first large-scale attempt to: (i) quantify the IFN-γ responses to a panel of TB-specific antigens using WBA and ELISA, (ii) identify other immune correlates of protection after

antigen stimulation using the Luminex technology and (iii) compare the two techniques as viable tools for researching TB, in a South African population.

CHAPTER 2

Screening of Novel MTB Antigens by IFN-γ ELISA in a population with a high incidence of LTBI

2.1 Antigen Screening

2.1.1 Background

An estimated one-third of the world's population is latently infected with tuberculosis, and the most effective intervention to counter spread of the disease would be the development of a competent postexposure vaccine. In 2003, the Grand Challenges in Global Health initiative was launched by the Bill and Melinda Gates Foundation to address pertinent research questions surrounding infectious diseases worldwide, and Grand Challenge 6-74 (GC6-74) was specifically targeted at TB research. The goal of this major study was to characterise the immune response to TB infection by identifying immune correlates of protection and host markers of active disease with prognostic potential. GC6-74 is a collaborative research effort, and one of the African field sites and research groups was established at Stellenbosch University in Cape Town.

This project was a study within the larger GC6-74 plan, and was designed to outline the natural protective immune profile of latently infected individuals by observing their IFN-γ responses to novel MTB antigens. A chosen panel of 78 antigens were used in the screening exercise, composed of 67 antigens from Leiden University Medical Centre

(LUMC, Netherlands) and the Max Planck Institute for Infection Biology (MPIIB, Germany), and 11 antigens from Statens Serum Institute (SSI, Denmark). These MTB proteins were produced using *in vitro* gene expression models that mimic the reaction of the bacillus under different stress conditions, a collaborative effort of the Schoolnik Lab (Stanford University) and SSI. The expression products were accordingly named latency, reactivation, resuscitation promoting factor (Rpf) or classic control antigens.

2.1.2 Setting

The epidemiological field site is situated in Cape Town, South Africa, which has one of the highest incidence rates of tuberculosis worldwide. In the surrounding suburbs, the annual risk of TB infection is 3.5% - 10 times the already high risk for sub-Saharan Africa (Walzl G. et al., 2005). Study participants were members of the Uitsig, Ravensmead, Adriaanse or Elsiesriver communities, which have long been recognised as specific areas with a high burden of TB infection, an incidence rate that is not overly-exacerbated by a high HIV prevalence. A typical ethnic profile shows these communities are largely inhabited by the Coloured (Brown) racial group. Participants were recruited from Tygerberg Hospital as well as smaller community clinics in the respective neighbourhoods.

2.1.3 Experimental Design

This study followed a cross-sectional study design, where prospective patients were recruited for the study over a total time-course of 18 months, following evaluation of their suitability for inclusion. The following study cohorts were identified according to their corresponding criteria:

1) TB Index cases - New TB cases were identified by research nurses through clinic registers and introduced to the study upon diagnosis with active TB. TB was diagnosed on the basis of at least 2 Zheil-Neelsen (ZN) positive sputum smears, and chest x-ray. After diagnosis, written informed consent was obtained for recruitment and patients were enrolled. The TB cases themselves were not investigated in the present study but were used to identify household contacts.
2) Household contacts (HHCs) of Index cases – After identification and recruitment of the Index cases, permission was obtained to visit their homes and recruit household members with whom they had close daily contact. Written informed consent was obtained for recruitment and household contacts were enrolled.

2.1.4 Participant Selection Criteria

Consent and Ethical Approval

Ethics approval from the Ethics Committee of Stellenbosch University was obtained prior to beginning the study, to allow patient participation. Trained research nurses thoroughly explained the study in basic terms to prospective participants. Informed, written consent was obtained from all persons aged 15 and older, or from the guardian of recruits who were younger than 15 years old. All study participants were tesed for HIV infection. Pre-

and post-test counselling was given to every subject, and his/her guardian where age appropriate.

Inclusion Criteria:

All participants had to be HIV-1 negative, confirmed by a HIV rapid test taken upon recruitment or proven test results not older than 3 months. For the skin test, 0.1ml or 5 tuberculin units (TU) of tuberculin purified protein derivative (PPD) was injected intradermally. All participants had to be available for measuring the TST induration after 48-72 hours and to have a posterior-anterior chest X-ray radiograph taken. Any household contacts that displayed symptoms of TB or lung disease were also excluded.

In addition, they also had to fulfil the following criteria:

- Be a community member living in the study area for over 3 months and have a permanent address
- Be aged 10 to 60 years,
- Be willing to have a chest X-ray, or have a previous, recent chest X-ray examined to clear the patient of active TB disease.

Exclusion Criteria:

Defaulting on any one or more of the following criteria was grounds for exclusion:

- Living in the study area for < 3 months and/or no permanent address,
- Previous or current treatment for TB or HIV-1,
- Current or recent (within 6 months) participation in a vaccine/drug trial,
- Concomitant cancer or diabetes mellitus,

- Chronic emphysema/ bronchitis/ asthma requiring systemic steroid therapy, or any steroid therapy within the past 6 months, and
- Pregnancy (current or within the past 3 months).

Subsequent to evaluating if a recruit was eligible for inclusion and obtaining written consent, venous blood was taken before administration of the PPD injection and transported to the laboratory strictly within 2 hours of collection. A sputum sample was also collected for smear and culture *M. tuberculosis* testing.

Final Participant Cohort

The final participant cohort selected for this study consisted of 2 groups of healthy, HIV negative household contacts of newly diagnosed pulmonary TB cases. A subset of 67 antigens from LUMC and MPIIB were tested on the first group of 38 and another set of 11 antigens from SSI were tested on the second group of 19.

Recruitment for the antigen screening study was a continuous process throughout the course of the project, but availability of the antigens was a major determinant with respect to when experiments could be designed and conducted. For this reason, the first group of 38 individuals were screened earlier with the available 67 antigens, and a second experiment for the SSI antigens was undertaken at a much later time with a smaller group.

For both the SSI antigen screening and the Luminex studies, 20 participants (group 2) were recruited. One participant was excluded very early on because of sample contamination. It was later discovered that 5 participants (including the first exclusion) were TST-negative and their results were not deemed useful for study purposes at that time. For the sake of completeness, the SSI ELISA data showed results for 19 people (only one exclusion) but all data for the Luminex and comparing ELISA and Luminex showed results for only 15 people (all 5 TST-negatives excluded).

2.2 Materials and Methods

2.2.1 Antigen Classification of Proteins provided by the LUMC and MPIIB

The antigens for screening were either recombinant proteins or peptide pools. All recombinant antigens were screened at a final concentration (after addition of blood) of 10µg/ml and peptide pools were tested at either 10µg/ml or 1µg/ml final concentration per peptide. Tables 1, 2, 3 and 4 show the range of antigens that were tested in the first round of the antigen screening experiment.

Table 1: List of the 6 Classical TB Vaccine* antigens tested.

ANTIGEN NAME	MTB GENE REGION	PROTEIN SIZE (a.a.)	DESCRIPTION
TB10.4 (Rv0288)	*esxH*	96	Low molecular weight protein Antigen 7; Classical Antigen
**Ag85A (Rv3804c)	*fbpA*	338	Secreted Antigen 85A; Classical Antigen
**Ag85B (Rv1886c)	*fbpB*	325	Secreted Antigen 85B; Classical Antigen
HSP 65 (Rv0440)	*groEL2*	540	Heat Shock Protein 65; Classical Antigen
TesatCFP10	fusion product	-	ESAT-6 and CFP-10 fusion protein; Classical Antigen
Rv3019	*esxR*	96	TB10.3; Secreted ESAT-6-like protein
ESAT-6	*esxA*	95	RD1 Protein; Classical Antigen

Tascon et al.; 1996 Nat. Med. 2 (8):888-92
Huygen et al.; 1996 Nat. Med. 2(8):893-8
Brandt et al.; 2000 Infect. Immun. 68(2):791-5
Olsen et al.; 2004 Infect. Immun. 72(10):6148-50
McShane et al.; 2004 Nat. Med. 10(11):1240-4
Brandt et al.; 2004 Infect. Immun. 72(11):6622-32
Skeiky et al.; 2004 J. Immunol. 172 (12):7618-28
Langermans et al.; 2005 Vaccine 23(21):2740-50
Williams et al.; 2005 Tuberc. 85(1-2):29-38
Irwin et al.; 2005 Infect. Immun. 73(9):5809-16

NB: All references cited refer to studies evaluating one or more of the classical TB antigens.

NB*: These proteins are all encoded by the RD1 or the DosR regulon of *M. tuberculosis*. Rv-numbers denote the names of the protein products. RD1 indicates the region of difference 1 and a.a indicates amino acid.

NB**: The two antigens of the Ag85 complex were screened as one, as the pooled peptide product Ag85A/B.

Table 2: List of the 35 DosR regulon Latency Antigens tested.

ANTIGEN NAME	MTB GENE REGION OR PROTEIN FUNCTION	PROTEIN SIZE (a.a.)
Rv2628	HP	120
Rv2626c	CHP	143
Rv2031c	*acr* (HSPX)	144
Rv1733c	Possible transmembrane protein	210
Rv2029c	*pfkB*	339
Rv2627c	CHP	413
Rv0569	CHP	88
RV2623	*TB31.7*	297
Rv0079	HP	273
Rv0080	HP	152
Rv0081	Transcriptional regulator	114
Rv0571c	CHP	443
Rv1738	CHP	94
Rv3134c	CHP-USPA motif	268
Rv3132c	*devS*	578
Rv3133c	*dosR*	217
Rv0570(Cpart)	*nrdZ*	
Rv0570(Npart)	*nrdZ*	combined 692
Rv0572c	HP	113
Rv0573c	CHP	463
Rv0574c	CHP	380
Rv1734c	HP	80
Rv1735c	CHP	165
Rv1736c(Cpart)	*narX*	
Rv1736c(Npart)	*narX*	combined 652
Rv1737c	*narK2*	395
Rv1812c	HP	400
Rv1813c	HP	143
Rv1996	CHP-USPA motif	317
Rv1997-C	*ctpF*	
Rv1997-N	*ctpF*	combined 905
Rv1998	CHP	258
Rv2003c	CHP	285
Rv2004c	CHP	498
Rv2005c	CHP-USPA motif	295

Voskuil *et al.* 2003,J.Exp.Med. 198(5): 705-13 (Schoolnik Lab)
Rosenkrands *et al.* 2002, Bacteriol. 184(13):3485-91 (Andersen Lab)
Leyten *et al.* 2005, Micr.Infect. 8(2006): 2052-2060

NB: Rv-numbers denote the names of the protein products. Italicised names designate the gene region and capitals the protein function. HSP indicates heat shock protein, and CHP and HP indicate conserved hypothetical and hypothetical proteins.

Table 3: List of the 5 Resuscitation-promoting factors (Rpf) tested.

ANTIGEN NAME	MTB GENE REGION	PROTEIN SIZE (a.a.)	DESCRIPTION
Rv0867c	*rpfA*	407	Possible Resuscitation-promoting factor RPFA
Rv1009	*rpfB*	362	Possible Resuscitation-promoting factor RPFB
Rv1884c	*rpfC*	176	Probable Resuscitation-promoting factor RPFC
Rv2389c	*rpfD*	154	Probable Resuscitation-promoting factor RPFD
Rv2450	*rpfE*	172	Probable Resuscitation-promoting factor RPFE

Mukamulova et al.; 1998 PNAS 95:8916-21

NB: Rv-numbers denote the names of the protein products. Italicised names designate the gene region and capitals the protein function, and a.a indicates amino acid.

Table 4: List of the 21 Reactivation Antigens, produced by the Wayne model, tested.

ANTIGEN NAME	MTB GENE REGION or PROTEIN FUNCTION	PROTEIN SIZE (a.a.)
Rv0140	CHP	126
Rv0246	Probable conserved integral membrane protein	436
Rv0251c	*hsp*	159
Rv0331	Putative dehydrogenase	388
Rv0384c	*clpB*	848
Rv0753c	*mmsA*	510
Rv1073	CHP	283
Rv1115	Possible Exported Protein	232
Rv1130	CHP	526
Rv1131	*gltA1*	393
Rv1471	*trxB*	123
Rv1717	CHP	116
Rv1874	HP	228
Rv1875	CHP	147
Rv2090	Probable 5'-3' Exonuclease	393
Rv2465c	*rpi*	162
Rv2466c	CHP	207
Rv3054c	CHP	184
Rv3223c	*sigH*	216
Rv3307	*deoD*	268
Rv3407 *	CHP	99

NB: Rv-numbers denote the names of the protein products. Italicised names designate the gene region and capitals the protein function, and a.a indicates amino acid. HSP indicates heat shock protein, and CHP and HP indicate conserved hypothetical and hypothetical proteins.

NB*: This was the only antigen kindly provided by the MPIIB.

2.2.2 Tissue Culture Assay Set-up

All standard tissue culture methods were strictly observed under thoroughly sterile conditions.

Antigen Reconstitution

Antigens were received in different amounts, either in lyophilised powder form (LUMC) or in solution (SSI). Lyophilised antigens were reconstituted to a stock concentration of 0.5mg/ml prior to being made up to a working dilution of 20µg/ml. To reconstitute, half of the total volume of sterile 1X PBS (Cambrex, Whitehead Scientific) required to solubilise the antigens to a concentration of 0.5mg/ml was initially added to the lyophilised proteins (dilution volume varied according to original concentration). The contents of the tubes were mixed well and placed in a 37^{o}C waterbath for 20 minutes. If the protein contents were not fully dissolved, the tubes were held in a sonicating waterbath for a few seconds intermittently until the powder went into solution. The solution was then transferred to a Greiner 15ml tube (LASEC) and the remaining 1X PBS was added. Finally, a volume of serum-free RPMI-1640 (Sigma) with L-glutamine (Sigma) at a 1% concentration was added to bring the solution to a working concentration of 20µg/ml. Antigens received already in solution (SSI) were directly made up to the same working dilution with RPMI + 1% L-glutamine.

Culture Plate Preparation

After reconstitution, the proteins or peptides at 20µg/ml were used to make frozen antigen plates for storage prior to whole blood assay. In a sterile laminar flow hood,

100µl/well of each antigen, in triplicate, was aliquoted onto labelled 96-well, U-bottom tissue culture plates (AEC Amersham). Staphylococcus enterotoxin B (SEB, Sigma) at 0.1µg/ml and/or phytohaemagglutinin (PHA; Sigma) at 5µg/ml were used as the positive stimulated culture controls on each plate, and plain RPMI with 1% L-glutamine as the unstimulated control. Empty wells were filled with distilled autoclaved water to prevent excessive evaporation from the plate during incubation.

2.2.3 Blood Processing

Blood Collection

Venous whole blood was aseptically collected from each participant into 10ml glass sodium heparin vacutainers (Becton Dickinson, Scientific Group). Blood tubes were transported to the laboratory at room temperature and processed strictly within 2 hours of collection in a laminar flow hood.

Whole Blood Assay (WBA)

Whole blood (2ml) was diluted 1 in 5 with RPMI-1640 with L-glutamine added to a 1% concentration. An aliquot of 100µl of blood/well was pipetted onto thawed antigen plates to make a final antigen concentration of 10µg/ml per well. Antigens were tested in triplicate. Culture plates were incubated for 7 days at 37^{o}C and 5% CO_2 in a humidified incubator. After the incubation period, the supernatant (SN) was carefully harvested off each well to avoid disturbing the layer of sedimented red blood cells. Replicate supernatants were pooled and mixed to ensure sample uniformity, and then separated into 3 aliquots: 210µl and 110µl aliquots of the pooled supernatant were stored on separate

96-well plates and another 110µl in 1.5ml Cap E™ eppendorf tubes (Merck). The 96-well storage plates were sealed with microtitre plate sealing tape (AEC Amersham) plus a lid. All tubes or plates were allocated a position on a computerised sample storage database before being frozen at – 80^{o}C until use.

2.2.4 IFN-γ Enzyme-linked Immunosorbent Assay (ELISA)

Materials and Reagents

Coating buffer: 0.1M $NaHCO_3$ in distilled water (dH_2O), pH 8.2

Blocking Buffer: 1X PBS with 10% heat inactivated foetal calf serum (HI FCS). FCS was heat inactivated for 30 minutes at 56^{o}C.

Wash buffer: 1X PBS with 0.05% Tween20 (Sigma)

Diluent solution: RPMI-1640 with 5% HI AB serum

Avidin peroxidise solution: 1/400 dilution of avidin peroxidise (Sigma) in blocking buffer

Capture antibody solution: 1/500 dilution of purified mouse α-human IFN-γ monoclonal antibody, clone NIB42 (BD Pharmingen) in coating buffer

Detection antibody solution: 1/500 dilution of biotinylated mouse α-human IFN-γ monoclonal antibody, clone 4S.B3 (BD Pharmingen) in blocking buffer

IFN-γ Standard solution: 1/250 dilution of 1µg/ml IFN-γ standard (Pharmingen) in diluent solution.

OPD FAST solution: 1 gold (urea peroxidise) and 1 silver (OPD) tablet (both Sigma), dissolved in 20ml dH_2O/ELISA plate.

ELISA

This protocol describes an indirect sandwich ELISA. Wells of the 96-well ELISA plates (AEC Amersham) were coated with 50µl/well of capture antibody solution and left to incubate overnight at 4^{o} C. The following day, plates were washed with wash buffer and blocked with 150µl of blocking buffer for 2 hours at room temperature (RT). After the blocking step, plates were washed again with wash buffer and 100µl/well of harvest supernatants were added in duplicate. IFN-γ standard solution and a positive internal control standard (pooled supernatant from a mitogen-stimulated WBA) were also added for the standard curve and internal positive control respectively. Plain diluent was added as the blank. Plates were incubated overnight at 4^{o} C. For detection, 100µl/well of detection antibody solution was added after plates were washed. After 1-hour incubation at RT, 100µl/well of avidin peroxidase solution was added, followed by another incubation period of 1 hour at RT, after which plates were washed. For the final detection step, 200µl/well of OPD FAST substrate was pipetted onto plates. Plates were incubated in the dark for 25 minutes at RT for colour development, and then optical density (OD) was read at 450nm on an ELISA plate reader.

The standard curve was generated using a serial dilution of the IFN-γ standard. There were two different ranges for the standard curves used for the ELISA, though the method for the assays was similar. Both were 8-point curves, but the top and bottom values were 4000 and 31.25pg/ml for one curve and 2000 and 15pg/ml for the other. This provided the distinction for the cut-off used to define positivity of the response, which was 30pg/ml for the LUMC antigens and 62.5pg/ml for the SSI antigens.

2.3 Results

2.3.1 Demographics of the Final Participant Groups

The two final participant groups, group 1 and group 2, both consisted of healthy, HIV negative adults that were HHCs of Tb Index cases, recruited from the nearby community study areas (Tables 5 and 6). Group 1 were tested on a panel of 67 antigens provided by Leiden University Medical Centre (LUMC) and Max Planck Institute for Infection Biology (MPIIB) and Group 2 were tested on a panel of 11 antigens provided by Statens Serum Institute in Denmark. Approximately three times more women than men were present in group 1 and four times more in group 2. All participants were of the Coloured (Brown) race except for one person in group 1.

Table 5: Participant demographic data for Group 1 (n = 38). All participants were TST positive.

GENDER SPREAD		AGE RANGE	TST READING (mm)	RACE
Male	Female			37 Coloured
11	27	18 - 56 years	≥ 10mm - 37mm	1 Black

Table 6: Participant demographic data for Group 2 (n = 19). 4 participants were tuberculin skin test (TST) negative (0mm) and 15 were TST-positive (>10mm).

