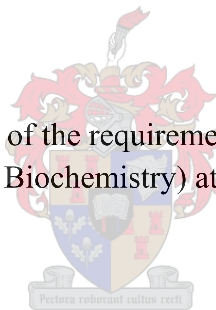
Distinct Immune Profiles of Recently Exposed Household Contacts in a Tuberculosis Endemic Setting in the Western Cape

Nokwanda Crystal Ngombane



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Declaration

I, the undersigned, hereby declare that the work contained in this thesis 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.

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Summary

Setting

This study was conducted in the Tygerberg district, in the Western Cape, South Africa.

Background

Tuberculosis is a major global health problem. Studies that follow individuals in close contact with active TB cases have provided a wealth of insight on TB transmission and on immune responses activated by *M. tuberculosis* infection, however, robust correlates of protection against TB are not known. The purpose of this study is to explore immunological profiles associated with TST conversion from negative to positive in household contacts of active TB cases in a high TB incidence area of the Western Cape.

Methods

Interferon gamma Elisa study

Whole blood of 17 of adult and adolescent TST negative recent contacts of smear positive active TB cases was stimulated with a panel of 22 *M.tb* specific antigens, which, were also used to stimulate whole blood of the same number of age and gender matched recruitment TST positive contacts and active TB cases. The 7-day culture supernatants were used to quantify and to compare IFN-γ production using the ELISA.

Luminex Assay study

Whole blood culture supernatants of 17 subjects that were initially TST negative, converting to TST positive by month 6, and that of 9 participants that had not converted 6 months into the study were used in the Luminex LINCO 27-plex assay. This was done to determine which

of the 27 cytokines may be associated with TST conversion after stimulation with 6 *M.tb* specific antigens at recruitment and 5 at month 6.

Results

Markedly different immune responses were observed between TST negative household contacts, TST positive household contacts and new active TB cases. IFN-γ production in response to *M.tb* PPD, Rv3019c, ESAT6/CFP10 fusion protein, TB10.4, Rv1737c, Rv1733c, Rv1735c, Rv2029c, Rv2450c, Rv0867c and Rv1009 antigens could discriminate between the not infected, *M.tb* infection and active TB.

This study showed that at study recruitment *M.tb* PPD induced high IP-10, IL-8, GM-CSF, IL-2, IL-5 and IL-13 levels in fast TST convertors and that when this group had become TST positive at month 6, all these cytokines were further enhanced as a result of this antigen. At month 6, ESAT6/CFP10 fusion protein produced significantly higher MCP-1, IP10 and IFN-γ concentrations in fast TST convertors only.

Dos-R antigens produced significantly higher VEGF, IL-12p70 and IL-10 at recruitment in slow convertors. At month 6, these antigens resulted in high IL-12p40 and IL-12p70levels in both fast and slow TST convertors. At this time point, Dos-R antigens produced high levels of MIP-1α, IL-1α and IL-1ra in fast convertors, whereas they resulted in decreased production of G-CSF, IL-10 and VEGF in slow TST convertors.

Conclusion

The capability of both Rv2029c and Rv2450c antigens to distinguish between infection and disease states and their positive association in the TST positive group only may be important in the identification of possible diagnostic or vaccine candidates. In TST negative contacts, *M.tb* PPD, Rv1737c or Rv2029c may be useful predictors of the rate of infection in TST

negative household contacts. The multiple cytokine profile studies may offer a valuable platform for understanding host-pathogen relations which could lead to the identification of potential vaccine or diagnostic candidates.

Opsomming

Instelling

Hierdie studie is uitgevoer in die Tygerberg-distrik, in die Wes-Kaap, Suid-Afrika.

Agtergrond

Tuberkulose is 'n groot globale gesondheid probleem. Studies wat volg individue in noue kontak met 'n aktiewe TB-gevalle het op voorwaarde dat' n rykdom van insig oor TB-oordrag en op immuun antwoorde geaktiveer word deur *M. tuberkulose*-infeksie, maar robuuste korrelate van beskerming teen TB is nie bekend nie. Die doel van hierdie studie is immunologiese profiele verband hou met TST sukses te verken van negatief na positief in die huishouding kontakte van aktiewe TB-gevalle in 'n hoë TB voorkoms gebied van die Wes-Kaap.

Metodes

Interferon gamma Elisa studie

Hele bloed van 17 van volwasse en adolessente TST negatiewe onlangse kontakte van die smeer positiewe aktiewe TB-gevalle was gestimuleer met 'n paneel van 22 TB spesifieke antigene wat ook gebruik word om te stimuleer hele bloed van dieselfde getal van ouderdom en geslag gekoppel werwing TST positiewe kontakte en aktiewe TB-gevalle. Die produksie metγ7-dag kultuur supernatants is gebruik om te kwantifiseer en IFN- behulp van die ELISA te vergelyk.

Luminex studie

Vol bloed kultuur supernatants van 17 vakke wat aanvanklik TST negatief is, herlei dit na die positiewe TST per maand 6 en dat van 9 deelnemers wat nie 6 maande omskep in die studie is gebruik in die Luminex LINCO 27-kompleks toets. Dit is gedoen om te bepaal watter van

die 27 cytokines kan geassosieer word met TST omskakeling na stimulasie met 6 TB spesifieke antigene op werwing en 5 by die maand 6.