GENDER SPREAD		AGE RANGE	TST READING (mm)	RACE
Male	Female			
8	11	12 - 51 years	0mm - 34mm	All Coloured

2.3.2 Gradient of Exposure

The gradient of exposure describes the level of contact between the recruited household contact and the diagnosed TB index case, taking note of the closeness of contact within a shared living area as well as the number of hours of exposure. Tables 7 and 8 show the gradient of exposure for group 1 and group 2 respectively. Exposure place describes the living area where the majority of exposure to an actively TB-infected person took place, with the yard being the lowest level of exposure and a shared room the highest. Exposure time was the total number of hours per day spent in the company of the infected person.

Table 7: Gradient of Exposure for Group 1.

	EXPOSURE PLACE			EXPOSURE TIME (hours)			
	Yard	House	Room	≤ 3	4-7hrs	8-11hrs	≥ 12hrs
Number of People	8	21	9	12	3	5	18

Table 8: Gradient of Exposure for Group 2.

	EXPOSURE PLACE			EXPOSURE TIME (hours)			
	Yard	House	Room	≤ 3	4-7hrs	8-11hrs	≥ 12hrs
Number of People	9	9	1	3	2	3	11

2.3.3 Validation of the IFN-γ ELISA using the Internal Positive Control

The internal positive control used as an ELISA positive standard was a pooled mitogen-stimulated supernatant from a 7-day WBA. The ELISAs were usually done in batches of 5-7 plates/batch. The performance of the positive control standard on each plate was used as an indicator of inter-well variation within a plate, and inter-plate variation. Figure 2 shows a representative spread of internal control values observed for 4 ELISA batches done over the course of the antigen screening study. In our lab, internal positive control values in the range of 950-1600pg/ml were generally accepted as normal.

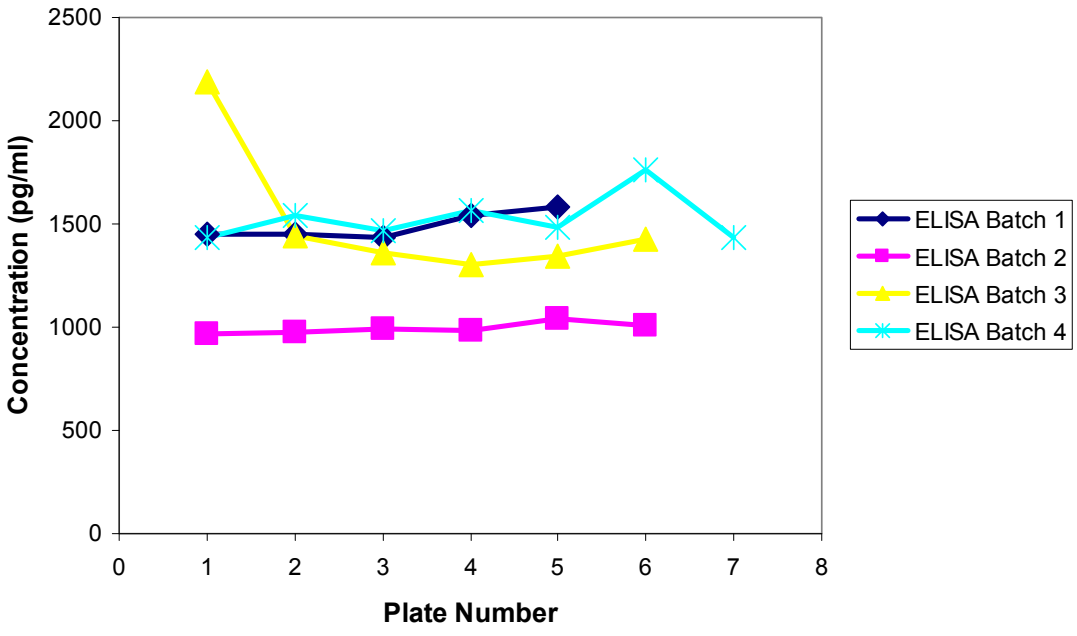


Figure 2: Comparison of the Internal Positive Control standard within ELISA batches. A typical ELISA batch consisted of 5 – 7 plates run on the same day. The internal positive control was added to each plate in duplicate, and the mean value (in pg/ml) of the wells was taken as the readout.

2.3.4 Statistical Analysis

To characterise the groups of responders according to levels of responses, response categories were defined and displayed in the figure key as follows: nil (0 – 30pg/ml), low (31 – 125pg/ml, indictaed by blue colour), intermediate (126 – 1000pg/ml, indictaed by purple colour) and high (> 1000pg/ml, indictated by cream colour). These categories were assigned in accordance with points on the standard curve. Grouping of the mean values and generation of graphs was done using basic Microsoft® Excel software.

2.3.5 Group 1 IFN-γ Responses to LUMC and MPIIB Antigens

2.3.5.1 Responses to Classic TB Antigens

IFN-γ responses to the classical TB antigens are shown in Figure 3. Generally, the highest percentage responders were observed to recombinant ESAT-6 and the fusion protein TesatCFP-10, with positive responses in 85% and 80% of individuals respectively. 61% of responses to ESAT-6 fell within the medium-to-high range (126 – 1000pg/ml) with 17% of responders producing over 1000pg/ml IFN-γ to this antigen. The frequency of response to TesatCFP-10 was more evenly divided, with 38% of positive responses in the moderate range and 26% in the high range. Individuals tended not to respond as well to Rv3019 (TB10.3), a secreted ESAT-6-like product, as they did to either ESAT-6 itself or the fusion protein, only half the number of participants (40%) being responsive to this antigen. Just over 31% showed a positive response to TB10.4, with approximately 50% showing moderately high IFN-γ production. Less than 10% of individuals responded to Ag85A/B and only 3% to HSP65. There was a high frequency

of positive responses (77%) to the mitogen phytohaemaglutinin (PHA), equally divided between moderate and high pg/ml values.

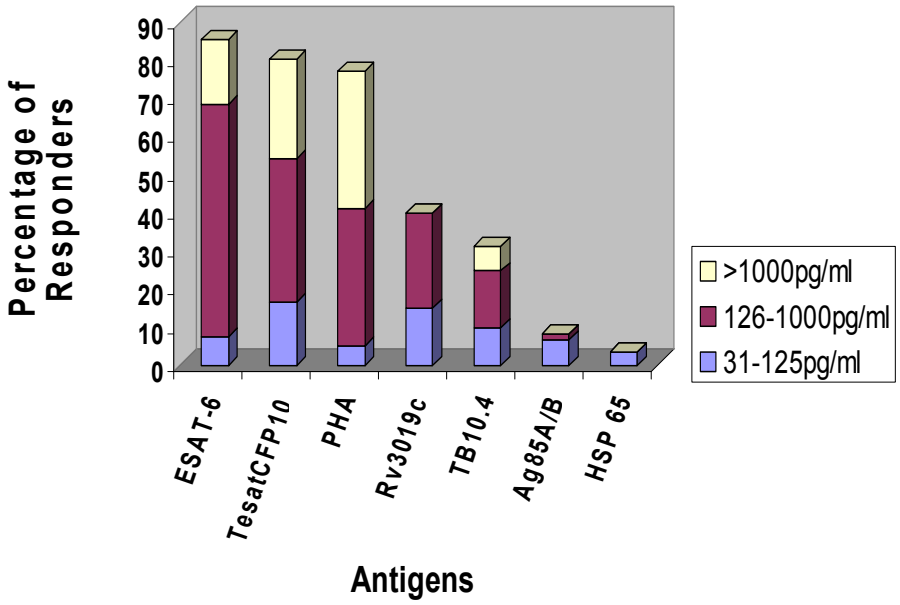


Figure 3: Percentage of positive responses to Classic TB antigens. ESAT-6, Rv3019c (a secreted ESAT-6-like protein), TesatCFP10, TB10.4 and Heat shock protein 65 (HSP65) were all recombinant antigens tested at a final concentration of 10µg/ml. The recombinant proteins of Antigen 85A and B were pooled (Ag85A/B) and both tested at 10µg/ml final concentration. Phytohaemaglutinin (PHA) was used as the positive control and tested at 5µg/ml. IFN-γ ELISA was performed on 7-day whole blood culture supernatants of TST positive, HIV negative patients (n = 38).

2.3.5.2 Responses to Latency Antigens

Figure 4 shows the frequency of responses to the latency antigens that elicited a positive IFN-γ response in the group tested. The two antigens eliciting the highest responses were Rv1733c and Rv1737c, with 45% and 43% of positive responders respectively. Four antigens, Rv1735c, Rv1736c, Rv0081 and Rv1996 produced a response in 28 – 38% of individuals. An additional four antigens elicited positive IFN-γ responses in 19% of those tested. Eight antigens (Rv1737c, Rv1735c, Rv1736-C, Rv1996, Rv1813c, Rv1997-C, Rv2031c and Rv0573c) elicited a moderate to high response (126 - 1000pg/ml) in over 50% of the people they were tested on, and an additional 4 antigens (Rv1733c, Rv0081, Rv1812c and Rv1997-N) did likewise in about 25% of responders. None of the latency antigens on the panel stimulated a positive response higher than 1000pg/ml.

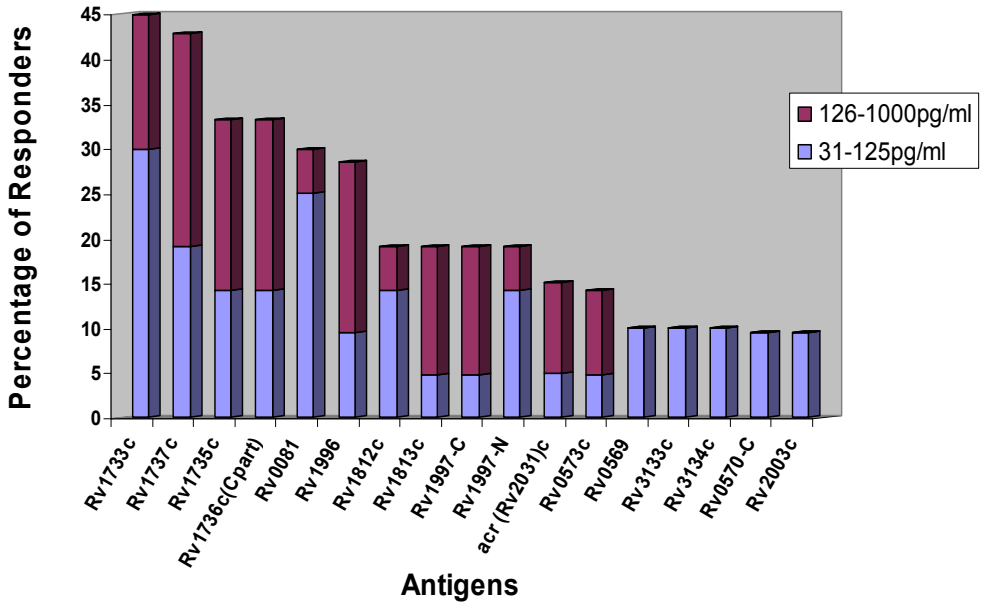


Figure 4: Percentage of responders to Latency antigens. IFN-γ ELISA was performed on 7-day whole blood culture supernatants of TST-positive, HIV-negative participants (n = 38).

2.3.5.3 Responses to Resuscitation Promoting Factor (Rpf) Antigens

The frequency of responses to all the RPF antigens is summarized in Figure 5. The overall response to the RPFs was second only to the response to most of the classical TB antigens, both in terms of the magnitude of response and the percent responders. At least 30% of participants produced a positive response to each individual RPF antigen. Positive IFN-γ responses were more or less equally divided between low and moderate-to-high levels in response to Rv0867, Rv2389, Rv2450c and Rv1009. In addition, 5% of responders (n = 3) produced IFN-γ levels in the high range (over 1000pg/ml) for these same four RPFs. The lowest levels of IFN-γ production (30 – 125pg/ml) were produced in response to Rv1884c, which also induced the lowest percent of responders.

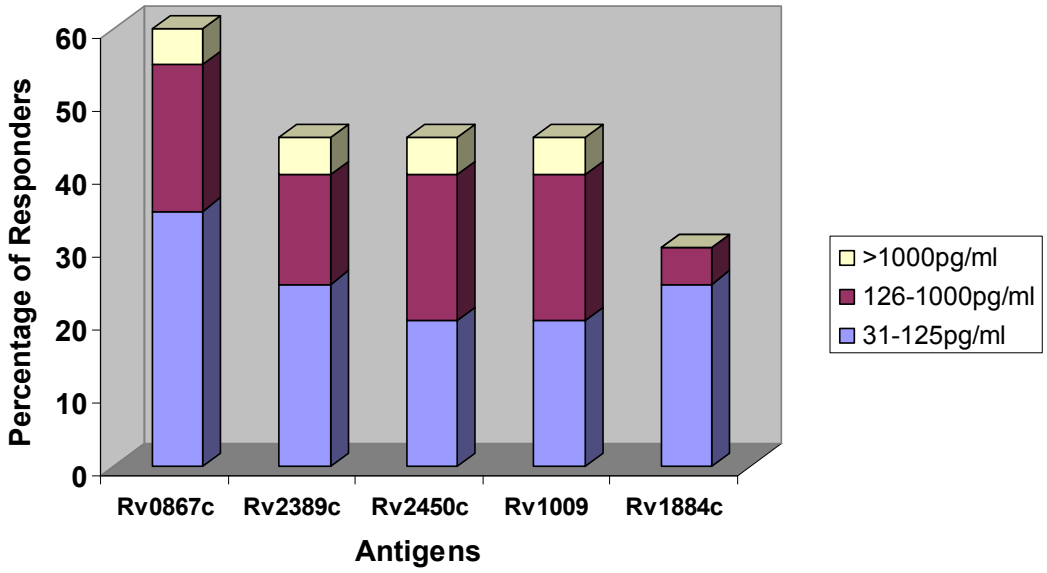


Figure 5: Percentage of responders to Resuscitation-promoting factor (RPF) antigens. IFN-γ ELISA was performed on 7-day whole blood culture supernatants of TST-positive, HIV-negative participants (n = 38).

2.3.5.4 Responses to Reactivation Antigens

Figure 6 shows the frequency of responses to the 13 out of 21 reactivation antigens tested that generated a response above 30pg/ml. In general, the profile of responses to the reactivation antigens was poor in comparison to the other types of antigens. The percentage of responders was only 5% for most of the antigens, and for 8 of the 21 proteins tested there was no response observed in any of the participants (results not shown here). IFN-γ responses above 125pg/ml were only produced by 5 reactivation proteins, the highest IFN-γ levels being induced by Rv1131 (24% of people produced both low and moderate/high level responses respectively) and Rv1115 (14% of people producing both low and moderate/high level responses respectively). In comparison, only 15% of participants responded to Rv3862, Rv0246 and Rv0331.

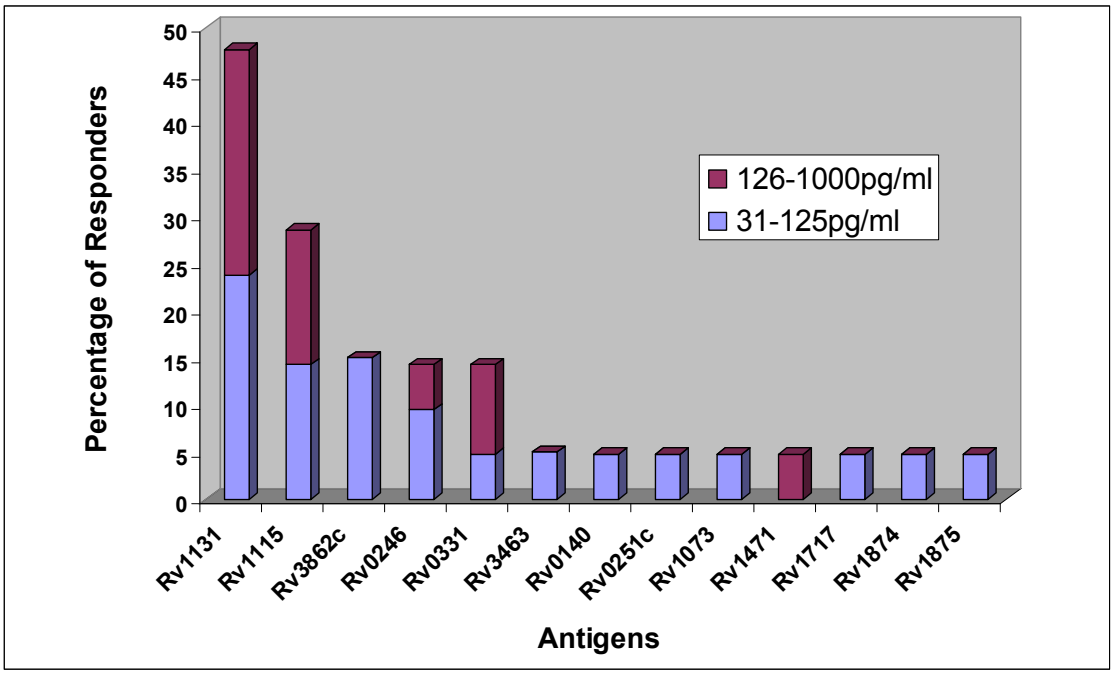


Figure 6: Percentage of responders to Reactivation antigens. IFN-γ ELISA was performed on 7-day whole blood culture supernatants of TST-positive, HIV-negative participants (n = 38).

2.3.6 Group 2 IFN-γ Responses to SSI Antigens

2.3.6.1 Antigens Used

A small panel of antigens was provided by SSI for testing. As much smaller quantities of protein were supplied, the antigens were only tested on a group of 19 people (group 2). The antigens tested were TB10.4 (peptide pool), ESAT-6 (peptide pool), Ag85B (recombinant), and peptide pools of Rv2654, Rv2659A, Rv2659B, Rv2659C, Rv2660, Rv2661, Rv2662 and Rv2663. The peptide pools arrived pre-pooled from SSI and were tested at a final concentration of 10 and 1μg/ml per peptide, whilst the recombinant protein were tested at 10μg/ml final concentration. In addition, SEB at 0.1μg/ml and PHA at 5μg/ml were used as the positive control stimulants. IFN-γ ELISA were performed on 7-day WBA supernatants of participants.

2.3.6.2 Responses to SSI Antigens

Figure 7 below shows the frequency of responses to the SSI panel of antigens. All participants showed a very strong response to the positive control stimulants SEB and PHA. The classical TB control antigens TB10.4 and ESAT-6 showed the highest frequency of responses in participants, which was comparable to responses observed in the Group 1 cohort. The peptide pools of these proteins induced responses more frequently at 10μg/ml than at 1μg/ml. Stimulation with TB10.4 and ESAT-6 at the higher concentration produced a positive response in 37% and 47% of responders respectively (n = 7 and n = 9), in comparison to 26% and 32% (n = 5 and n = 6) respectively at the

1µg/ml concentration. Recombinant Ag85B, which was tested at 10µg/ml, induced responses in only 2 participants (10.5% of responders).

In general, there was a low frequency of responses observed to the other peptide pool antigens (data not shown here). Only Rv2654 and Rv2659C stimulated notable positive reactions in participants (37% and 32% of responders respectively). Each of the other 6 non-control antigens, Rv2569A, Rv2659B, Rv2660, Rv2661, Rv2662 and Rv2663, elicited low responses in only one or two participants.

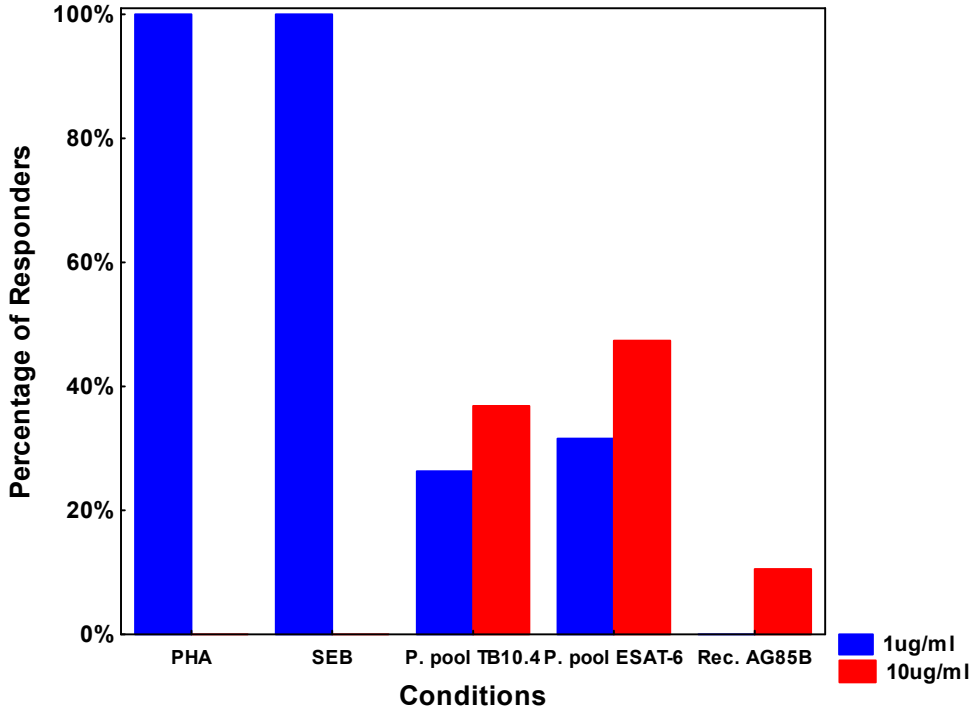


Figure 7: Frequency of positive responses to SSI antigens. ELISA was performed on 7-day whole blood culture supernatants of HIV-negative participants of cohort Group 2 (n = 19). The blue bars indicate peptide pool antigens tested at 1µg/ml and the red bars, with the exception of Ag85B, indicate peptide pools tested at 10µg/ml. Recombinant Ag85B was also tested at 10µg/ml final concentration. Positivity of the assay was defined as greater than twice the lower limit of detection (62.5pg/ml in this case) of the IFN-γ ELISA.

2.4 Discussion

The factors influencing the interaction between the host and the infective agent *M. tuberculosis* are hypothesized to collectively play a role in what defines latent and active infection. Little is known about the cytokine profile of latent infection in humans, and it is not yet fully understood what causes latent infection to persist in the majority of exposed individuals as opposed to what causes reactivation of TB disease in the minority. The RD1 gene region of mycobacteria has yielded valuable information about the different genes of interest with regard to TB research. The DosR regulon of *M. tuberculosis* is expressed *in vitro* under simulated *in vivo* conditions of nutrient starvation, hypoxia and low-dose NO (Voskuil M.I et al., 2003). This genomic region has demonstrated increasing potential in recent years, as it has been realized that proteins encoded by this region could provide vital information as to what constitutes protective immunity against TB in humans. This study evaluates the human *in vitro* T cell responses of latently infected individuals to 78 RD1 or DosR regulon encoded antigens by quantifying their IFN-γ production by ELISA.

The results of this study have shown that all tested proteins, whether peptide or recombinant, were able to induce T cell responses, demonstrated by varying IFN-γ levels. As has been demonstrated by other studies, the IFN-γ responses to classical TB proteins were observed in a large number of participants (Lalvani A. et al., 2001; Weldingh K. et al., 2005; Porsa et al., 2006; Hoff S.T et al., 2007). An overwhelming majority of participants responded to ESAT-6 and TesatCFP-10, 80% and 85% respectively. Interestingly, other groups have found that patients with past or active TB preferentially

recognize these two secreted antigens over other latency antigens (Demissie A. et al., 2006; Leyten E.M.S et al., 2006; Lin M.Y et al., 2007). This finding could suggest that a significant number of latently infected individuals that responded to ESAT-6 and TesatCFP-10 in this study could be people on the verge of progressing to active TB. It could also suggest that high IFN-γ responses to these antigens could be more indicative of latent as opposed to active infection in a South African population. TB10.4 induced responses in over 31% of responders, and this antigen has also been established to be another prime vaccine candidate (Hervas-Stubbs S. et al., 2006). There were some responses to proteins of the Ag85 complex (10% of responders) but responses to Ag85 proteins fused to ESAT-6 have been shown to be stronger and more TB-specific in mice and macaques, indicating this protein may confer better protection as a fusion product than on its own (Langermans J.A.M et al., 2004; Radošević K. et al., 2007).

Of the 35 latency antigens tested, 17 induced detectable responses in participants. The most notable 6 antigens, Rv1733c, Rv1737c, Rv1735c, Rv1736c, Rv0081 and Rv1996 were most frequently recognized by at least 30% of responders. In addition to Rv2029c which was recognized by 5% of responders (not shown on figure 4), Rv1733c and Rv2031 have been closely associated with what may be a typical latent infection profile in humans and mice (Demissie A. et al., 2006; Leyten E.M.S et al., 2006; Lin M.Y et al., 2007). The results obtained here concur with the finding that latently infected individuals preferentially recognize certain latency antigens over others. Rv2627c and Rv2628c have also been found to be strongly recognized by T cells in mice (Roupie V. et al., 2007), but these two antigens performed poorly in our WBA, producing responses in 0% and 5% of

responders respectively. New antigens that have never been tested before and have produced strong TB-specific IFN-γ responses here (for example Rv1737c, Rv1735c, Rv1736c, Rv0081 and Rv1996) show that more latency factors are at play during the course of dormant infection, and these proteins could be further evaluated as vaccine candidates.

Many studies that have undertaken to test panels of TB-specific antigens for immunogenicity have only endeavoured to screen DosR latency or classic control, RD1-encoded vaccine candidates. Though the production of antigens associated with reactivation and resuscitation has been well described, few research efforts have attempted to characterize the immunoreactive and vaccine properties of these proteins *in vivo* or *in vitro*. The present study demonstrated that 5 of the reactivation antigens (Rv1131, Rv1115, Rv3862c, Rv0246 and Rv0331) tested produced responses in 15% or more of responders and that generally, the RPFs were highly immunogenic in participants. One study has shown similar results in mice (Yeremeev V.V et al., 2003) where the capacity for RPF-like proteins to induce significantly high *M. tuberculosis*-specific T cell proliferation *in vivo* was observed, as well as high antibody titres that correlated with a humoral response. It is generally accepted that a competent vaccine against TB would provide protection by inducing a strong cellular Th1-biased response (De Groot et al., 2005; Gupta D.G et al., 2007) but a humoral, immune response may also be of benefit. It was also observed that vaccination with RPFE or Rv2450 provided a significant degree of protection against TB challenge in mice, and correlated strongly with decreased bacterial burden in the lungs and spleen and increased survival times

(Yeremeev V.V et al., 2003). Although the responses to RPFs and reactivation antigens were merely quantitative here, there is strong supportive evidence that these proteins are potentially protective against reactivated TB. It would be an attractive feature for a TB vaccine to prime the host to recognize proteins associated with resuscitation to aid in combating the conversion to active disease. Including RPFs in vaccine design may be important for this reason.

The question of the types of antigens provided was also tackled. Most (86) of the antigens were recombinant*, E. coli* vector-produced proteins whilst 10 were peptide pools. There was a clear difference in the level of responses to recombinant versus peptide pooled antigens tested at the same concentration, with the recombinant proteins inducing higher response levels in a markedly greater proportion of participants. Although relatively large amounts of protein can be generated in a bacterial construct and the production and purification steps can be optimized, there is the very real problem of contamination by vector components that can lead to high background and even false positive readouts in some cases. This can make the interpretation of strong positive results somewhat more difficult (Tom Ottenhoff at LUMC, personal communication). The obvious advantage of peptide pools over recombinants is the lack of vector contaminants in the protein, but they are more costly to make and the pooling of several peptides in a well might lead to interference (for example, MHC receptor site-competition) with the process of antigen presentation, leading to false negatives (May Young Lin and Tom Ottenhoff at LUMC, personal communication). Possible solutions for these problems would be additional purification steps, optimizing concentration dosage for recombinant proteins, and

minimizing the pool size and final concentration to prevent interference in the case of peptides. Keeping these limitations in mind, more comparisons between the two types of antigens need to be done to fully define and carefully interpret results. Nevertheless, preliminary results of large screening exercises such as the one described here provide important information giving valuable insight into quantitative protective immune responses to latent TB infection.

CHAPTER 3

Cytokine Evaluation of WBA Supernatants by Luminex Assay

3.1 Luminex study

3.1.1 Background

This study was designed to complement the results generated by the conventional IFN-γ ELISA method used throughout the antigen screening. Although ELISA is an invaluable tool for measuring immune responses, it has been well recognised that IFN-γ is not the only cytokine generated during tuberculosis infection. In order to effectively outline an infection profile, a variety of other immune regulators need to be identified. Cytokine expression profiling (CEP) methods are relatively new immunoassay systems that have revolutionised the analysis of multiple cytokines in a given sample volume.

These Multiplex assays like the Luminex® system employ fluorescent bead-based luminex technology. This immunoassay operates on the capture/detection sandwich-type model largely similar to the conventional ELISA method, only it has the potential to measure up to 100 different cytokines in a 50µl sample. This makes the system capable of detecting a cocktail of other analytes besides IFN-γ in culture supernatants and serum. These assays are thus potentially powerful methods of monitoring up- and down-regulation of cytokines and hence providing valuable time-points of immune status. The time-saving advantages, ease of use, and utilisation of small volumes of often precious samples, makes the Luminex® technology ideal for screening purposes in large-scale

cross-sectional studies. The LINCO® 21-plex Human Cytokine Pre-mixed kit was used in this study to identify other biomarkers potentially present in human WBA supernatants.

3.1.2 Experimental Design

The setting, and participant selection and inclusion/exclusion criteria used for this study were identical to those used in the antigen screening study. Consent and ethical approval was granted by the Stellenbosch University Ethics Committee.

Final Participant Cohort

The participants used in this study were the same as in Group 2 of the antigen screening study, and initially consisted of 20 healthy HIV negative household contacts of newly diagnosed pulmonary TB cases. Five participants were excluded because their TST induration was later discovered to be 0mm. A posterior-anterior chest X-ray radiograph was also taken of all participants and none showed early signs of TB or lung disease development.

Antigen Selection

The scope of the experimental design for the Luminex study was restricted by the number of kits that were available. Experiments using the LINCO-plex assay is a costly venture, and the five assay kits used were kindly provided by collaborators at the Max Planck Institute for Infectious Biology (MPIIB). As a result, the number of antigens that could be tested in total was limited to 10. The 8 test conditions were chosen from a final antigen rank list compiled by 3 African field sites that are part of the Gates Tuberculosis

Consortium (GC6-74), sites that have previously screened most of the antigens mentioned in this body of work in other African populations. In order to complement the antigen screening study, the antigens were selected on the criterion that they, as a group, were a representative spread of most of the antigen ranks (high, intermediate and low) at all the sites. Table 9 shows the site and overall rankings for the chosen panel.

Table 9: Field site and overall rankings for the chosen panel of Luminex antigens. This table is an excerpt of the rankings generated at three of the African field sites, Stellenbosch University (SUN, South Africa), MRC (Medical Research Council, Gambia) and Case (Case Western Reserve University, Uganda). The two conditions not shown are the negative control (RPMI-1640 with 1% glutamine) and TesatCFP-10, a classic vaccine antigen that performed well at all sites and was used as the positive control stimulant.

Antigen Name	SUN Rank	MRC Rank	CASE Rank	Overall Ranking
Rv 2450	2	1	6	1
Rv 1733c	11	4	1	2
Rv 1131	8	10	4	3
Rv 0081	17	19	2	4
Rv 1737c	6	13	8	5
Rv 1735c	7	41	3	6
Rv 0569c	57	48	5	19
Rv 2029c	66	3	75	43

Samples Used

The samples used were aliquots of 7-day, antigen- stimulated WBA supernatants that had been previously harvested and stored at -80^{o} C for prospective studies such as this one. For each participant, one 110µl aliquot of antigen-stimulated supernatant was thawed at

room temperature, and then spun at 1000*g* for 10 minutes to sediment cellular debris before use.

3.1.3 The LINCO® 21-plex Human Cytokine Pre-mixed kit

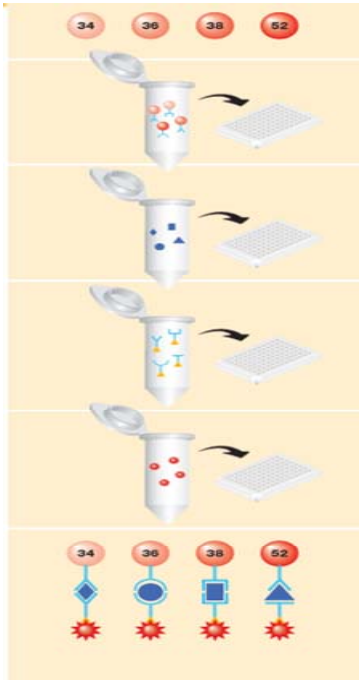
This is a Multiplex assay kit that is manufactured by LINCO Research, Inc for research diagnostics on cell culture supernatants or serum samples. It consists of pre-mixed antibody-immobilised bead sets that can routinely be used for the simultaneous quantitative determination of human cytokines and chemokines. The kits are available as a 13-, 21- and 29-plex, and the methodology below describes the use of the 21-plex kit. The array of cytokines measured is shown below in Table 10 and Figure 8 illustrates the basic methodology behind the Multiplex assay.

Table 10: The premix antibody-immobilised bead set for the LINCO 21-plex kit. All cytokines are designated a #-number for the purpose of reading the fluorescent beads using the multiplex software.

21-plex Premix Beads	
#01 Human IL-1β	#28 Human IL-15
#03 Human IL-2	#30 Human IL-17
#09 Human IL-4	#32 Human IL-1α
#10 Human IL-5	#35 Human IFNγ
#12 Human IL-6	#37 Human G-CSF
#13 Human IL-7	#39 Human GM-CSF
#20 Human IL-8	#40 Human TNFα
#23 Human IL-10	#44 Human Eotaxin
#25 Human IL-12p70	#46 Human MCP-1
#26 Human IL-13	#57 Human MIP-1α
	#65 Human IP-10

Figure 8: Basic methodology of the Multiplex bead-based immunoassay. Illustration (without annotation) appears courtesy of Bio-Rad Laboratories Inc. website, www.bio-rad.com.

- The technology employs the use of flourescent-dyed beads to which biomolecules are bound.
- Antibody specifically directed against cytokines of interest are covalently coupled to colour-coded polystyrene beads.
- The antibody-coupled beads are allowed to react with a sample (supernatant) containing an unknown concentration of cytokine.
- After a series of washes to remove unbound protein, a biotinylated detection antibody, specific for a different epitope on the cytokine, is added to the beads. This results in the formation of a sandwich of antibodies around the cytokine.
- The reaction mixture is detected by streptavadin-phycoerythrin (streptavadin-PE), which binds to the biotinylated detection antibodies.
- The well contents are drawn up into the Luminex suspension array system, which identifies and quantitates each specific reaction based on bead colour and flourescence. Unknown cytokine concentrations are also automatically calculated by the system's software.



LINCO 21-plex Assay Procedure

The premix bead kit was used as per the manufacturer's instructions with a few alterations. Briefly, culture supernatants were thawed and spun down at 1500rpm for 10 minutes to precipitate cellular debris. To generate a 5-point standard curve, the reconstituted cytokine standard cocktail was serially diluting into 2000, 400, 80, 16, and 3.2pg/ml concentrations. For the quality controls, the two control buffers (QC-I and QC-II) were used as directed by the kit. In addition, 25µl of control buffers was also added to aliquots of the sample supernatants (supernatant stimulated for 7 days with the chosen

antigens and pooled) and these were used as spiked controls. The filter plate was blocked with 200µl of assay buffer, left at RT on a standard plate shaker for 10 minutes. The buffer was then removed by vacuum aspiration and 25µl of assay diluent was added to sample wells whilst RPMI-1640 with 1% glutamine was added to standard wells. Following the suggested vertical sample placement on the plate layout, 25µl of either standard, control, spiked control or neat supernatant solution was added to the appropriate wells, followed by the addition of 25µl antibody-coated fluorescent beads. After 1-hour incubation on a plate shaker at RT, the fluid was gently aspirated. Biotinylated secondary antibody was then added, followed by streptavidin- labelled phycoerythrin antibody, with alternate washing and incubation steps between addition of each antibody. Finally, 100µl of sheath fluid was put into each well and the beads were resuspended gently for 5 minutes, after which the plate was read on the Bio-plex® array reader. Plates were read at high and low RP1 target to generate broad range and high sensitivity plots. Preliminary data analysis was done using the Bio-Plex Manager software (Bio-Rad Laboratories) with a five-parametric regression curve (5-PL) fitting.

Assay Characteristics and Standard definitions

Reading: The reported fluorescence of the sample/well measured in pg/ml. All readings higher than the observed background sample reading are taken as positive.

Recovery: This is the ratio of the observed (actual reading) over the expected (manufacturer's guideline) concentration in a sample, expressed as a percentage. Recoveries falling in the range of 70 - 130% are considered acceptable for this assay.

Sensitivity: The minimum detectable concentrations of each evaluated cytokine in pg/ml, depending on the amount of time lapsed immediately after when the plate should be read. The sensitivity is determined by two sets of standard curve ranges, the broad (1.95 – 32 000 pg/ml) and the high sensitivity (0.2 - 3.2pg/ml).

Linearity: This ratio describes the observed amount of cytokine in a sample as compared to the observed total amount of cytokine in an undiluted sample of the same type. This ratio is multiplied by the coefficient of dilution and expressed as a percentage.

RP1: The RP1 is the channel through which the fluorescent beads flow for quantitation on the assay reader. Low RP1 is the fluorescent channel recommended to assay a wide range of cytokine concentrations or when the range of expected concentrations is unknown. Hence, low RP1 gives rise usually to a broad range standard curve. High RP1 conversely is ideal for quantifying low cytokine levels and provides for greater assay sensitivity, and therefore makes use of the high sensitivity standard curve.

3.2 Statistical Analysis

Box plots were used to visually display log expressions for different cytokines and antigens. A factor analysis was done to determine the factor structure underlying all the cytokine variables. The scree plot was used to determine the number of factors, and factor loadings were calculated using the varimax rotation.

3.3 Results

3.3.1 Quality Control Assessment

The quality control (QC) evaluation is carried out as a standard procedure during the course of the run for each LINCO-plex plate, to validate that the run was successful and any large variations in the results was not due to defects in the kit. The two supplied controls are premixed, lyophilized human cytokine cocktails called QC-I and QC-II. Each analyte of the 21-plex assay is expected to produce a read-out that lies within a range for both controls. Figures 9 and 10 are graphical representations of expected and observed outcomes for QC-I and QC-II. In addition, the control buffers were spiked with participant supernatant and the effects of the spiked controls, QC-I-sup and QC-II-sup, are also shown in Figures 11 and 12.