Resultate

Opmerklik verskillende reaksies tussen hierdie drie studiegroepe is waargeneem. Elf antigene kan diskrimineer tussen die nie besmet is, *M.tb* infeksie en aktiewe TB insluitende *M.tb* PPD, Rv3019c, ESAT6/CFP10 fusie-proteïen, TB10.4, Rv1737c, Rv1733c, Rv1735c, Rv2029c, Rv2450c, Rv0867c en Rv1009.

Hierdie studie het getoon dat studie-werwing *M.tb* PPD veroorsaak hoë IP-10, IL-8, GM-CSF, IL-2, IL-5 en IL-13-vlakke in 'n vinnige TST omsetters en dat wanneer hierdie groep geword het TST positief maand 6, is verder al hierdie cytokines versterk as gevolg van hierdie antigeen. By die maand 6, ESAT6/CFP10 fusie-proteïen geproduseer aansienlik hoër MCP-1, IP10 en IFN- γ konsentrasies in 'n vinnige TST converters alleen. Dos-R antigene wat aansienlik hoër VEGF, IL-12p70 en IL-10 by werwing in stadige omsetters. By die maand 6, hierdie antigene het gelei tot hoë IL-12p40 en IL-12p70levels in beide 'n vinnige en stadige TST omsetters. In en IL-1ra α, IL-1 α hierdie tyd punt, Dos-R antigene wat hoë vlakke van MIP-1 in 'n vinnige converters, terwyl hulle gelei tot verminderde produksie van G-CSF, IL-10 en VEGF in slow TST omsetters.

Sluiting

Die vermoë van beide Rv2029c en Rv2450c antigene om te onderskei tussen infeksie en siekte state en hul positiewe assosiasie in die TST positiewe groep slegs van belang kan wees in die identifisering van moontlike diagnostiese of entstof kandidate. In TST negatiewe kontakte, kan M.tbPPD, Rv1737c of Rv2029c nuttig wees voorspellers van die tempo van infeksie in TST negatiewe huishoudelike kontakte. Die meervoudige cytokine profiel studies

kan waardevolle platform aanbod vir die begrip van die gasheer-patogeen Aangeleenthede wat kan lei tot die identifisering van potensiële entstof of diagnostiese kandidate.

TABLE OF CONTENTS

Declaration ii

Summary iii

Opsomming vi

Table of Contents ix

List of Abbreviations xvii

Acknowledgements xx

1. CHAPTER 1: INTRODUCTION 1

1.1. The epidemiology of tuberculosis (TB) 1

1.2.TB prevention 2

1.3.TB Detection diagnosis and treatment 3

1.3.1. Detection 3

1.3.2. Diagnosing TB disease 3

1.3.3. TB treatment 6

1.4.Diagnosing Latent TB infection (LTBI) 7

1.5.Latent TB infection in household contacts of active TB cases 10

1.6. The immune system 10

1.6.1. The innate immune system 10

1.7.The adaptive immune response 12

1.8.The host immune response to *M.tb* 14

1.9.The *M.tb* antigens – proteomics 14

1.10. Cytokine bio-signatures – Luminex technology 15

1.11. The current study 16

2. CHAPTER 2: INTERFERON GAMMA RESPONSES TO NOVEL *M.TB* ANTIGENS IN ACTIVE TB CASES AND TB CONTACTS WITH DIFFERENT SKIN REACTIONS 17

2.1. Introduction 17

2.2. Study Design and Methods 20

2.2.1. Setting 20

2.2.2. The tuberculin skin test (TST) 20

2.2.3. Study population 20

2.2.4. Blood Samples and HIV testing 25

2.2.5. Whole blood assay 25

2.2.6. Antigens 27

2.2.7. IFN-γ ELISA response cut-off 30

2.2.8. Statistics 32

2.2.9. Research Questions 32

2.3. Results 33

2.3.1. Comparison of the clinical and demographic information 33

2.3.2. Reproducibility control 37

2.3.3. Standardization and normalization of the IFN-γ ELISA results 37

2.3.4. 2.3.4 Responses to negative and positive controls 40

2.3.5. Comparing response frequencies to classical *M.tb* antigens between study groups at recruitment 40

2.3.6. Comparison of responses to DosR regulon encoded antigens within and between study groups at recruitment 46

2.3.7. Comparison of responses to resuscitation promotion factors between study groups TST negative household contacts 49