Results of Kit Validation and Quality Control (QC)

Generally, the outcome of the validation and the QC evaluation revealed that the analytes performed at acceptable levels and the supplied kit was in good working condition. Recoveries for all the 21 cytokines were within the 70 – 130% acceptable range. All observed values for QC-I fell within the expected ranges for each cytokine, as well as for QC-II, with the exception of 5 cytokines for the latter. Of the 5 observed out-of-range values for QC-II, only MCP-1 was of significant concern, yielding a result of 2600pg/ml which was extremely high (figure 10). The observed values for the other four cytokines, IL-2, IL-5, IL-8 and MIP1-α, only exceeded their expected ranges by an average of about 100pg/ml (figure 10).

When the QC buffers were spiked with human supernatant to test its effect on their performance, it was also shown that in general, the addition of supernatant did not affect the QC expected value. For QC-I, the most notable difference was that the added sample appeared to lower the action of the buffer to below what was expected. This was observed for 9 out of the 21 cytokines, but only 5 of the 9 (IL-6, IL-8, MCP-1, MIP1-α and IP-10) showed a considerable decrease (figure 11). QC-II spiked with supernatant showed that two cytokines, IL-8 and MCP-1, fell considerably out of their expected ranges by about 400pg/ml (figure 12).

Figure 9: Quality control assessment of each analyte of the LINCO 21-plex using QC-I. The upper and lower bars of the concentration range denote the highest and lowest expected values, and the open circle indicates where the observed value fell for each analyte.

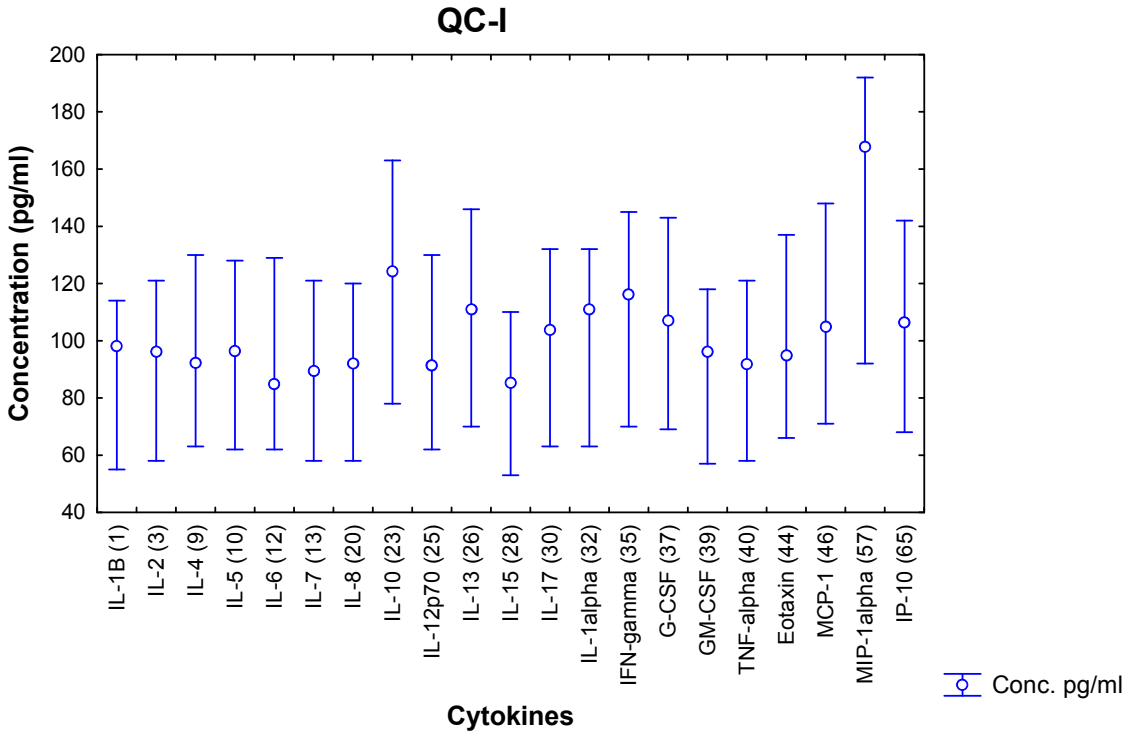
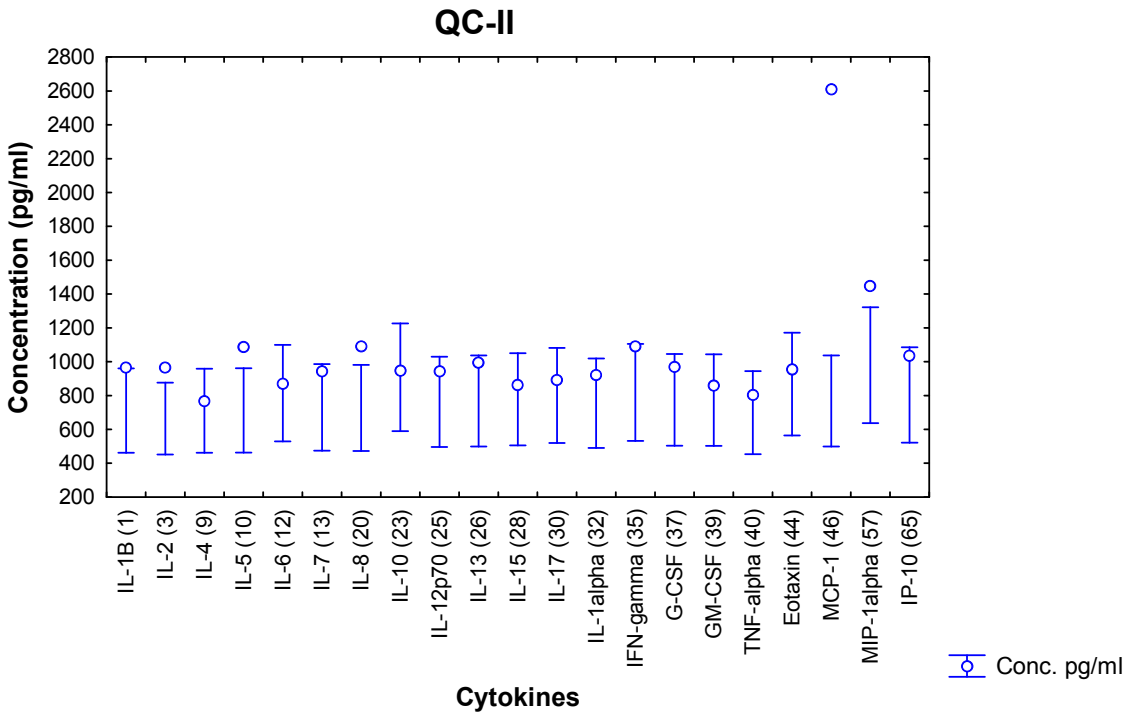
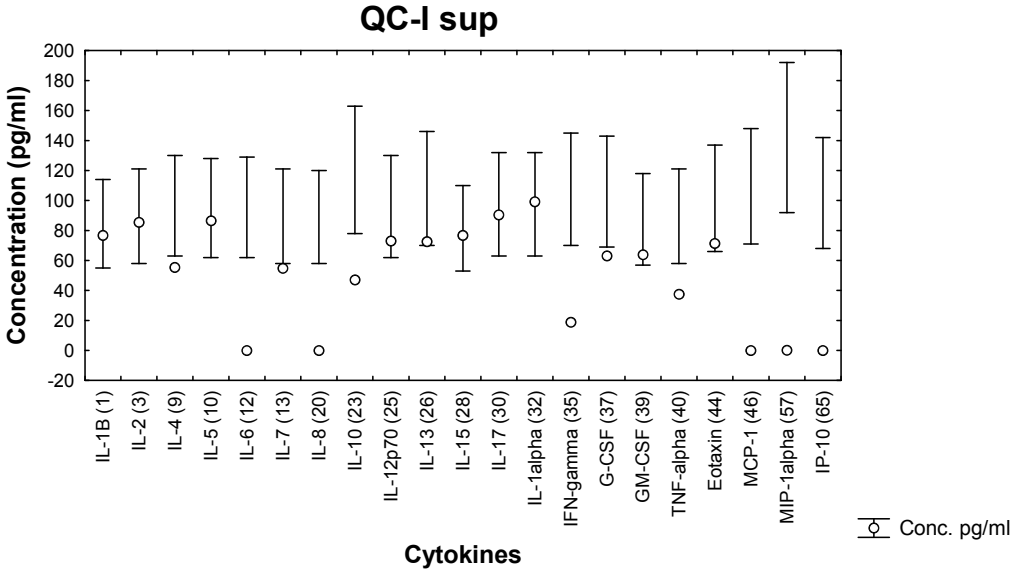
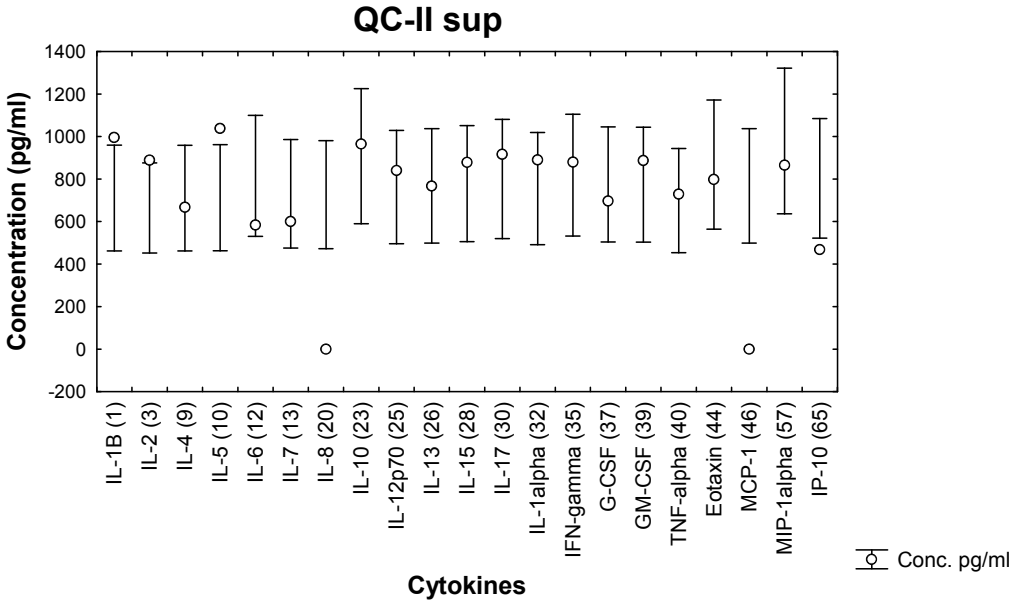


Figure 10: Quality control assessment of each analyte of the LINCO 21-plex using QC-II. The upper and lower bars of the concentration range denote the highest and lowest expected values, and the open circle indicates where the observed value fell for each analyte.







Figures 11 and 12: Quality control assessment of each analyte of the LINCO 21-plex, using supernatant-spiked controls, QC-I-sup and QC-II-sup respectively. The upper and lower bars of the concentration range denote the highest and lowest expected values, and the open circle indicates where the observed value fell for each analyte.

3.3.2 Evaluation of the Assay by Antigen

Negative Control

To standardize the LINCO-plex assay with the ELISA, RPMI with 1% glutamine, which was used as the negative control in the 6-day whole blood assay, was also used here as the unstimulated control. As expected, most of the cytokines showed a very low response to the unstimulated control, with values in the range of 2 – 10pg/ml (figure 13). Two cytokines, IL-8 and MCP-1, showed unexpectedly higher responses to the negative control, each with a median value of about 50pg/ml.

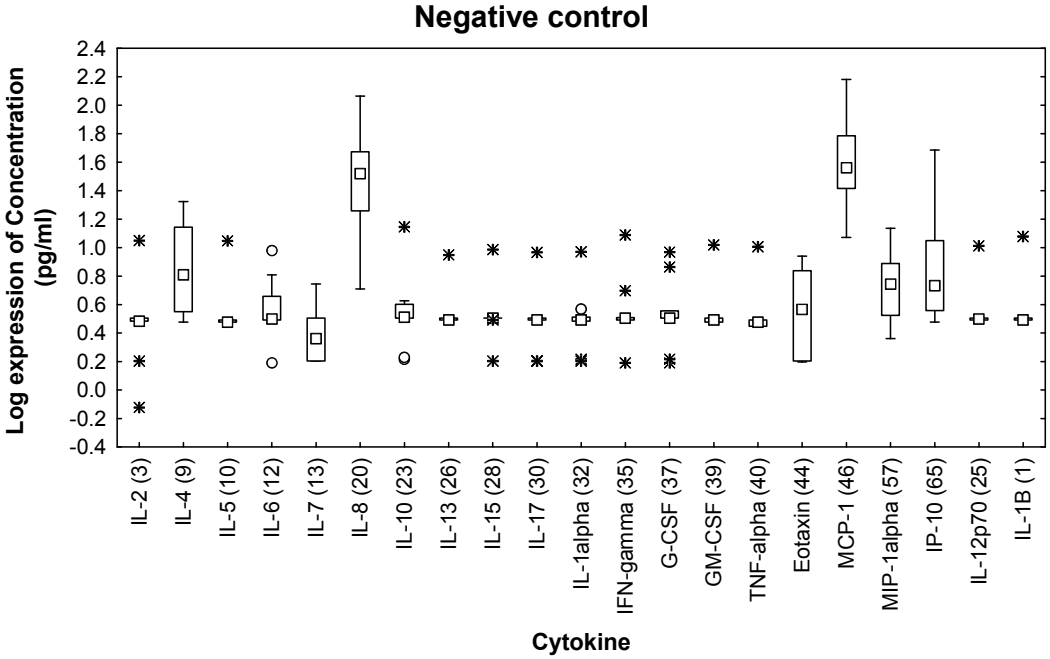


Figure 13: Box plot of the negative control condition using the LINCO 21-plex assay. 7-day WBA supernatant stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST-positive, HIV-negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

TesatCFP-10

The fusion protein of ESAT-6 and CFP-10, TesatCFP-10, was used as the positive control stimulant. Most of the cytokines responded favourably to the positive control. Lower median values were around 30 – 75pg/ml for IL-1B, IL-2, IL-5, IL-10, IL-13, IL-17, IL-4, G-CSF, IL-1α and eotaxin (figure 14). Moderately high median values were recorded for TNF- α (200pg/ml), GM-CSF (500pg/ml), and MIP-1α (500pg/ml). Higher concentrations, above 1000pg/ml, were observed for IL-6, IL-8, IFN-γ, MCP-1 and IP-10. IL-4, IL-15 and IL-12p70 produced no responses to the positive control.

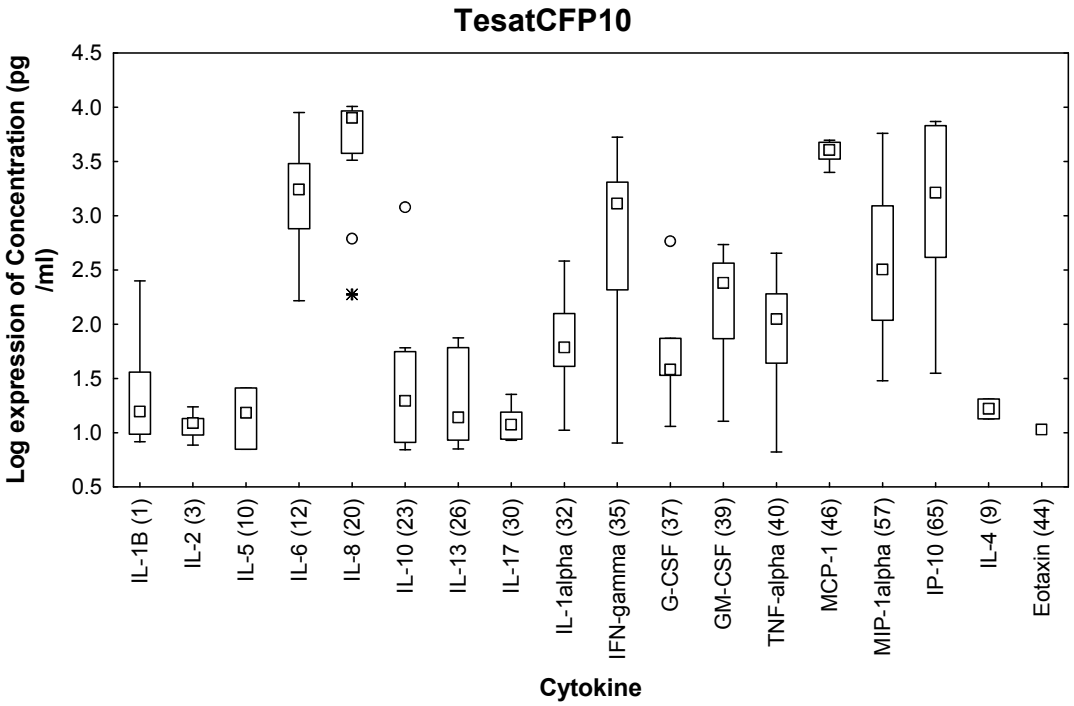


Figure 14: Box plot of the positive control condition TesatCFP-10, using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST- positive, HIV-negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv2450

Most of the cytokine responses to Rv2450 were low to moderately high, ranging from 50 – 250pg/ml (figure 15). High median responses to the antigen were produced by IL-6 (over 1000 pg/ml), MCP-1 (5000 pg/ml) and IL-8 (about 10 000pg/ml). Seven cytokines (IL-2, IL-4, IL-5, IL-7, IL-13, IL-15 and IL-12p70) showed no reaction to Rv2450.

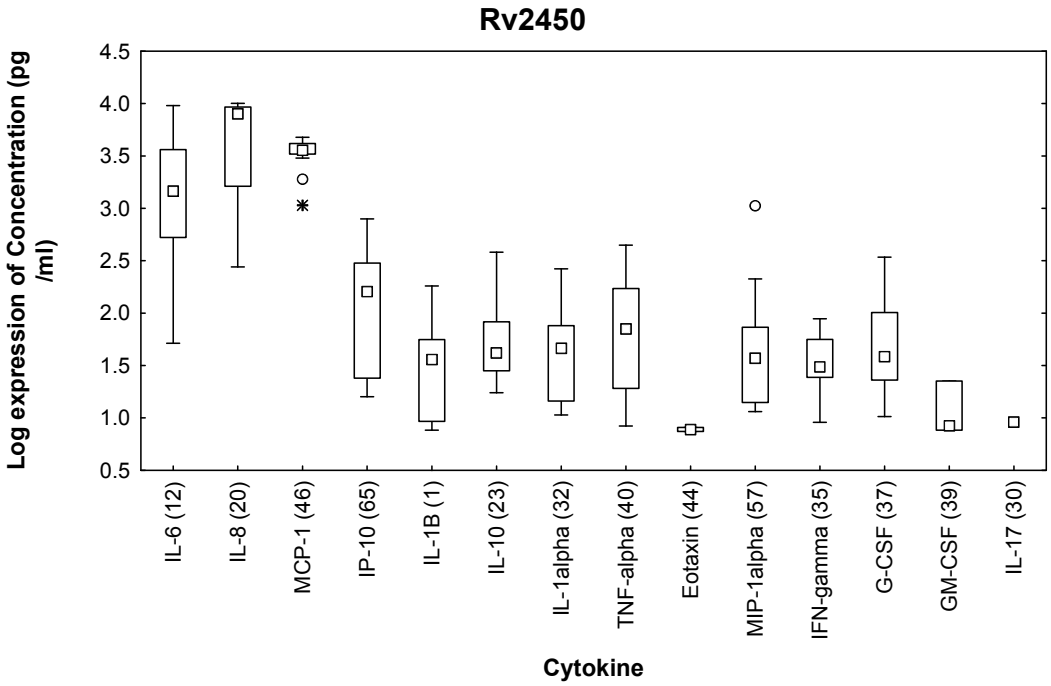


Figure 15: Box plot of antigen Rv2450 using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample partcipant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv1733c

Generally, median concentration responses to Rv1733c were moderately high, ranging from 100 – 600pg/ml on average (figure 16). High median readouts above 5000pg/ml were observed for IL-6, IL-8 and MCP-1. IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17 and eotaxin were not produced in response to Rv1733c.

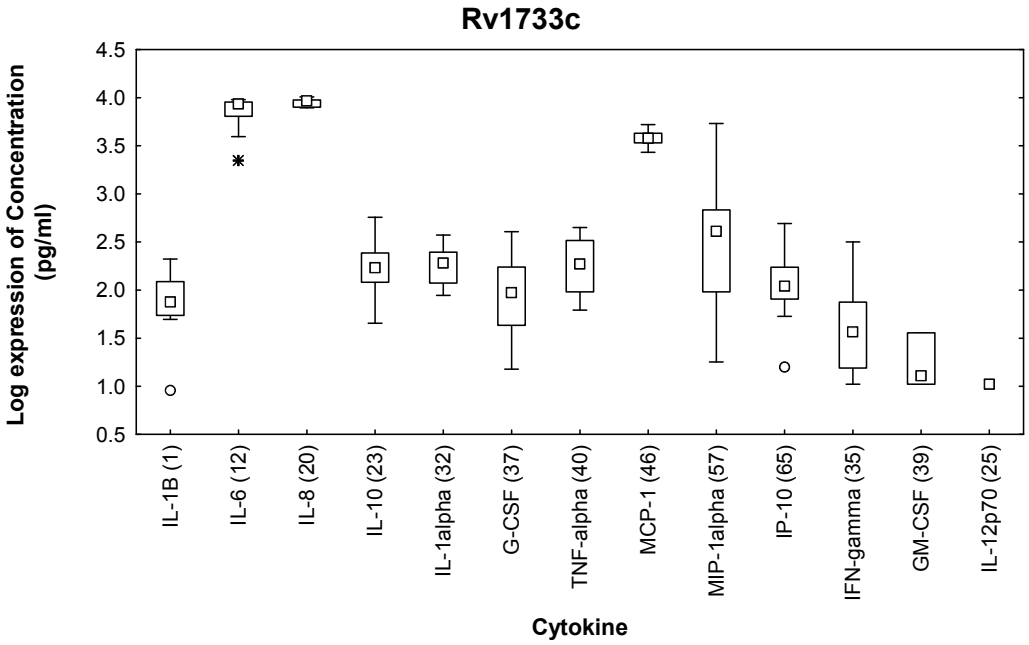


Figure 16: Box plot of antigen Rv1733c using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv 1131

Lower cytokine responses were produced to Rv1131 (figure 17). With the exception of IL-6, IL-8 and MCP-1 which produced responses between 500 – 5000pg/ml, most of the other cytokines trailed behind with low values in the range of 10 – 100pg/ml. Only one participant produced low levels of GM-CSF, IL-1B and G-CSF (not the same participant for each cytokine). Eight cytokines were not produced following stimulation with Rv1131.

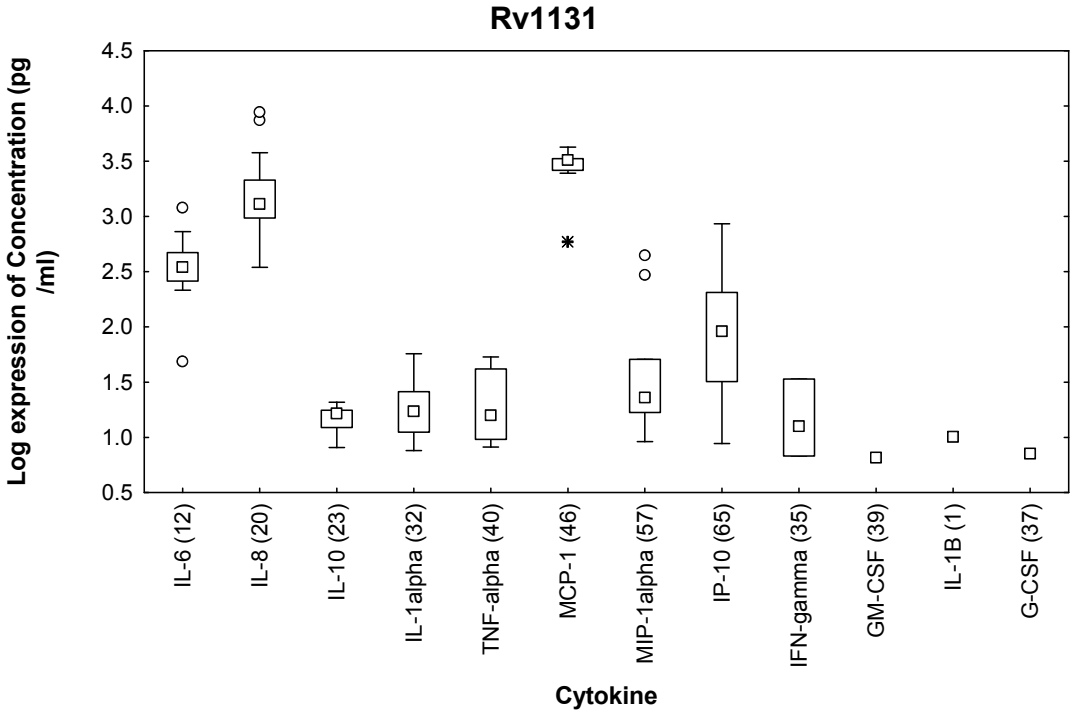


Figure 17: Box plot of antigen Rv1131 using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv0081

Of the 21 cytokines in the assay, only 7 were produced after stimulation with Rv0081 (figure 18). IL-1α, TNF- α, MIP-1α, IL-6 and IL-8 were produced in low levels, in the range of 10 – 70pg/ml. Higher quantities of IL-8 and MCP-1 (about 500p/ml) were produced.

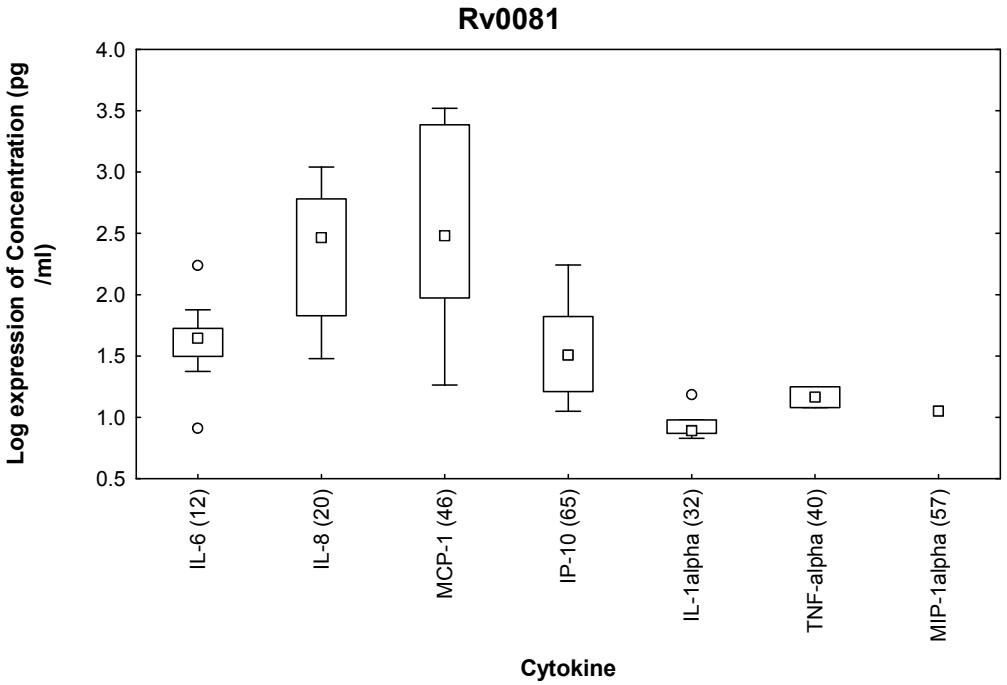


Figure 18: Box plot of antigen Rv0081 using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv 1737c

The median cytokine levels to Rv1737 were mostly in the low to moderate range of 25pg/ml (GM-CSF) to 300pg/ml (IP-10). Again, IL-6, IL-8 and MCP-1 were generated in high levels, above 5000pg/ml (figure 19).

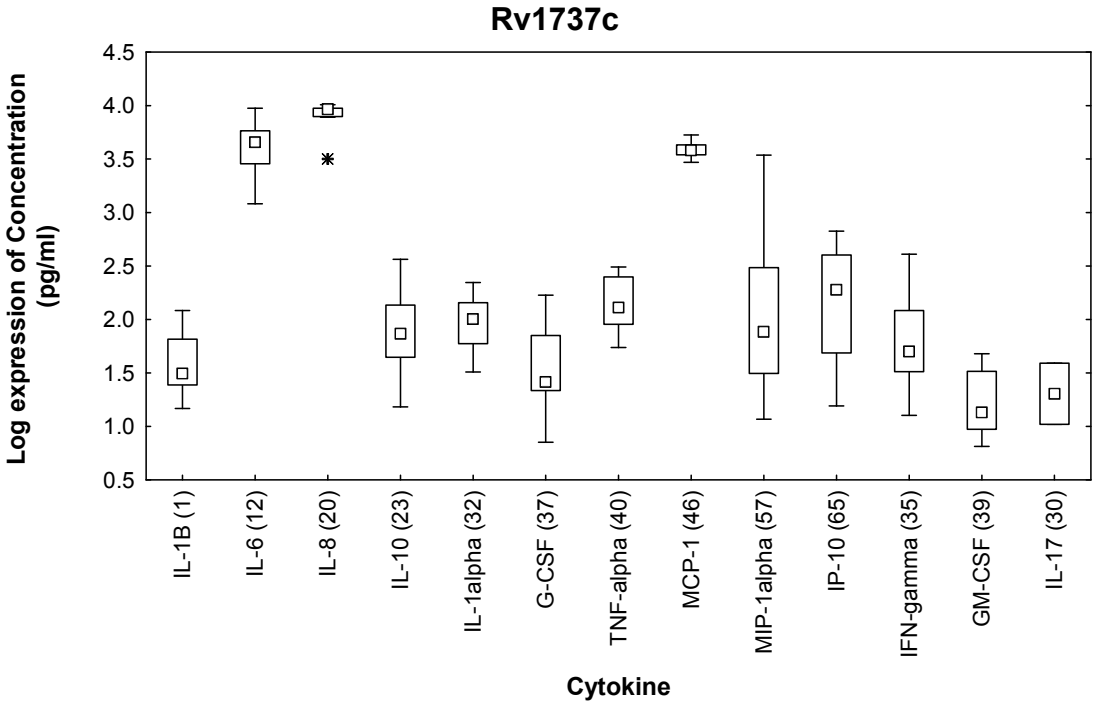


Figure 19: Box plot of antigen Rv1737c using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv1735c

Most cytokines expressed at low to moderate levels after stimulation with Rv1735c (figure 20). Concentrations were in the range of 20 – 80pg/ml for nine of the thirteen cytokines expressed, and IP-10 showed more moderate-to-high level expression at about 200pg/ml. High level cytokine expression was observed for IL-6, IL-8 and MCP-1 (about 4000 – 9000pg/ml).

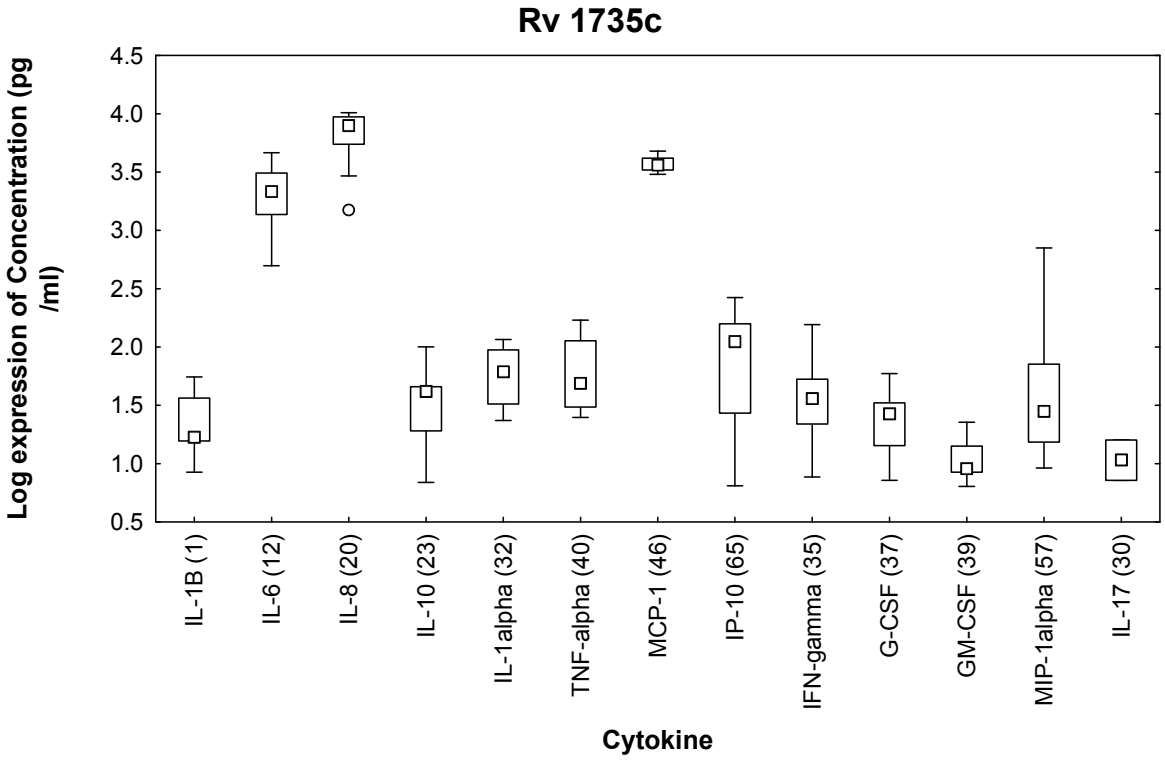


Figure 20: Box plot of antigen Rv1735c using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv0569c

IL-1B, IL-10, IFN-γ, GM-CSF, MIP-1α and G-CSF were produced at low levels of between 10 – 70pg/ml (figure 21). IL-1α and TNF-α were expressed at moderately high levels (at or slightly above 100pg/ml) and the high-level expressors were IL-6, IL-8 and MCP-1 (above 5000pg/ml). Eotaxin, IL-17 and IL-4 performed poorly, each showing a weak reaction in only one participant.

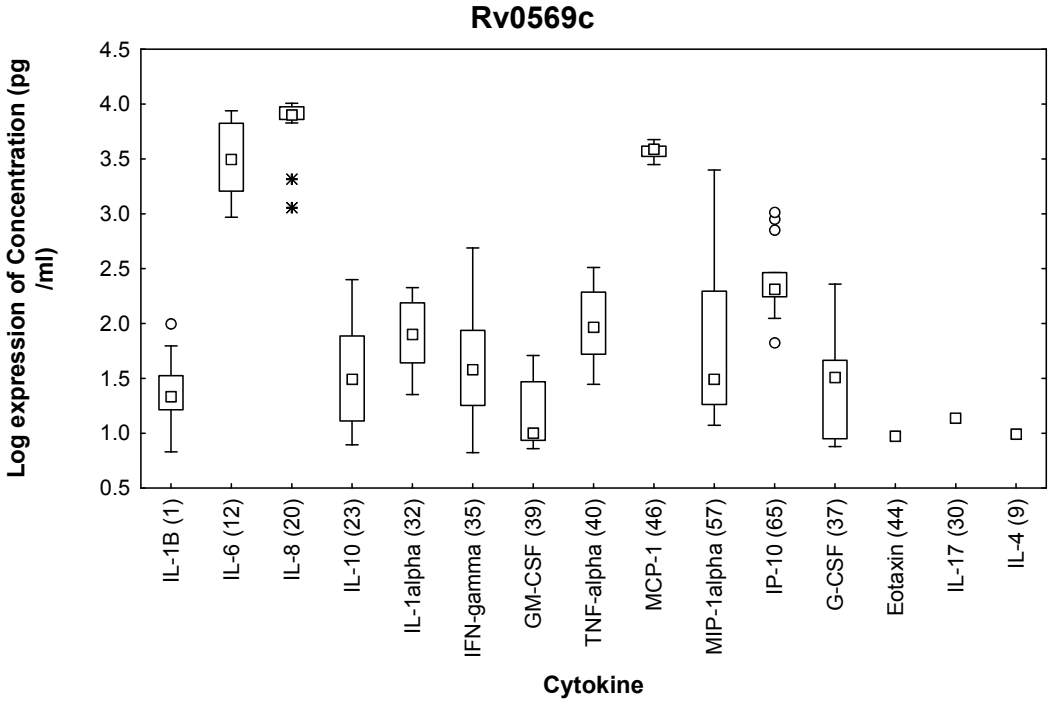


Figure 21: Box plot of antigen Rv0569c using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

Rv 2029c

Low-level expression cytokines for Rv2029c were IL-1B, IL-10, IL-1α, GM-CSF, IL-17, MIP-1α and G-CSF, in the range of 20 – 100pg/ml (figure 22). Moderately high levels of IFN-γ and IP-10 (400 – 600pg/ml) and high to very high levels of IL-6, IL-8 and MCP-1 (2000 – 8000pg/ml) were recorded.