2.3.8. Comparison of responses to reactivation antigens 51

2.3.9. Associations between IFN-γ responses to classical and novel *M.tb* antigens 51

2.4. Discussion 59

3. CHAPTER 3: MULTI-PLEX CYTOKINE ANALYSIS IN ADULT HIV NEGATIVE FAST AND SLOW TST CONVERTORS

3.1. Introduction 70

3.2. Materials And Methods 72

3.2.1. Study population 72

3.2.2. The Luminex Assay 75

3.2.3. Results of Kit Validation and Quality Control (QC) 80

3.2.4. Statistical Analysis 82

3.3. Research Questions 83

3.4. Results 83

3.4.1. Magnitude of response in fast versus slow TST convertors at recruitment 83

3.4.1.1. Negative control at recruitment 83

3.4.1.2.Fast versus slow TST convertors at recruitment 84

3.4.2. Magnitude of response in fast and slow TST convertors over time 89

3.4.2.1.Negative control at 6 months 89

3.4.2.2. Cytokines that change over time in fast convertors only 91

3.4.2.3. Cytokines that change over time in slow convertors only 97

3.4.2.4. Cytokines that change over time in both fast and slow convertors 97

3.4.3. Percent responders: fast TST convertors versus slow TST convertors 98

3.4.3.1.Classical TB antigens: *M.tb* PPD 98

3.4.3.2.Classical TB antigens: ESAT6/CFP10 Fusion Protein 102

3.4.3.3.DosR regulon encoded antigens: Rv1733c 102

3.4.3.4.DosR regulon encoded antigens: Rv1737c 104

3.4.3.5.DosR regulon encoded antigens: Rv2029c 104

3.4.3.6.Resuscitation promotion factors: Rv2450c 107

3.4.4. Correlations between cytokines 109

3.4.4.1.Correlations between cytokines in fast TST convertors at recruitment 109

3.4.4.2.Correlations between cytokines in slow TST convertors at recruitment 110

3.4.4.3.Correlations between cytokines in fast TST convertors at month 6 110

3.4.4.4.Correlations between cytokines in slow TST convertors at month 6 111

3.4.5. Comparison of the ELISA and Luminex methods 111

3.5. Discussion 113

4. CHAPTER 4: MAIN FINDINGS AND CONCLUSIONS 118

REFERENCE LIST 124

LIST OF FIGURES

Figure 2.1 TST status at recruitment, 6 months and 18 months in study participants enrolled into the GC6 household contact study 23

Figure 2.2 Acid-fast bacilli sputum smear grade of TB index cases in contact with TST negative and TST positive study participants 36

Figure 2.3 Interferon gamma level in internal positive controls 1 and 2, per plate 39

Figure 2.4 Negative control and positive controls used in the interferon γ ELISA 42

Figure 2.5 Interferon gamma responses to classical *M.tb* antigens 44

Figure 2.6 Comparison of IFN-γ responses to DosR regulon-encoded latency antigens in TST negative household contacts, TST positive household contacts and TB cases 45

Figure 2.7 Comparison of TST negative subjects, TST positive subjects and TB index cases in response to stimulation with resuscitation promotion factors 48

Figure 3.1 Luminex Assay Quality control assessment _QCI and QCII 79

Figure 3.2: Cytokine profile in supernatant from unstimulated 7-day whole blood at study recruitment 81

Figure 3.3 Cytokine responses to *M.tb* PPD at recruitment: fast versus slow TST convertors 85

Figure 3.4 Cytokine responses to *M.tb* derived antigens at recruitment: fast versus slow TST convertors 86

Figure 3.5 IL-10 responses to Rv2029c at recruitment: fast versus slow TST convertors 87

Figure 3.6 Cytokine profile in supernatant from unstimulated 7-day whole blood at month 6 88

Figure 3.7 Cytokines that increase over time in response to classical *M.tb* antigens only and in fast convertors only 90

Figure 3.8 Cytokines that increase over time in response to DosR Regulon-encoded antigens only and in fast convertors only 92

Figure 3.9 Cytokines that increase over time in response to classical *M.tb* antigens and DosR regulon-encoded antigens in fast convertors only 93

Figure 3.10 Cytokines that decrease over time in response to DosR Regulon-encoded antigens only and in slow convertors only 95

Figure 3.11 Cytokines that increase over time in response to DosR Regulon-encoded antigens only in fast and slow convertors 96

Figure 3.12 Cytokines that change over time in response to classical or DosR Regulon- encoded *M.tb*antigens in fast and slow convertors 97

LIST OF TABLES

Table 2.1 GC6-74 Clinical Case Definition For TB diagnosis 24

Table 2.2 Clinical and demographic information of study population 26

Table 2.3.1 Classical *M.tb* antigens 28

Table 2.3.2 Dos-R regulon antigens 29

Table 2.3.3 Resuscitation Promotion Factors 30

Table 2.3.4 Reactivation Antigens 30

Table 2.4 TB Contact Score of Household Contacts To TB Index Cases 34

Table 2.5: Correlations between *M.tb* antigens in TST negative contacts 50

Table 2.6: Correlation between the clinical data and *M.tb* antigens in TST positive contacts 53

Table 2.7: Correlation between the *M.tb* antigens in TST positive contacts 56

Table 2.8: Correlations between demographic data and *M.tb* antigens in TB cases 57

Table 2.9: Correlations between *M.tb* antigens in TB cases 57

Table 3.1 Clinical and demographic data of the study population 73

Table 3.2.1 Cytokines and chemokines included in the 27-plex Luminex kit and secreted by cells of the innate immune system 74

Table 3.2.2 Cytokines and chemokines included in the 27-plex Luminex kit and secreted by cells of both the innate and the adaptive immune systems 77

Table 3.2.3 Cytokines and chemokines included in the 27-plex Luminex kit and
secreted by cells of the adaptive immune system 78

Table 3.3 Cut-off point per cytokine/chemokine in the 27-plex Luminex kit 99

Table 3.4.A. Percentage of responders to classical TB antigen *M.tb* PPD
at recruitment and at month 6 100

Table 3.4.B. Percentage of responders to classical *M.tb*antigen ESAT6/CFP10
fusion protein at recruitment and at month 6 101

Table 3.4.C. Percentage of responders to Dos-R regulon encoded antigen Rv1733c
at recruitment and at month 6 103

Table 3.4.D. Percentage of responders to Dos-R regulon encoded antigen Rv1737c
at recruitment and at month 6 105

Table 3.4.E. Percentage of responders to Dos-R regulon encoded antigen Rv2029c
at recruitment and at month 6 106

Table 3.4.F. Percentage of responders to RPF antigen Rv2450c at recruitment only 108