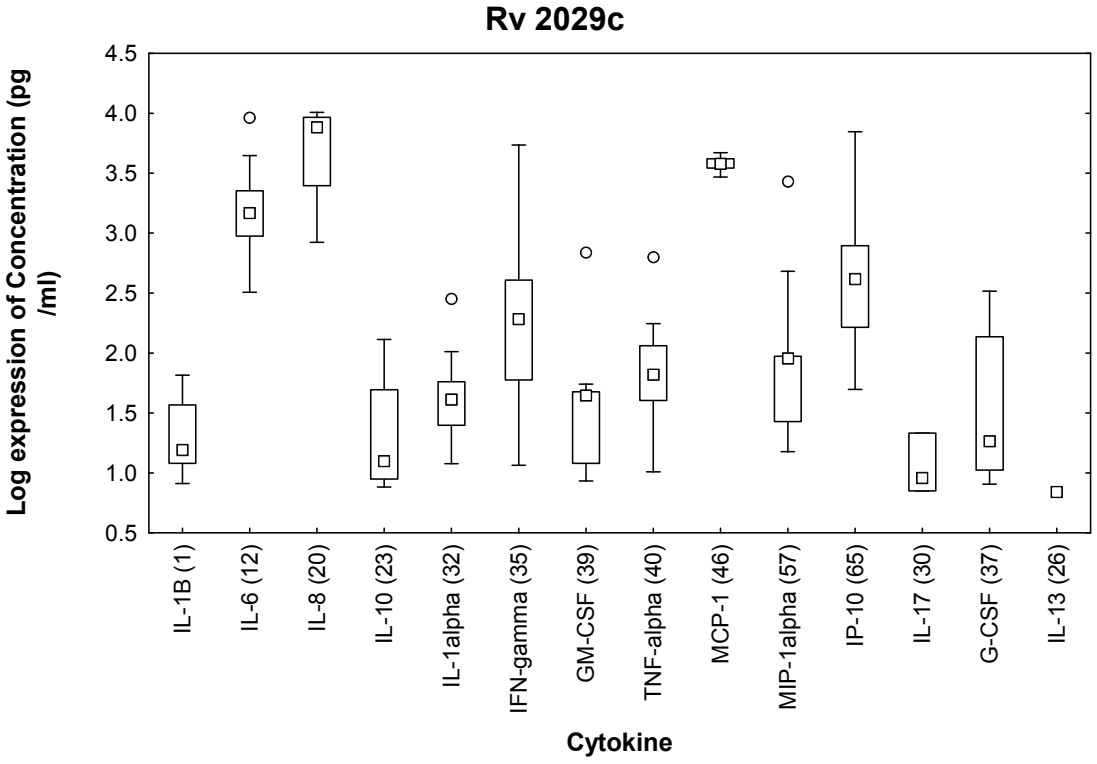


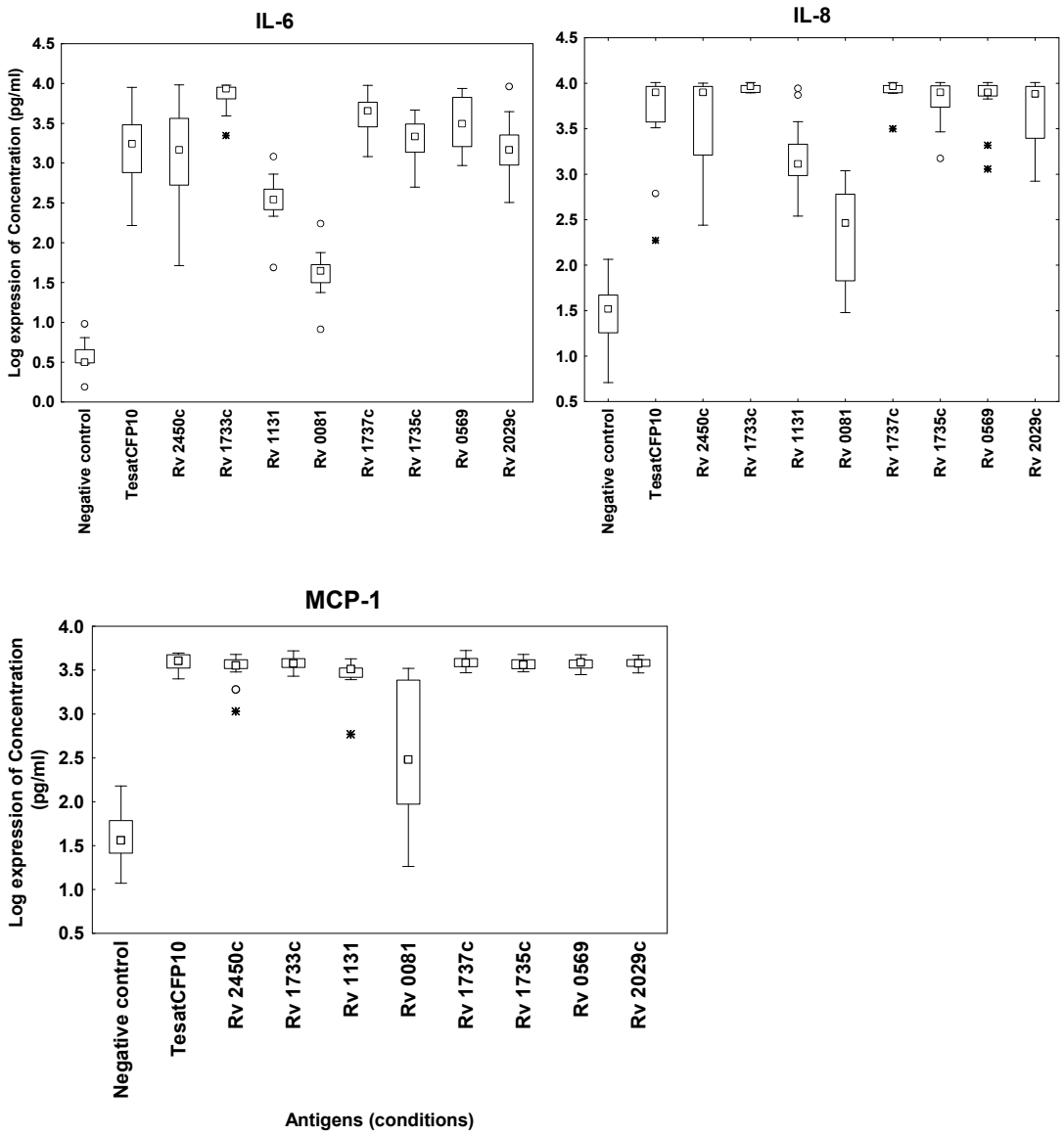
Figure 22: Box plot of antigen Rv2029c using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts. The upper and lower limits of the box denote the 25 – 75percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

3.3.3 Evaluation of the Lincoplex Assay by Cytokine

For ease of evaluation, the performance of each cytokine after stimulation with the ten conditions was grouped according to their levels of expression. As shown on the previous graphs, the upper and lower limits of the box denote the 25 – 75 percentile and the whisker lines denote the non-outlier range. Open squares denote the medians, and open circles and asterisks indicate outliers and extremes.

High to Very High-Level Expression Cytokines

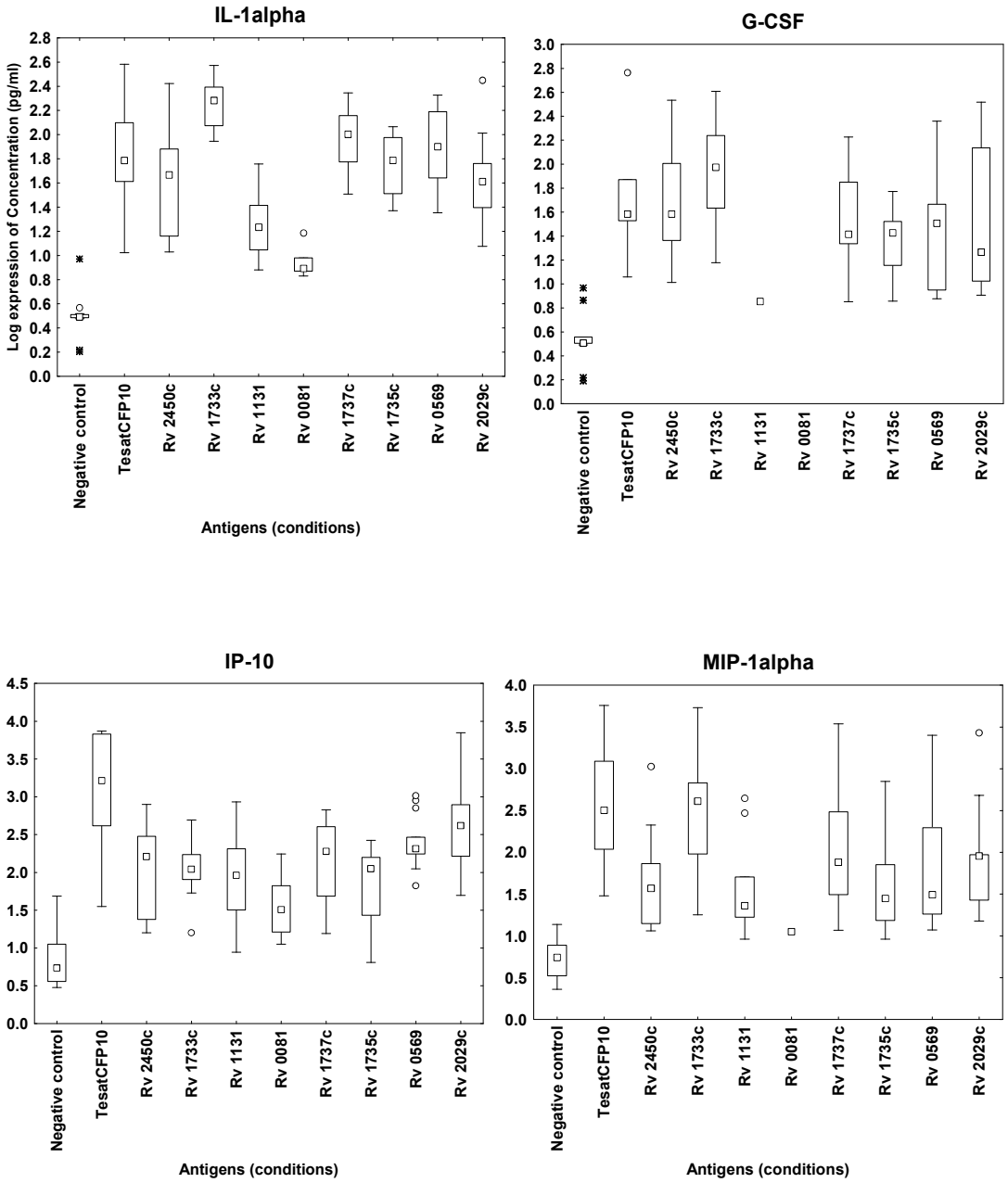
Figures 23, 24 and 25 show the 3 cytokines, IL-6, IL-8 and MCP-1, that consistently expressed at the highest levels after stimulation with the nine antigen conditions (excluding the negative control). TesatCFP-10, the positive control, induced high cytokine levels (about 3000- 9000pg/ml) of all three cytokines. Antigen Rv0081 overall induced the lowest cytokine levels, with median values for all 3 cytokines ranging from 50 – 500pg/ml, followed by Rv1131 which was lower in IL-6 (median of 500pg/ml) and IL-8 (median of 1000pg/ml). The other 7 antigens produced similar cytokine levels, producing median readouts of about 3000pg/ml for IL-6 and above 5000pg/ml for both IL-8 and MCP-1.



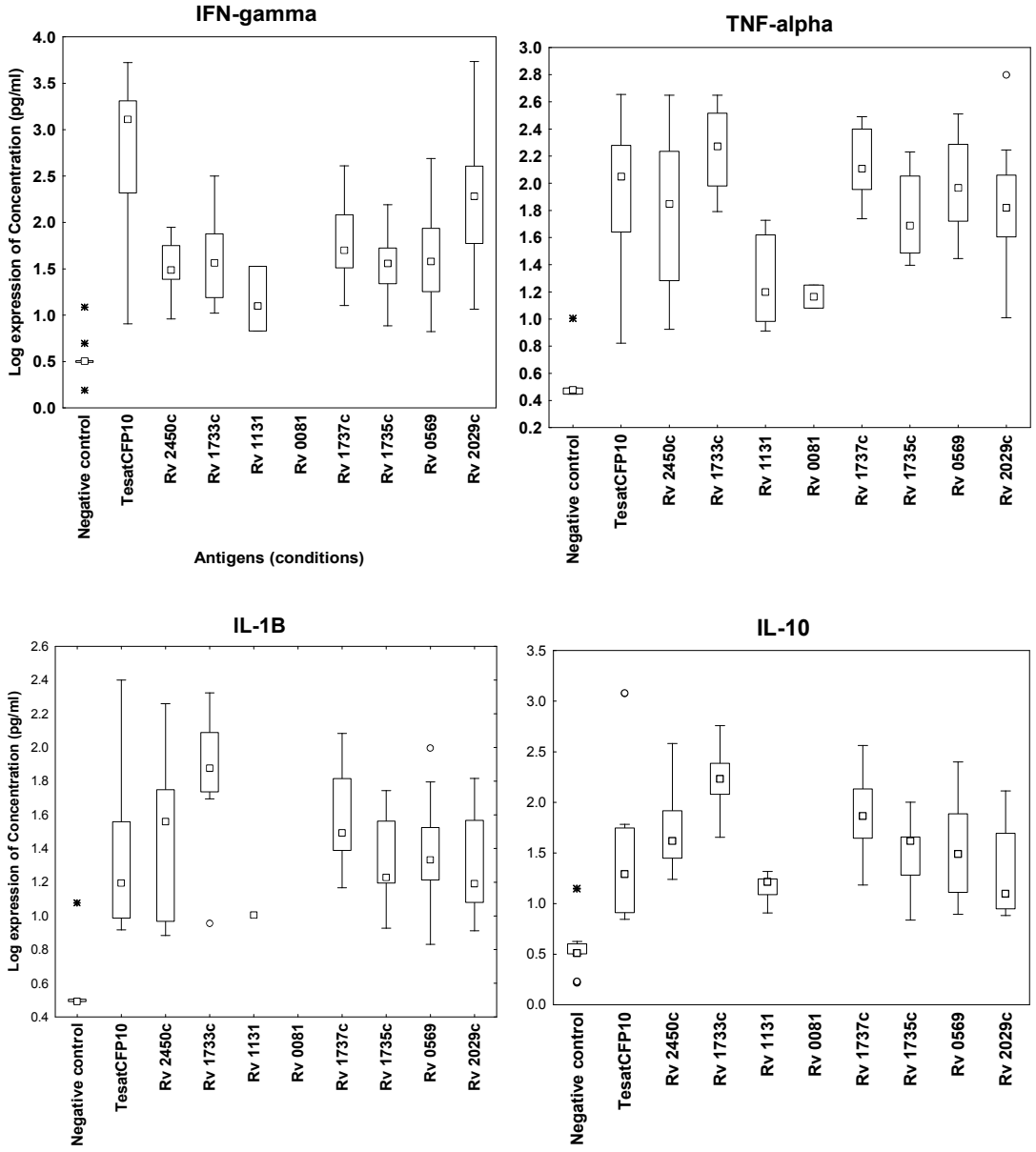
Figures 23 - 25: Box plots of IL-6, IL-8 and MCP-1 expression levels using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST-positive, HIV-negative Household contacts.

Moderately High-Level Expression Cytokines

Figures 26 – 33 show the 8 cytokines (IL-1α, G-CSF, IP-10, MIP-1α, INF-γ, TNF-α, IL-1B and IL-10) that were induced at moderate levels after stimulation with the nine antigen conditions. TesatCFP-10 positively induced cytokine expression of all the 8 cytokines, at lower levels for IL-1α, G-CSF, TNF-α, IL-1B and IL-10 with median values of 20 – 100pg/ml, and at higher levels for IP-10, MIP-1α and INF-γ with median values of 500 – 3000pg/ml. Rv1733c induced, on average, higher cytokine levels than the other 7 non-control antigens, its induction level of IL-1α, MIP-1α, TNF-α, IL-1B and IL-10 being significantly higher than levels of the same cytokines for the other antigens. Rv2450c, Rv1737c, Rv1735c, Rv0569 and Rv2029c stimulated similar patterns of cytokine production, with median value ranges of 30 – 200pg/ml for each cytokine. Again, Rv0081 and Rv2450 were low cytokine stimulators, showing little or no induction of cytokine expression across the board.



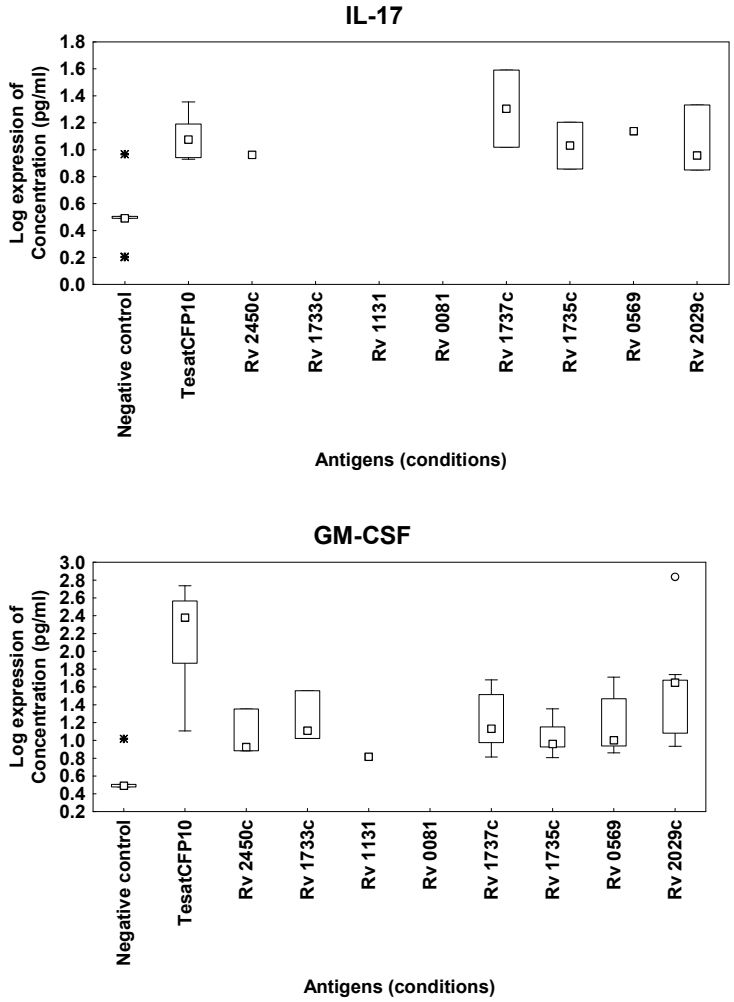
Figures 26 - 29: Box plots of IL-1α, G-CSF, IP-10 and MIP-1α expression levels using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST-positive, HIV-negative Household contacts.



Figures 30 - 33: Box plots of INF-γ, TNF-α, IL-1B and IL-10 expression levels using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST-positive, HIV-negative Household contacts.

Low-Level Expression Cytokines

Figures 34 and 35 are of IL-17 and GM-CSF, the two analytes that were expressed at very low levels, or not at all for some antigens, during the experiment. With the notable exception of TesatCFP-10 which induced relatively high levels of GM-CSF, median values for both cytokines trailed in the 10 – 40pg/ml range.



Figures 34 and 35: Box plots of IL-17 and GM-CSF expression levels using the LINCO 21-plex assay. 7-day WBA supernatants stimulated with ten "antigen" conditions were tested against an array of 21 cytokines. The sample participant group (n=15) consisted of TST positive, HIV negative Household contacts.

No Cytokine Expression except for in TesatCFP-10

Following stimulation, IL-2, IL-4, IL-5, IL-13, IL-12p70 and eotaxin were either expressed at very low levels or not at all for any of the antigens except for TesatCFP-10 (data not shown here). The positive control itself was only able to induce sporadic expression in few or single participants at a time, with levels reaching only 20pg/ml.

No Cytokine Expression at all

Throughout the experiment, IL-7 and IL-15 expression was nil for all antigen conditions, including the negative and positive controls (data not shown here).

Comparison of the ELISA and Luminex methods

A final correlation between the two assays performed for the antigen screening (ELISA) and the multiplex cytokine assay (Luminex) was done where the assays were statistically compared. Using only the IFN-γ data, which was the shared readout value of both techniques, intra-class correlation (ICC) co-efficients were generated between the assays, displayed in Table 10 below. It was demonstrated that though there was only some agreement and consistency between how the different antigens fared within each technique, overall there was a strong positive correlation between the performance of the two assays.

Table 10: Intra-class correlation co-efficients between the two cytokine assays, Luminex and ELISA. Only results generated from IFN-γ expression was used for the correlation. The two-way ICC was calculated where all subjects were statistically rated by the same judges.