Table 3.5 Correlation co-efficient between the ELISA and the Luminex Assay 112

Appendix 1.1 – 1.21: Table of cytokine correlations 139

LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
Ag85a/b	Antigen85 a/b of M. tuberculosis
APC	Antigen presenting cell
BCG	*Mycobacterium bovis* bacillus Calmette Guérin
CCL	Chemokine ligand
CCR	Chemokine receptor
CD#	"cluster of differentiation" nomenclature for cell surface molecules
CO_2	Carbon dioxide
CSF	Colony stimulating factor
DC	Dendritic cell
DosR	Dormancy survival regulator
DTH	Delayed Type Hypersensitivity Reaction
E.coli	*Escherichia coli*
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESAT6_CFP10	6 kDa early secretory antigenic target_culture filtrate protein 10 fusion protein of *M. tuberculosis*
FCS	Foetal calf serum
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIV	Human immunodeficiency virus
HHC	Household contact
HspX	Heat shock protein X
IFNγ	Interferon gamma
IGRA	Interferon gamma release assay
IL	Interleukin
IL	Interleukin-1 receptor antigen
IP10	Interferon inducible protein 10
kDa	kilodalton
LTBI	Latent tuberculosis infection

LUMC	Leiden University Medical Centre
MDR-TB	Multi-drug resistant tuberculosis
M.tb	*Mycobacterium tuberculosis*
MHC	Major histocompatibility complex
MCP	Monocyte chemotactic protein-1
MIP	Macrophage inflammatory protein
NO	Nitric oxide
NK	Natural killer cell
NRP	Non-Replicating Persistence
NTM	Non Tuberculous Mycobacteria
OD#	optical density, # indicates wavelength in nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemaglutinin from Phaseolus vulgaris
PPD	Purified protein derivative of *M. tuberculosis*
RD	Regions Of Difference
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RPF	Resuscitation promoting factor
RT	Room temperature
SEB	Staphylococcus enterotoxin B
sCD40L	Soluble CD 40 Ligand
TB	Tuberculosis
TGFα	Transforming growth factor alpha
Th1	T-helper cell type 1
Th17	T-helper cell type 17
Th2	T-helper cell type 2
TNFα	Tumour necrosis factor alpha
TLR	Toll like receptor

TST	Tuberculin skin test
UNAIDS	United Nations AIDS
VEGF	Vascular endothelial growth factor
WBA	Whole Blood Assay
WHO	World Health Organization
XDR	Extremely resistant drug TB

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"God is my helper; the Lord is the sustainer of my life" Ps54:4.

CHAPTER 1: INTRODUCTION

1.1. The epidemiology of tuberculosis (TB)

Mycobacterium tuberculosis *(M.tb)* is one of the oldest human pathogens known, and causes tuberculosis (TB), which continues to cause a major global health problem. In 2008 there were 9.4 million new TB cases detected, with 1.2 million reported fatalities across the world (WHO, 2009a). The incidence of TB in South Africa has continued to rise from 109 000 in 1990 to 461 000 in 2007 (Kritzinger *et al.,* 2009; Shanaube *et al.,* 2009; WHO, 2009a). There are many factors that drive the TB epidemic. Individuals who are infected with HIV are at greater risk of developing active TB (Narain *et al.,* 1992). South Africa has an estimated 5.7 million people infected with HIV (UNAIDS, 2008) and in 2008, 72% of TB cases were co-infected with HIV (WHO, 2009a). TB is also associated with several other serious illnesses including diabetes mellitus, renal failure and drug and/or alcohol abuse (American Thoracic Society, 2000). It has been observed that the risk of developing TB increases with decreasing social capital (van Rie *et al.,* 1999; Holtgrave & Crosby, 2004; Baker *et al.,* 2008; Harling *et al.,* 2008). It is believed that between 18% and 22% of South Africans are chronically poor (Aliber, 2003). South Africa takes 5th place in the 22 countries that carry 80% of the global burden of TB (WHO, 2009a). It is probably the country's prevailing socioeconomic conditions that have contributed to South Africa being in such an unenviable position.

The problematic global TB situation has remained unresolved because of various other limitations, which include high rates of latent TB infection, lack of understanding of the relationship between the mycobacteria and the host, emergence of drug resistant bacilli and poor adherence to treatment (Ruhwald & Ravn, 2009; Tsara *et al.,* 2009; Lönnroth *et al.,* 2010).

1.2 TB prevention

The structural challenges that are found in health systems have contributed to the continued scourge of TB. Also previous infection with *M.tb* is not protective. The currently used TB prevention methods, diagnostic tools as well as treatment regimens are old and inadequate (Lönnroth *et al.,* 2010). Koch unsuccessfully tried to develop a TB vaccine soon after discovering *M.tb* in 1881(Brock, 1988). Bacillus Calmette Guérin (BCG), an attenuated live *Mycobacterium bovis* vaccine that is used in several countries including South Africa was developed by Calmette and Guérin and was first used in humans in 1921 (Clarke & Rudd, 1992). Whereas BCG is capable of preventing disseminated forms of TB in infants and children (Rodrigues *et al*., 1993; Trunz *et al*., 2006) it has been found to be ineffective in adults (Fine, 1995). Guwatudde *et al*., (2003) have shown that BCG vaccination is associated with reduced risk of active TB among contacts. Knowledge about what constitutes an appropriate and effective immune response to *M.tb* is limited and there are presently no robust immune biomarkers of protection against TB. Lin and Ottenhoff (2008) argue that the current vaccine development initiatives have to overcome the challenges that are posed by pathogen strain heterogeneity and have to take account of the global host diversities. Several anti-TB vaccines have already undergone phase III clinical trials and others are in the pipeline. Recently, a promising BCG boosting recombinant novel vaccine that comprises Ag85A, Ag85B, and TB10.4 was tested on BCG vaccinated adults and has been found to be safe, immunogenic as well as capable of inducing polyfunctional CD4+ cells as well as enduring CD8+ cells (Abel *et al*., 2010). MVA85A, which expresses Ag85A, is another BCG boosting vaccine that has been shown to induce polyfunctional CD4+ cells in adolescents and children (Scriba *et al*., 2010). Whether this translates to protection is yet to be discovered when the efficacy studies have been done.

1.3 TB Detection, diagnosis and treatment

Early detection, diagnosis and effective treatment is central to the control of TB because it decreases transmission and the force of infection.

1.3.1 Detection

Several socio-economic barriers to early TB detection and treatment have been identified. These include alcohol and/or substance abuse, poverty, low access to health care facilities, old age, cultural beliefs and poor knowledge about TB (Storla *et al.,* 2008). Some argue that delays in TB detection could be reduced through active case finding (Sekandi *et al.,* 2009). Previously, it has been shown in one high incidence South African rural community that there were two undiagnosed TB cases for every nine cases of sputum positive pulmonary tuberculosis being treated at any one time (Pronyk *et al.,* 2001).

1.3.2 Diagnosing TB disease

The importance of timely and quality diagnostic results in TB control cannot be over-emphasized. Ineffective diagnostic tools as well as inadequate follow-up routines are central to delays in treatment initiation and once diagnosis was ascertained, treatment was promptly initiated (Storla *et al.,* 2008).

Clinical Signs and symptoms

At the point of patient care, the identification of presenting clinical symptoms such as fever, chronic cough, loss of appetite, night sweats, and malaise among others by the health personnel is an important first line of TB diagnosis (American Thoracic Society, 2000). However, symptoms alone are not sufficient to diagnose and treat TB as other illnesses including bacterial and viral upper and lower respiratory tract infections, mycoses,

paragonimiasis and non-infectious conditions such as chronic obstructive pulmonary disorder may also cause some of these symptoms (Rosen, 2006).

Chest X-ray

The lungs are the primary sites of *M.tb* infection (Monack *et al.,* 2004). Post-primary TB disease is characterized by the development of cavities in the lungs (Kritski & Fiuza de Melo, 2007). The presence of cavitary disease in TB cases is associated with bacillary burden as well as with the clinical characteristics of the index case (Guwatudde *et al.,* 2003). Other pulmonary abnormalities that may develop include infiltrations as well as calcification, which may be detected by a chest X-ray (American Thoracic Society, 2000). However, a study in the Gambia observed that chest examinations alone were not wholly reliable in the diagnosis of TB as approximately 1% of sputum smear positive TB cases were reported to have a normal chest X-ray (Rathman *et al.,* 2004).

Microscopy using acid fast stain

The identification of mycobacterial bacilli in sputum is critical for the diagnosis of active TB when not using culture (American Thoracic Society, 2000). *M.tb* and non tuberculous mycobacteria (NTM) are called acid-fast because they have an ability to resist de-colourization with acid-alcohol solutions after staining with arymethane dyes such as carbol fuchsin because of the mycolic acid residues (Barrera, 2007). Acid fast bacilli (AFB) are detected from a sputum by a process that is initiated by the fixation of the smear, followed by the staining with carbol-fuchsin (a pink dye), and decolorization with acid-alcohol and counterstaining with methylene-blue with the AFB appearing pink in a contrasting background (Eisenstadt & Hall, 1995). Preliminary confirmation of mycobacteria through acid fast sputum smear is an easy, fast investigative tool that also provides a semi-quantitative indication of the bacterial load even though it does not confirm *M.tb* because all

NTMs are acid fast and thus has to be complemented by culture; other limitations include the high number of bacilli required (at least 5 000 bacteria/mL), the high number of fields that have to be counted per specimen (at least 300 fields), which could lead to fatigue and error in high work-load situations (de Waard & Robledo, 2007). Sputum quality is also central to the success of the diagnosis. Poor quality sputum can lead to false negative findings (Hirooka, 2004) while improved results may be attained through an induced sputum or a high-quality sputum (Alisjahbana, 2005).

Culture

In order to confirm the presence of *M.tb*, the sputum has to be cultured in solid or liquid media, a process which also involves decontamination, antimicrobial susceptibility testing and confirmatory biochemical analysis or polymerase chain reaction (PCR) (Eisenstadt & Hall, 1995). While the benefits of sputum culture cannot be over-emphasized because of greater sensitivity, *M.tb* grows slowly, takes up to 48 days, with resultant delays in the confirmation of diagnosis. In addition the processes are costly, require complex laboratory settings and are potentially bio-hazardous. Furthermore there is always a possibility of cross-contamination which would produce false positive results (de Waard & Robledo, 2007).

Nucleic Acid Amplification Tests (NAA*T*)

Nucleic acid amplification tests are quick and highly specific diagnostic tools that differentiate *M.tb* from other NTMs and involve a two-step, PCR method that is based on the genomic regions of difference (RD) corresponding to the presence of the different *M. tuberculosis* complex members (Warren *et al.*, 2006). The PCR methods such as such as the GenoType® MTBDRplus assay have facilitated fast detection of resistant strains enabled (Evans, 2009).