	Rater 1	Rater 2	ICC Agreement	ICC Consistency
TesatCFP10	Luminex	ELISA	0.56	0.64
Rv2450	Luminex	ELISA	0.25	0.41
Rv1733c	Luminex	ELISA	0.4	0.58
Rv1131	Luminex	ELISA	0.24	0.36
Rv0081	Luminex	ELISA	-6.26	-6.59
Rv1737c	Luminex	ELISA	0.45	0.66
Rv1735c	Luminex	ELISA	0.34	0.53
Rv0569	Luminex	ELISA	0.55	0.67
Rv2029c	Luminex	ELISA	0.95	0.97
Overall	Luminex	ELISA	0.75	0.77

3.3.4 Factor Analysis, Eigenvalues and Factor Loadings

A factor analysis is a statistical undertaking that is performed when a large amount of data, or set of variables, has been analysed, and endeavours to explain the data in a competent, and this case biologically relevant, manner. The purpose of the analysis is to put the variables into groups called factors, and ascertain if how they interact within these factors can explain a significant portion of the data set. Factor loadings describe how the original variables, cytokines in this case, load or correlate to the ascribed factors. The STATISTICA software programme was used here to arrive at a factor analysis for the Luminex data.

Scree Plot

A scree plot is drawn to first reduce the many variables into a few factor categories to examine their combined effect within that factor category, and then examine how many factors affect the whole data set. The scree plot therefore takes into account how many of the factors, as a composite, reliably explains most of the data. The first factor, or eigenvalue, has the most weight and therefore should account for most of the relevance of the experiment. All subsequent factors/eigenvalues have decreasing significance and explain less of the data, with the last value having the least impact. The size of the eigenvalue means how much variance in the data is explained by a specific factor/eigenvalue, and the first factor usually has the largest size, followed by the second factor, etc. Figure 36 below shows the scree plot that was constructed for the Luminex analysis, and Tables 11 and 12 show how many factors were considered to explain the greatest portion of the data, and the factor loadings.

3.5.4.1 Results of the Factor Analysis

It was shown that the first 4 factors accounted for most of the data (72%), and the rest of the eigenvalues thereafter had low or negligible significance. Taken alone, factor 1 (which loaded 7 cytokines) accounted for about 35% of the variance, factor 2 (8 cytokines) for 20%, factor 3 (3 cytokines) for 11% and factor 4 (2 cytokines) for 6%.

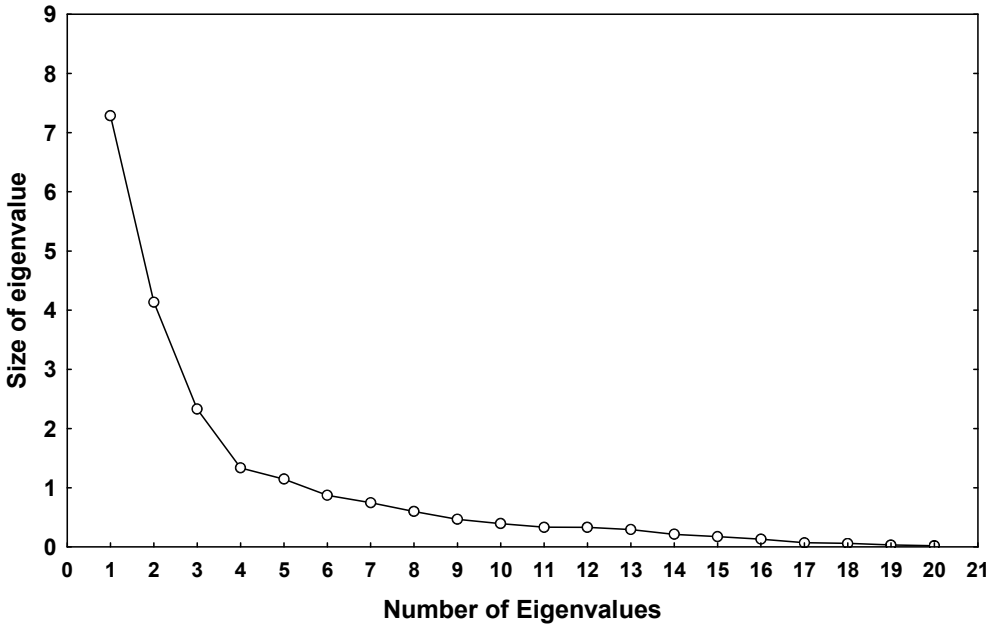


Figure 36: Scree Plot of Eigenvalues for the Luminex study. The total number of eigenvalues considered relevant for this study is shown on the x-axis, and the size of eigenvalues on the y-axis shows how much variance is explained by each factor. STATISTICA™ was used to construct this graph.

Table 11: Factor analysis for the Luminex study. The 21 cytokines loaded on the first 4 factors, which were considered the most important as they explained a cumulative 71.85% of the data.

Factor	Size of Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative %
1	7.29	34.69	7.29	34.70
2	4.14	19.69	11.42	54.40
3	2.33	11.09	13.75	65.50
4	1.34	6.36	15.09	71.85

Table 12: Factor loadings for the Luminex assay. The factor loading was varimax normalized, and all marked loadings are > 0.600. Cytokines marked in red loaded on factor 1, yellow in factor 2, blue on factor 3 and green on factor 4. Marked in pink is one cytokine, IL-17, which did associate with a factor (0.508) but not as strongly as other cytokines.

Factor Loadings (Varimax normalized) (Marked loadings are >0.70)				
Variable (cytokine)	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**
IL-1B (1)	**0.899**	0.169	-0.170	0.126
IL-2 (3)	0.022	**0.699**	0.327	0.083
IL-4 (9)	0.099	**0.720**	0.288	0.011
IL-5 (10)	-0.016	**0.868**	0.264	0.040
IL-6 (12)	**0.859**	0.142	-0.068	0.259
IL-7 (13)	0.317	**0.716**	-0.048	0.110
IL-8 (20)	0.506	-0.008	0.047	**0.751**
IL-10 (23)	**0.675**	0.004	0.221	0.030
IL-12p70 (25)	0.353	**0.734**	-0.254	-0.156
IL-13 (26)	-0.058	**0.661**	0.472	0.196
IL-15 (28)	-0.082	**0.743**	-0.107	-0.406
IL-17 (30)	-0.053	**0.508**	0.266	0.397
IL-1alpha (32)	**0.890**	0.066	0.177	0.249
IFN-gamma (35)	0.319	0.046	**0.843**	-0.047
G-CSF (37)	**0.885**	0.023	0.063	-0.001
GM-CSF (39)	0.181	0.300	**0.899**	0.103
TNF-alpha (40)	**0.835**	0.232	0.250	0.256
Eotaxin (44)	0.086	**0.623**	0.077	0.057
MCP-1 (46)	0.243	0.040	0.034	**0.815**
MIP-1alpha (57)	**0.618**	-0.009	0.313	0.018
IP-10 (65)	0.064	0.232	**0.830**	0.088

3.4 Discussion

Scientific research has greatly expanded our knowledge of the functions, complexity and synergy of cytokines in immune profiling, and great strides have also been taken with associated technology to keep pace with these advances. The Luminex multi-analyte profiling system (Lab-MAP) has proven to be of extensive use in the simultaneous detection of an array of cytokines present in body fluids like serum and supernatant, and the clinical applications of this technology are manifold. One of the first studies to validate this new generation of cytokine assay was conducted in 1998 by researchers from the Luminex Corporation, who set out to measure four cytokines in human cell culture supernatants and compare the assay to a standard ELISA (Oliver K.G et al., 1998). Subsequent studies also went on to give credence to bead-based multiplex assays, detecting a cocktail of cytokines in serum and PBMCs of mice and humans (Carson R.T and Vignali D.A, 1999; Kellar K.L et al., 2001; Prabhakar U. et al., 2002). This study reports on the Luminex bead assay being used to detect cytokines in antigen-stimulated human culture supernatants. The results show this assay produces results with a much wider scope than a conventional single-cyokine ELISA, and it also has high sensitivity and accuracy.

In this study, a pre-mixed Luminex kit for quantitating 21 human cytokines in culture supernatants was evaluated and essentially yielded very comparable results to a standard IFN-γ ELISA. The recoveries of each cytokine was found to be well within the acceptable range of 70 – 130%, and the quality control and spiking experiments deemed the assay fit for cytokine quantification. Although the spiking experiments of the quality

control did show that the addition of supernatant to the QC buffers did have a tendency to throw the observed value outside of the acceptable range, in general this did not indicate that the quality of supernatant would adversely affect the performance of most cytokines during the experiment.

The panel of 21 cytokines was varied in scope in terms of covering the range of analytes thought to play a role in human immune responses, and they were representative of non-specific, as well as Th1 and Th2-associated mediators. The result of the factor analysis showed that the tone of the response observed here was largely a Th1-biased one, based on the associated cytokines that explained 47% of the data (combination of cytokines loading on factor 1 and 3). IFN-γ, TNF-α, IP-10, MIP1-α and G-CSF were all Th1-biased cytokines inducers that expressed at encouraging levels after stimulation with the antigens, especially TesatCFP10. The induction of the pro-inflammatory cytokines IFN-γ and TNF-α gives credence to the view that the immune profile after mycobacterial infection is a primarily a cytotoxic T cell-mediated one generated in response to an intracellular pathogen, a response which stimulates the activation of macrophages, lymphocytes and NK cells (Flynn J.L and Chan J., 2001, Kaufmann S.H.E et al, 2006).

MIP1-α, IP-10 and G-CSF are also chemoattractants that play a role in the CMI response, by inducing directed chemotaxis in nearby responsive cells and attracting them to the site of infection. After exposure to *M. tuberculosis*, these chemokines are produced largely by T cells. They have the effect of increasing the production, differentiation and clonal expansion of phagocytic macrophages, NK cells and dendritic cells, all of which are

important in phagocytosis, bacterial killing and antigen presentation via the MHC I and II pathways. IL1-1α and IL-1B tend to be classified as non-specific or pleiotropic cytokines, as they are readily produced by and have effects on a variety of cells in the body, and their induction here could well be associated with that of the Th1 cytokines. IL-10 is an anti-inflammatory mediator that suppresses IFN-γ production and thus represses the pro-inflammatory response (Raja A., 2004). It is an important mediator in controlling what would otherwise be a hyperactive and ultimately destructive expression of Th1 cytokines like IL-12 and IFN-γ, and its detection here may suggest that its presence is necessary for the modulation of the protective response after mycobacterial infection.

The failure to see a strong induction of IL-2, IL-15 and IL-12p70, which loaded with other low expressors onto factor 2, could be explained by the fact that these cytokines, particularly IL-2, are early postexposure Th1 cytokines. IL-2 and IL-12p70 (like IL-12) are key players in the differentiation and proliferation of CD8 and CD4 T cells respectively, and IL-15 has similar functions to IL-2 (Ladel C.H et al., 1997; Blanchard D.K et al., 1989). They were not measured in generous quantities in this case because their production had probably peaked and then tailed off by the end of the 7-day WBA, rendering them practically undetectable. It is also likely that these cytokines were degraded over time by proteases in the supernatant, or that receptor-bound cytokine was spun out of the supernatant when protein debris was removed by centrifugation. A shorter time course experiment to measure when levels of these cytokines are optimal would most likely reveal they are present in appreciable amounts in the first 1 – 3 days of the assay.

Following antigen stimulation, IL-7, IL-4, IL-5, IL-13 and eotaxin were very poorly expressed across the board, being produced only in very low levels in a few cases following stimulation with TesatCFP10. IL-7 encourages the differentiation of naïve B and T progenitors to differentiate into mature immune cells, and its absence may be indicative that either it was not detected because it is an early-induced molecule like IL-2 or that it is not necessary as a regulator during infection with *M. tuberculosis*. IL-4, IL-5, IL-13 and eotaxin are well-defined Th2 regulators, and early production of IL-4 particularly favours the proliferation of B cells, mast cells and eosinophils, and subsequent antibody production. The clear failure to detect any contributing Th2 cytokine in this evaluation lends further support to the theory that the Th1 response predominates during mycobacterial infection and confers immune protection (Flynn J.L et al., 1993; Lin Y. et al., 1996; Cooper A.M et al., 1997), but sheds no light as to how the Th2 response can contribute protection against disease. The characterisation of this aspect of TB immunity requires further elucidation.

IL-6, IL-8 and MCP-1 showed a tendency to express at levels 2- to 3-log higher than the other cytokines. The pattern of expression of these three Th2 cytokines was somewhat unexpected and discordant from what was expected: surprising, in that these Th2 cytokines unfailingly expressed at remarkably high levels after antigen stimulation when a clear Th1 profile was anticipated, and discordant in that their magnitude of expression also did not correlate with the low levels of their other Th2-associated counterparts. Although IL-6 has been established as a Th2 cytokine closely linked to B cell activation

and antibody production, it also activates T cells and stimulates the production of acute phase proteins and IFN-γ, thus contributing to the inflammation that is characteristic of early infections (VanHeyningen T.K et al., 1997; Saunders B.M et al., 2000). It is therefore understandable to see this cytokine's expression linked to that of the Th1/factor 1 components. IL-8 and MCP-1 act as chemoattractants after infection, activating and mobilizing neutrophils, monocytes and lymphocytes; IL-8 prompting neutrophils and naïve T cells and MCP-1 guiding monocytes to leave the bloodstream and migrate to surrounding tissues. The action of the two cytokines is also usually accompanied by acute inflammation, which can occur during both Th1 and Th2-biased infections, which would explain why they were still measured in large quantities in the Luminex assay. The action of these two cytokines is also strongly complementary *in vivo*, and the findings here support their syngergistic effects (both cytokines loaded strongly on factor 4, which accounted for 6% of data variance). A high level of IL-6, IL-8 and MCP-1 in the supernatants is also indicative of the presence of bacterial endotoxins. Antigens produced in bacterial vectors, as the recombinant antigens tested here were, are known to contain appreciable quantities of endotoxins. Lipopolysaccharide (LPS), a cell wall component of other non-tuberculous bacteria like *Escherichia coli*, has been known to over-stimulate the production of a distinct group of cytokines and chemokines, of which IL-6, IL-8 and MCP-1 are a part (Zhang Y. et al., 1994; Christodoulides M. et al., 2002). Mycobacteria also contain a glycolipid called lipoarabinomannan (LAM), which is functionally similar to LPS. It is difficult to distinguish whether the high productionof these 3 immune factors here is specific to *M. tuberculosis* or indicates antigenic contamination with interfering bacterial-vector components.

This study has produced multiplex results for TB-specific antigens that correlate well with what was found in the antigen screening study, as well as what has been found when similar screening studies have been undertaken worldwide. It has been shown here that the fusion product of ESAT-6 and CFP10 (TesatCFP10), which has long been recognized as a classic TB antigen, is strongly immunogenic in people infected with latent tuberculosis and living in an area of high TB incidence. This finding corroborates those of other groups that also demonstrated that significant antibody responses to ESAT-6, CFP10 and the fusion protein are consistent with latent infection as well as active disease (Weldingh K. et al., 2005; Porsa et al., 2006; Hoff S.T et al., 2007). This antigen appears to be very important for use in new serodiagnostic assays and vaccines being developed against TB, as it is now generally recognized that latently infected individuals are strongly positive for ESAT-6 and CFP10 in both antibody- and CMI-based assays (Lalvani A. et al., 2001; Hoff S.T et al., 2007). Three other antigens that were well-recognised and induced significant cytokine levels were Rv1733c, Rv0569 and Rv2029c. These latency antigens have also been found to be frequently recognized as TB-specific in the sera and supernatant of latently and active infected TB patients (Leyten E.M.S et al., 2006; Lin M.Y et al., 2007).

The Luminex study has shown the multiplex assay to not only be highly comparable to the ELISA ‘gold standard’ method of cytokine detection, but also accurate and reproducible. This fluorescent bead-based technique has the obvious advantage of being able to detect multiple analytes in a single sample, and the aliquot size needed for a single

run can be up to an eighth of the volume required to run an ELISA sample in duplicate. One limiting feature of the multiplex assay is the mixing and quantification of multiple analytes in a single well. Cross-reactivity between receptors and non- or less-specific molecules can result in false positive readings, whilst interference from other blocking antibodies or contaminating proteins can result in false negatives (de Jager W. and Rijkers G.T, 2006). Optimisation of the assay can overcome these issues, and the increasing popularity of multiplex immunoassays marks a trend to integrate their use in more research and clinical diagnostic studies such as this one.

CHAPTER 4

General Discussion and Conclusion

The rate of infection with tuberculosis has reached pandemic proportins worldwide, and our current methods of diagnosing the disease are not highly specific or effective. One critical step to curbing the spread of TB would be the development of an effective method to diagnose latent tuberculosis in the early post-exposure phase. For over 100 years, TB infection has been detected using the tuberculin skin test, but issues of cross-reactivity with non-tuberculous mycobacteria, errors in administering the intradermal injection and anergy in immunosuppressed individuals have negatively affected the reliability of the test (Desem N. and Jones S.L, 1998). Many TB diagnostic assays also rely heavily on the production of IFN-γ to measure the level of response in affected people, but it has been established that other immune mediators play a significant role in characterizing the natural protective immune response to TB. There is therefore urgent need not only for other immune correlates of protection to be identified in humans, but also for tools and assays that would enable the immune profile to be well characterized to be developed and optimized.

This study has demonstrated that a novel panel of MTB-specific antigens screened in a population with a high incidence of latent TB infection could produce detectable cytokine responses, not just of IFN-γ but a host of other cytokines and chemokines. The antigen screening project was a relatively large-scale endeavour, and to my knowledge, covered the largest and widest range of MTB-specific antigens that has been screened in an African population. There were a few interesting aspects of the study that were not fully

addressed, such as the correlation of the responses with TST induration or gradient of exposure. The profile of the responses seen here would also be interesting after participants were followed up at certain time-points over a longer period, to see how many progressed to active disease and how many did not, and how this correlated with their . Nevertheless, the results generated here represent a useful and novel characterization profile of the immune response to tuberculosis. It has been shown that IFN-γ is an important correlate produced after exposure to TB, as are many other cytokines. It has also been shown that the Multiplex assay is a simple, sensitive and versatile method that could be valuable as a prognostic tool in outlining the course of infection with *M. tuberculosis*.

Reference List

(1) Adams LB, Dinauer MC, Morgenstern DE, Krahenbuhl JL. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. Tuber Lung Dis 1997; 78(5-6):237-246.

(2) Algood HM, Chan J, Flynn JL. Chemokines and tuberculosis. Cytokine Growth Factor Rev 2003; 14(6):467-477.

(3) Altare F, Durandy A, Lammas D, Emile JF, Lamhamedi S, Le DF et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science 1998; 280(5368):1432-1435.

(4) Andersen P. Vaccine strategies against latent tuberculosis infection. Trends Microbiol 2007; 15(1):7-13.

(5) Andersen P, Doherty TM, Pai M, Weldingh K. The prognosis of latent tuberculosis: can disease be predicted? Trends Mol Med 2007; 13(5):175-182.

(6) Armstrong JA, Hart PD. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J Exp Med 1971; 134(3 Pt 1):713-740.

(7) Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD et al. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. J Immunol 1999; 162(6):3504-3511.

(8) Beatty WL, Rhoades ER, Ullrich HJ, Chatterjee D, Heuser JE, Russell DG. Trafficking and release of mycobacterial lipids from infected macrophages. Traffic 2000; 1(3):235-247.

(9) Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol Microbiol 2002; 43(3):717-731.

(10) Blanchard DK, Michelini-Norris MB, Friedman H, Djeu JY. Lysis of mycobacteria-infected monocytes by IL-2-activated killer cells: role of LFA-1. Cell Immunol 1989; 119(2):402-411.