Immunological diagnostic tools

Markers of immune responses to *M.tb* antigens may provide invaluable diagnostic information. It is well established that after inhalation, *M.tb* or its products are recognized by macrophage and dendritic cell (DC) toll like receptors (TLR), with a resulting process that induces intricate inflammatory responses including the secretion of cytokines and chemokines (van Crevel *et al.,*2002). The discovery of an immunological bio-signature resultant from *M.tb* infection may present an opportunity for the finding of diagnostic tools if specific enough.

Serologic tests are simple to use and are relatively inexpensive and are based on antibody production to *M.tb* or some of its antigens is yet to be proven (Lighter *et al.,* 2009a). Most serologic tests have proved disappointing (Pai *et al.,* 2009).

1.3.3 TB treatment

Treatment of active TB is based on the World Health Organisation (WHO) guidelines which advocate for the use of fixed-dose combination drugs for 6 months and passive case finding (WHO, 2009b). In South Africa a combination of potent anti-tuberculosis isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol is used, with an initial intensive phase of 2-3months, and a continuation phase of 4-7 more months .Recently, another South African study conducted in a rural setting emphasizes the importance of active case finding as a strategy for timely treatment, especially in HIV infected individuals (Houlihan *et al.,* 2010), but active case finding has not been implemented. The efficacy of TB treatment however, depends on the susceptibility of the mycobacterial strain to the prescribed anti-TB drugs. TB control programmes are faced with the growing problem of multi-drug resistant TB which usually necessitates intensive and toxic drugs (Tsara *et al.,* 2009). Inappropriate chemotherapy, poor adherence to treatment and prolonged periods of infectiousness due to

delays in susceptibility testing have been found to play a role in the spread of MDR-TB (Victor *et al.,* 2007).The center for diseases control and prevention defines multidrug-resistant TB (MDR TB) as TB that is resistant to at least two anti-TB drugs, isoniazid and rifampicin which are first-line drugs in the treatment of TB disease; and Extensively drug resistant TB (XDR TB) is described as is a type of MDR TB which in addition to being ris resistant to isoniazid and rifampin, it is also resistant to any fluoroquinolone and at least 1 of 3 injectable second-line drugs (http://www.cdc.gov/tb/publications/factsheets/drtb.htm). Singh and co-workers (2007) argue that the emergence of XDR-TB in South Africa is a failure of the health system. The South African government states that MDR is both expensive to manage with a morbidity rate of at least 30% (The National Department of Health (1999). A number of South African patients with active MDR-TB have been found to have had a prior TB episode (Calver *et al.,* 2010). It is possible that these patients may have not adhered to treatment protocol or may have been re-infected with drug resistant strains.

1.4 Diagnosing Latent TB infection (LTBI)

M.tb is transmitted in aerosols from person to person. Close contact with an active TB case increases vulnerability to infection (Guwatudde *et al.,* 2003; Hussain *et al.,* 2007). It has been observed that the duration of contact with an active TB case is associated with risk of disease (Guwatudde *et al.*, 2003). Approximately one third of the world's population has asymptomatic sub-clinical and non-contagious TB infection (Dye *et al.,* 1999). It is established that between 5%–10% of HIV negative individuals with LTBI will develop active TB in their lifetime (Horsburgh *et al.,* 2004). These figures are higher in TB endemic areas. A recent study set in a crowded Western Cape community in South Africa reported a latent TB infection rate of 28% in the 5-10 year old group, increasing up to 88% among HIV negative individuals between 31 and 35 years of age (Wood *et al.,* 2010). Hanifa *et al.,* (2009) have also observed LTBI rates of 89% among South African miners.

In order to reduce the pool of latently infected persons, the WHO recommends that countries should implement policies which enable the tracing and diagnosis of people who are at risk of acquiring LTBI and concludes that citizens of those countries have benefitted from a significantly reduced prevalence of tuberculosis (WHO, 2009b). It has been suggested that TB endemic countries would also benefit from programmes that trace contacts of TB patients as an essential aspect of identifying and treating latent TB infection and to thereby reduce the incidence of TB (Morrisson *et al.,* 2008). At present, tests that are available for LTBI diagnosis are based on demonstrating immunologic memory to mycobacteria (Ruhwald & Ravn, 2009). Because of the large numbers of people with LTBI and the pressing need to treat TB only children under 5 years who test TST positive are given a chemoprophylaxis 3 months in South Africa (National Department of Health, 2000).

The tuberculin skin test (TST)

The tuberculin skin test (TST), which is commonly used to diagnose past or present tuberculosis infection, utilizes a purified protein derivative (PPD) of the tubercle bacilli. PPD, which is also known as tuberculin, is introduced into the skin by intradermal injection and the resulting delayed type hypersensitivity reaction (DTH; type IV) causes an induration to appear on the surface of the skin 48-72 hours later ((MMWR, 2000). In most studies that use the TST to determine infection with *M.tb* in HIV negative people an induration of $\geq$ 10mm is taken as a positive result. It has been observed that there is higher risk of being TST positive in household contacts of TB cases with higher smear grades (Lienhardt *et.al*, 2003; Okada *et al.,* 2008). Cells and cytokines of both the innate and the adaptive immune systems are known to play a role in mounting the DTH reaction (Kobayashi *et al.,* 2001). Many of the antigens that are present in PPD are also found in BCG and other mycobacteria, rendering TST non-specific (Lalvani & Pareek, 2009). A study that investigated household contacts in Gambia found that BCG vaccination may have an influence on TST (Lienhardt *et al.*, 2003).

TST sensitivity and specificity is thus determined by a cut-off point; with a lower cut-off point resulting in higher sensitivity but lower specificity for *M.tb* (Mack *et al.,* 2009). There are several other disadvantages to a TST. Individuals have to make 2 visits to a health facility which may be expensive and inconvenient. Furthermore the intradermal administration of tuberculin and the measurement of the DTH reaction induration are subjective (MWWR, 2010).

Interferon gamma release assays (IGRAs)

Although mainly used in developed nations, interferon gamma release assays (IGRAs) are LTBI diagnostic tests that have recently gained widespread use. These diagnostic tools are also based on cell-mediated immune recognition of mycobacterial antigens and measure *in vitro* interferon gamma (IF*N-γ)* production as part of effector immune response induced by stimulation with *M.tb-* specific antigenic such as 6 kilodalton (kDa) early secretory antigenic target (ESAT-6) and 10kDA culture filtrate protein 10 fusion protein (CFP-10) (Mack *et al.,* 2009; Ruhwald & Ravn, 2009). The main advantages of the IGRA are the availability of results within 24 hours without the need for a second visit as well as the objectivity in the results (MWWR, 2010). IGRAs are becoming accepted as accurate indicators of LTBI in low TB prevalence settings (Diel *et al.,* 2008). IGRAs have been found to be more specific when compared to TSTs in populations that use BCG vaccination (Mack *et al.,* 2009). While there is not enough evidence available about the utility of these instruments in high prevalence settings, researchers that used ELISPOT to diagnose LTBI in Gambia advise that a negative ELISPOT result in the diagnosis of *M.tb* infection should be treated with caution (Hill *et al.*, 2008). The Gambia has a TB prevalence rate of 404/100 000 (WHO, 2008). A major drawback of the IGRAs is that they require a blood sample to be tested within 6 hours of collection and in settings where the distances between the laboratory and the sample

collection points are large, delays in processing may decrease IGRA accuracy (Al-Orainey, 2009).

1.5 Latent TB infection in household contacts of active TB cases

Studies of TB contacts offer an invaluable research platform in the understanding of the natural progression of *M.tb* infection. Although neither TST nor IGRA can distinguish between active and latent tuberculosis (Mack *et al.,* 2009), the difference in their sensitivities for predicting progression to active TB was not found to be statistically significant (MWWR, 2010). Consequently, the recent guidelines for detection of LTBI in vulnerable persons in the United States of America recommend the utilization of either a TST or IGRA for diagnostic purposes. Large longitudinal research studies into Human immunodeficiency Virus (HIV) negative household contacts of TB index cases in the developing world have found progression rates to active TB to be between 4% and 6.7% within 2 years of the initial contact (Teixeira *et al., 2001*; Wang & Lin, 2000; Hussain *et al.,* 2007).

1.6 The immune system

1.6.1 The innate immune system

The presence of a pathogen is recognized by immune receptors (pattern recognition receptors), which are present on surfaces of various cells including macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils and natural killer (NK) cells, triggering the innate immune system (Janeway & Medzhitov, 2002). The main purpose of these pattern recognition receptors is to incite inflammatory responses including opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (Medzhitov & Janeway, 1997). In TB, as an example, *M.tb* binds monocytes/macrophages via the complement receptors, the mannose

receptors and this process is facilitated by lipoarabinomannan, a glycolipid found on the mycobacterial surface which leads to phagolysosome fusion (Raja, 2004). A number of other receptors, such as Toll-like receptors (TLRs), recognize conserved pathogen associated molecular patterns (PAMPs) that are present among groups of microorganisms leading to the activation of the host innate inflammatory response (Flynn & Chan, 2001; Takeda *et al.,* 2003). TLR 2 is one of the most important TLRs in TB and gene polymorphisms in this TLR have been associated with increased susceptibility to TB (Yim *et al.,* 2006; Chen, *et al.,* 2010).

Circulating precursor dendritic cells (DCs) enter tissues as immature DCs or directly encounter pathogens that induce secretion of cytokines which, in turn, can activate eosinophils, macrophage and natural killer (NK) cells (Banchereau *et al.,* 2000). DCs are the initiators as wells as the modulators of the immune response and are involved in a complex system which begins with the recognition and capture in peripheral tissues of antigens which bind to the molecules of the major histocompatibility complex (MHC) that are on their surfaces (Banchereau & Steinman, 1998). These allow for the selection of rare circulating antigen-specific lymphocytes. After encounter with *M.tb*, DCs undergo maturation after-which they migrate to the draining lymph nodes where they play a critical antigen presenting function (Bhatt & Salgame, 2007). DCs are not only vital for T-cell stimulation as well as for B-cell growth and immunoglobulin secretion, but are also critical in preventing autoimmunity (Banchereau & Steinman, 1998). The microenvironment determines the effector function of the DCs. They may differentiate and become involved in enhanced antigen presentation; or escalate antigen degradation; induce either immunity or tolerance; or may lead to the polarization of T cell responses towards type 1 or type 2.

Macrophages are the first line of non-specific host defenses; recognize invading pathogen or antigen via cell-surface receptors; binding them and ingesting them in a process called

phagocytosis and producing a number of cytokines and chemokines which in turn influence the immune responses (Bhatt & Salgame, 2007).