(11) Boom WH, Canaday DH, Fulton SA, Gehring AJ, Rojas RE, Torres M. Human immunity to M. tuberculosis: T cell subsets and antigen processing. Tuberculosis (Edinb) 2003; 83(1-3):98-106.

(12) Boshoff HI, Barry CE, III. Tuberculosis - metabolism and respiration in the absence of growth. Nat Rev Microbiol 2005; 3(1):70-80.

(13) Bosio CM, Gardner D, Elkins KL. Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology. J Immunol 2000; 164(12):6417-6425.

(14) Brandt L, Elhay M, Rosenkrands I, Lindblad EB, Andersen P. ESAT-6 subunit vaccination against Mycobacterium tuberculosis. Infect Immun 2000; 68(2):791-795.

(15) Brandt L, Skeiky YA, Alderson MR, Lobet Y, Dalemans W, Turner OC et al. The protective effect of the Mycobacterium bovis BCG vaccine is increased by coadministration with the Mycobacterium tuberculosis 72-kilodalton fusion polyprotein Mtb72F in M. tuberculosis-infected guinea pigs. Infect Immun 2004; 72(11):6622-6632.

(16) Brewer TF, Colditz GA. Relationship between bacille Calmette-Guerin (BCG) strains and the efficacy of BCG vaccine in the prevention of tuberculosis. Clin Infect Dis 1995; 20(1):126-135.

(17) Campos-Neto A, Ovendale P, Bement T, Koppi TA, Fanslow WC, Rossi MA et al. CD40 ligand is not essential for the development of cell-mediated immunity and resistance to Mycobacterium tuberculosis. J Immunol 1998; 160(5):2037-2041.

(18) Carson RT, Vignali DA. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J Immunol Methods 1999; 227(1-2):41-52.

(19) Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. J Immunol 1999; 162(9):5407-5416.

(20) Chan J, Fujiwara T, Brennan P, McNeil M, Turco SJ, Sibille JC et al. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci U S A 1989; 86(7):2453-2457.

(21) Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J Exp Med 1992; 175(4):1111-1122.

(22) Christodoulides M, Makepeace BL, Partridge KA, Kaur D, Fowler MI, Weller RO et al. Interaction of Neisseria meningitidis with human meningeal cells induces the secretion of a distinct group of chemotactic, proinflammatory, and growth-factor cytokines. Infect Immun 2002; 70(8):4035-4044.

(23) Cohen-Gonsaud M, Barthe P, Bagneris C, Henderson B, Ward J, Roumestand C et al. The structure of a resuscitation-promoting factor domain from

Mycobacterium tuberculosis shows homology to lysozymes. Nat Struct Mol Biol 2005; 12(3):270-273.

(24) Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998; 393(6685):537-544.

(25) Collins FM. Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome. Clin Microbiol Rev 1989; 2(4):360-377.

(26) Cooper AM, Magram J, Ferrante J, Orme IM. 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):39-45.

(27) Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science 1993; 259(5102):1739-1742.

(28) De Groot AS, McMurry J, Marcon L, Franco J, Rivera D, Kutzler M et al. Developing an epitope-driven tuberculosis (TB) vaccine. Vaccine 2005; 23(17-18):2121-2131.

(29) de JW, Rijkers GT. Solid-phase and bead-based cytokine immunoassay: a comparison. Methods 2006; 38(4):294-303.

(30) de JW, te VH, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003; 10(1):133-139.

(31) Demissie A, Leyten EM, Abebe M, Wassie L, Aseffa A, Abate G et al. Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with Mycobacterium tuberculosis. Clin Vaccine Immunol 2006; 13(2):179-186.

(32) Desem N, Jones SL. Development of a human gamma interferon enzyme immunoassay and comparison with tuberculin skin testing for detection of Mycobacterium tuberculosis infection. Clin Diagn Lab Immunol 1998; 5(4):531-536.

(33) Ding A, Nathan CF, Graycar J, Derynck R, Stuehr DJ, Srimal S. Macrophage deactivating factor and transforming growth factors-beta 1 -beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. J Immunol 1990; 145(3):940-944.

(34) Doherty TM, Andersen P. Vaccines for tuberculosis: novel concepts and recent progress. Clin Microbiol Rev 2005; 18(4):687-702.

(35) dupont NC, Wang K, Wadhwa PD, Culhane JF, Nelson EL. Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: determinations of a panel of nine cytokines in clinical sample culture supernatants. J Reprod Immunol 2005; 66(2):175-191.

(36) Dye C, Watt CJ, Bleed DM, Hosseini SM, Raviglione MC. Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. JAMA 2005; 293(22):2767-2775.

(37) Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995; 346(8986):1339-1345.

(38) Flesch I, Kaufmann SH. Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of Mycobacterium tuberculosis. J Immunol 1987; 138(12):4408-4413.

(39) Fletcher HA. Correlates of immune protection from tuberculosis. Curr Mol Med 2007; 7(3):319-325.

(40) Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001; 19:93-129.

(41) Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med 1993; 178(6):2249-2254.

(42) Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ et al. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity 1995; 2(6):561-572.

(43) Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B et al. Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med 2003; 197(1):7-17.

(44) Gong JH, Zhang M, Modlin RL, Linsley PS, Iyer D, Lin Y et al. Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. Infect Immun 1996; 64(3):913-918.

(45) Gonzalez-Juarrero M, Turner OC, Turner J, Marietta P, Brooks JV, Orme IM. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis. Infect Immun 2001; 69(3):1722-1728.

(46) Gordon AH, Hart PD, Young MR. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature 1980; 286(5768):79-80.

(47) Goren MB, D'Arcy HP, Young MR, Armstrong JA. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 1976; 73(7):2510-2514.

(48) Gupta UD, Katoch VM, McMurray DN. Current status of TB vaccines. Vaccine 2007; 25(19):3742-3751.

(49) Hampshire T, Soneji S, Bacon J, James BW, Hinds J, Laing K et al. Stationary phase gene expression of Mycobacterium tuberculosis following a progressive nutrient depletion: a model for persistent organisms? Tuberculosis (Edinb) 2004; 84(3-4):228-238.

(50) Hart PD, Armstrong JA, Brown CA, Draper P. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect Immun 1972; 5(5):803-807.

(51) Harth G, Horwitz MA. An inhibitor of exported Mycobacterium tuberculosis glutamine synthetase selectively blocks the growth of pathogenic mycobacteria in axenic culture and in human monocytes: extracellular proteins as potential novel drug targets. J Exp Med 1999; 189(9):1425-1436.

(52) Henderson RA, Watkins SC, Flynn JL. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. J Immunol 1997; 159(2):635-643.

(53) Hervas-Stubbs S, Majlessi L, Simsova M, Morova J, Rojas MJ, Nouze C et al. High frequency of CD4+ T cells specific for the TB10.4 protein correlates with protection against Mycobacterium tuberculosis infection. Infect Immun 2006; 74(6):3396-3407.

(54) Hingley-Wilson SM, Sambandamurthy VK, Jacobs WR, Jr. Survival perspectives from the world's most successful pathogen, Mycobacterium tuberculosis. Nat Immunol 2003; 4(10):949-955.

(55) Hoff ST, Abebe M, Ravn P, Range N, Malenganisho W, Rodriques DS et al. Evaluation of Mycobacterium tuberculosis--specific antibody responses in populations with different levels of exposure from Tanzania, Ethiopia, Brazil, and Denmark. Clin Infect Dis 2007; 45(5):575-582.

(56) Howard AD, Zwilling BS. Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines. Clin Exp Immunol 1999; 115(3):428-434.

(57) Huygen K, Content J, Denis O, Montgomery DL, Yawman AM, Deck RR et al. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nat Med 1996; 2(8):893-898.

(58) Irwin SM, Izzo AA, Dow SW, Skeiky YA, Reed SG, Alderson MR et al. Tracking antigen-specific CD8 T lymphocytes in the lungs of mice vaccinated with the Mtb72F polyprotein. Infect Immun 2005; 73(9):5809-5816.

(59) Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med 1998; 188(12):2199-2204.

(60) Kaufmann SH. Tuberculosis: back on the immunologists' agenda. Immunity 2006; 24(4):351-357.

(61) Kaufmann SH, Baumann S, Nasser EA. Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis. Int J Tuberc Lung Dis 2006; 10(10):1068-1079.

(62) Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ et al. Infection by Mycobacterium tuberculosis promotes human alveolar macrophage apoptosis. Infect Immun 1997; 65(1):298-304.

(63) Kellar KL, Kalwar RR, Dubois KA, Crouse D, Chafin WD, Kane BE. Multiplexed fluorescent bead-based immunoassays for quantitation of human cytokines in serum and culture supernatants. Cytometry 2001; 45(1):27-36.

(64) Kendall SL, Movahedzadeh F, Rison SC, Wernisch L, Parish T, Duncan K et al. The Mycobacterium tuberculosis dosRS two-component system is induced by multiple stresses. Tuberculosis (Edinb) 2004; 84(3-4):247-255.

(65) Ladel CH, Szalay G, Riedel D, Kaufmann SH. Interleukin-12 secretion by Mycobacterium tuberculosis-infected macrophages. Infect Immun 1997; 65(5):1936-1938.

(66) Lalvani A, Nagvenkar P, Udwadia Z, Pathan AA, Wilkinson KA, Shastri JS et al. 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):469-477.

(67) Langermans JA, Doherty TM, Vervenne RA, van der LT, Lyashchenko K, Greenwald R et al. Protection of macaques against Mycobacterium tuberculosis infection by a subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. Vaccine 2005; 23(21):2740-2750.

(68) Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE et al. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of Mycobacterium tuberculosis. Microbes Infect 2006; 8(8):2052-2060.

(69) Lichtenauer-Kaligis EG, de BT, Verreck FA, van VS, Hoeve MA, van d, V et al. Severe Mycobacterium bovis BCG infections in a large series of novel IL-12

receptor beta1 deficient patients and evidence for the existence of partial IL-12 receptor beta1 deficiency. Eur J Immunol 2003; 33(1):59-69.

(70) Lin MY, Geluk A, Smith SG, Stewart AL, Friggen AH, Franken KL et al. Lack of immune responses to Mycobacterium tuberculosis DosR regulon proteins following Mycobacterium bovis BCG vaccination. Infect Immun 2007; 75(7):3523-3530.

(71) Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominent Th2 cytokine response in human tuberculosis. Infect Immun 1996; 64(4):1351-1356.

(72) MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997; 15:323-350.

(73) MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci U S A 1997; 94(10):5243-5248.

(74) Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. J Bacteriol 1996; 178(5):1274-1282.

(75) Majlessi L, Simsova M, Jarvis Z, Brodin P, Rojas MJ, Bauche C et al. An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis. Infect Immun 2006; 74(4):2128-2137.

(76) McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med 2004; 10(11):1240-1244.

(77) Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB. A bacterial cytokine. Proc Natl Acad Sci U S A 1998; 95(15):8916-8921.

(78) Mukamolova GV, Turapov OA, Young DI, Kaprelyants AS, Kell DB, Young M. A family of autocrine growth factors in Mycobacterium tuberculosis. Mol Microbiol 2002; 46(3):623-635.

(79) Muller I. CSWHKSHE. Impaired resistance to Mycobacterium tuberculosis infection after selective *in vivo* depletion of L3T4+ and Lyt2+ T cells. Infect Immunol 1987; 55:2037-2041.

(80) Munoz-Elias EJ, McKinney JD. Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. Nat Med 2005; 11(6):638-644.

(81) Murray PJ, Wang L, Onufryk C, Tepper RI, Young RA. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. J Immunol 1997; 158(1):315-321.

(82) Mustafa AS. Recombinant and synthetic peptides to identify Mycobacterium tuberculosis antigens and epitopes of diagnostic and vaccine relevance. Tuberculosis (Edinb) 2005; 85(5-6):367-376.

(83) Mwandumba HC, Russell DG, Nyirenda MH, Anderson J, White SA, Molyneux ME et al. Mycobacterium tuberculosis resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. J Immunol 2004; 172(7):4592-4598.

(84) Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N Engl J Med 1996; 335(26):1941-1949.

(85) Nicholson S. B-AMSJRLNCXQ-WMRWJRCJGJBNLCRWHJL. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. J Exp Med 1996; 184:2293-2302.

(86) Oliver KG, Kettman JR, Fulton RJ. Multiplexed analysis of human cytokines by use of the FlowMetrix system. Clin Chem 1998; 44(9):2057-2060.

(87) Olsen AW, Williams A, Okkels LM, Hatch G, Andersen P. Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model. Infect Immun 2004; 72(10):6148-6150.

(88) Orne I. CF. Adoptive protection of the *Mycobacteria tuberculosis*-infected lung. Cell Immun 1984; 84:113-120.

(89) Porsa E, Cheng L, Seale MM, Delclos GL, Ma X, Reich R et al. Comparison of a new ESAT-6/CFP-10 peptide-based gamma interferon assay and a tuberculin skin test for tuberculosis screening in a moderate-risk population. Clin Vaccine Immunol 2006; 13(1):53-58.

(90) Prabhakar U, Lipshutz D, Bartus JO, Slivjak MJ, Smith EF, III, Lee JC et al. Characterization of cAMP-dependent inhibition of LPS-induced TNF alpha production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor. Int J Immunopharmacol 1994; 16(10):805-816.

(91) Radosevic K, Wieland CW, Rodriguez A, Weverling GJ, Mintardjo R, Gillissen G et al. Protective immune responses to a recombinant adenovirus type 35 tuberculosis vaccine in two mouse strains: CD4 and CD8 T-cell epitope mapping and role of gamma interferon. Infect Immun 2007; 75(8):4105-4115.

(92) Raja A. Immunology of tuberculosis. Indian J Med Res 2004; 120(4):213-232.

(93) Rojas M, Olivier M, Gros P, Barrera LF, Garcia LF. TNF-alpha and IL-10 modulate the induction of apoptosis by virulent Mycobacterium tuberculosis in murine macrophages. J Immunol 1999; 162(10):6122-6131.

(94) Rook GA. Macrophages and Mycobacterium tuberculosis: the key to pathogenesis. Immunol Ser 1994; 60:249-261.

(95) Rosenkrands I, Slayden RA, Crawford J, Aagaard C, Barry CE, III, Andersen P. Hypoxic response of Mycobacterium tuberculosis studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. J Bacteriol 2002; 184(13):3485-3491.

(96) Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL et al. Immunogenicity of eight dormancy regulon-encoded proteins of Mycobacterium tuberculosis in DNA-vaccinated and tuberculosis-infected mice. Infect Immun 2007; 75(2):941-949.

(97) Ruscetti F, Varesio L, Ochoa A, Ortaldo J. Pleiotropic effects of transforming growth factor-beta on cells of the immune system. Ann N Y Acad Sci 1993; 685:488-500.

(98) Russell DG, Mwandumba HC, Rhoades EE. Mycobacterium and the coat of many lipids. J Cell Biol 2002; 158(3):421-426.

(99) Samanich K, Belisle JT, Laal S. Homogeneity of antibody responses in tuberculosis patients. Infect Immun 2001; 69(7):4600-4609.

(100) Samanich KM, Keen MA, Vissa VD, Harder JD, Spencer JS, Belisle JT et al. Serodiagnostic potential of culture filtrate antigens of Mycobacterium tuberculosis. Clin Diagn Lab Immunol 2000; 7(4):662-668.

(101) Saunders BM, Frank AA, Orme IM, Cooper AM. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection. Infect Immun 2000; 68(6):3322-3326.

(102) Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J et al. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. J Exp Med 2000; 192(3):347-358.

(103) Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nat Med 2003; 9(8):1039-1046.

(104) Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM et al. Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. J Exp Med 2003; 198(5):693-704.

(105) Shiloh MU, Nathan CF. Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. Curr Opin Microbiol 2000; 3(1):35-42.

(106) Shleeva M, Mukamolova GV, Young M, Williams HD, Kaprelyants AS. Formation of 'non-culturable' cells of Mycobacterium smegmatis in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. Microbiology 2004; 150(Pt 6):1687-1697.

(107) Skeiky YA, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, Dillon DC et al. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol 2004; 172(12):7618-7628.

(108) Stenger S, Niazi KR, Modlin RL. Down-regulation of CD1 on antigen-presenting cells by infection with Mycobacterium tuberculosis. J Immunol 1998; 161(7):3582-3588.

(109) Styblo K. The impact of HIV infection on the global epidemiology of tuberculosis. Bull Int Union Tuberc Lung Dis 1991; 66(1):27-32.

(110) Tascon RE, Colston MJ, Ragno S, Stavropoulos E, Gregory D, Lowrie DB. Vaccination against tuberculosis by DNA injection. Nat Med 1996; 2(8):888-892.

(111) Timm J, Post FA, Bekker LG, Walther GB, Wainwright HC, Manganelli R et al. Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. Proc Natl Acad Sci U S A 2003; 100(24):14321-14326.

(112) Ting LM, Kim AC, Cattamanchi A, Ernst JD. Mycobacterium tuberculosis inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. J Immunol 1999; 163(7):3898-3906.

(113) Toossi Z, Gogate P, Shiratsuchi H, Young T, Ellner JJ. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. J Immunol 1995; 154(1):465-473.

(114) VanHeyningen TK, Collins HL, Russell DG. IL-6 produced by macrophages infected with Mycobacterium species suppresses T cell responses. J Immunol 1997; 158(1):330-337.

(115) Voskuil MI. Mycobacterium tuberculosis gene expression during environmental conditions associated with latency. Tuberculosis (Edinb) 2004; 84(3-4):138-143.

(116) Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR et al. Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. J Exp Med 2003; 198(5):705-713.

(117) Walker L, Lowrie DB. Killing of Mycobacterium microti by immunologically activated macrophages. Nature 1981; 293(5827):69-71.

(118) Walzl G, Beyers N, van HP. TB: a partnership for the benefit of research and community. Trans R Soc Trop Med Hyg 2005; 99 Suppl 1:S15-S19.

(119) Wang CH, Liu CY, Lin HC, Yu CT, Chung KF, Kuo HP. Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. Eur Respir J 1998; 11(4):809-815.

(120) Wang J, Xing Z. Tuberculosis vaccines: the past, present and future. Expert Rev Vaccines 2002; 1(3):341-354.

(121) Warner DF, Mizrahi V. Tuberculosis chemotherapy: the influence of bacillary stress and damage response pathways on drug efficacy. Clin Microbiol Rev 2006; 19(3):558-570.

(122) Wayne LG, Lin KY. Glyoxylate metabolism and adaptation of Mycobacterium tuberculosis to survival under anaerobic conditions. Infect Immun 1982; 37(3):1042-1049.

(123) Wayne LG, Sohaskey CD. Nonreplicating persistence of mycobacterium tuberculosis. Annu Rev Microbiol 2001; 55:139-163.

(124) Wayne LG, Sramek HA. Antigenic differences between extracts of actively replicating and synchronized resting cells of Mycobacterium tuberculosis. Infect Immun 1979; 24(2):363-370.

(125) Weldingh K, Rosenkrands I, Okkels LM, Doherty TM, Andersen P. Assessing the serodiagnostic potential of 35 Mycobacterium tuberculosis proteins and identification of four novel serological antigens. J Clin Microbiol 2005; 43(1):57-65.

(126) Yeremeev VV, Kondratieva TK, Rubakova EI, Petrovskaya SN, Kazarian KA, Telkov MV et al. Proteins of the Rpf family: immune cell reactivity and vaccination efficacy against tuberculosis in mice. Infect Immun 2003; 71(8):4789-4794.

(127) Zhang Y, Broser M, Rom WN. Activation of the interleukin 6 gene by Mycobacterium tuberculosis or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF-kappa B. Proc Natl Acad Sci U S A 1994; 91(6):2225-2229.