NK cells are bone-marrow derived lymphocytes that have been shown to lyse tumor-derived, virus-infected cells in the absence of previous sensitization or activation (Trinchieri, 1989). They are mainly activated by interleukin-12 (IL-12) and play an important role in the innate immune system (Trinchieri, 1995), producing large quantities of IFN-γ, Tumor Necrosis Factor α (TNF α) as well as Macrophage inflammatory protein (MIP-1) chemokines (Biron *et al.,* 1999). NK cells are activated via two types of receptors which either recognize MHC class I–like ligands or do not and thus have multiple recognition systems that are able to respond to diverse abnormalities arising from various situations (Raulet *et al.,* 2001).

Neutrophils are cells of the innate immune system that are granular and have large vacuoles. The pathogens are killed and digested by the protein found in the granules which is released by the neutrophils after they have phagocytozed the microbe (Segal, 2005).

Although gamma delta (δγ) T cells are plastid and play a role in innate immunity, antigen presentation and regulation of the DC function (Casetti & Martino, 2008) they are thought play an important bacterical role in *M.tb* (Dagna *et al.*, 2002).

1.6.2 The adaptive immune response

There are 3 different Th cells: Th1, Th2 and Th17 cells. IL-12 is an important factor for the differentiation of naive T cells into IFN-γ-producing Th1 cells. Both IL-12 and IL-23 are critical in linking the innate and the adaptive immune responses and continue to exert their effects after the adaptive immune system has been activated either through the classical Th1-type response or the IL-23/IL-17 immune pathway to create Th17 cells (Langrish *et al.,* 2004).

It is now established that after T cell receptor activation has been triggered by the antigen-presenting cells (APCs), the cytokine milieu decides the direction of CD4 T cell differentiation to Th1, Th2, Th17 or induced iTreg effector cells through a complex process that is determined by several transcription factors, master regulators, as well as STAT family proteins; Th1, Th2, and Th17 cells are important for eradicating intracellular pathogens, helminthes, and extracellular bacteria/fungi, respectively (Zhu *et al.,* 2010). The expansion of complex T cell populations after bacterial infection is accompanied by an increase in their overall affinity for the antigen (Busch & Pamer, 1999).

CD4 T cells are critical for the outcome of any infection because they direct the ongoing immune response through the secretion of cytokines; and for a long time were classified into Th1 and Th2 subsets until a third set, Th17 cells was discovered (Szabo *et al.,* 2003). Th1 and Th17 cells are also involved in many types of autoimmune diseases, whereas Th2 cells contribute to allergic responses (Zhu & Paul, 2008). In TB, it has recently been shown by Scriba and co-workers (2008) that there was a population of CD4+ T cells that expressed either IL-17 or IL-22 and that IL-17 producing cells were found to be lower in TB patients compared to persons that have been exposed to mycobacteria.

There are several populations of regulatory T cells (Tregs) that play a role in the maintenance and control of immune responses by regulating the magnitude of effector immune responses and are thus able to control the intensity of secondary responses to infections. These include IL-10 producing Tregs as well as Foxp3+ Tregs, which are critical in maintaining self-tolerance and in modulating immune responses to infections (Belkaid & Tarbell, 2009).

CD8+ T cells often play a major role in immunity to viral and intracellular bacterial infections (Zinkernagel *et al.,* 1996) and identify pathogen-derived peptide epitopes which are presented by MHC class I molecules on the surface of infected cells (Germain, 1994).

Antigen-naive CD8+ T cells that are exposed to stimuli and increase chemokine receptor 5 (CCR5) expression, wait for the DC to present the appropriate antigen, adhere and begin to secrete Chemokine ligand 3 (CCL3) and CCL4 leading to attraction of more DCs as well as CD8+ cells (Castellino & Germain, 2006).

1.7 The host immune response to M.tb

The tipping of the balance during the interaction between *M.tb* and its human host determines the outcome of infection. It is understood that although several factors may be responsible, the ability of the host's innate immune system to contain the initial infection is critical (van Crevel *et al.,* 2002). IFN-γ alone is not sufficient to protect against *M.tb* but this cytokine does play an important role in the fight against tuberculosis (Flynn *et al.,* 1993; van Crevel *et al.,* 2002; MacMicking *et al.,* 2003; Abebe *et al.,* 2005; Flynn & Chan, 2005). Once *M.tb* is established within alveolar macrophages, it is thought that an interplay between TNF-α and IFN-γ induces the infected macrophages to differentiate into epitheliod cells which fuse together to form multinucleated cells. The resulting giant cells form the centre of the granuloma, a system that is still poorly understood, but believed to be maintained by chemokines and cytokines (Ruhwald & Ravn, 2009). These mediators of inflammation are thus important tools in the understanding of the *M.tb* infection and their measurement could be used as a biomarker or biosignature for infection.

1.8 The M.tb antigens – proteomics

The advent of genomics has brought with it the possibility of advancing knowledge in the sphere of TB prevention, diagnosis and treatment. Consequently, 16 genetic regions of differences (RD1 to RD16) between *M. tuberculosis* and *M. bovis* have been elucidated (Behr & Small*,* 1999). It has been observed through the use of synthetic peptide pools that proteins encoded by some regions of difference may play a role in immune protection against TB,

while other regions may be responsible for pathogenesis (Al-Attiyah & Mustafa, 2008). Proteins that are encoded by RD1 (ESAT-6 and CFP-10) and RD11 (TB7.7) have been used successfully as LTBI diagnostic tools (Mack *et al.,* 2009).

Several studies have attempted to simulate the micro-environment of *M.tb* under different stress conditions, including oxygen depletion, nutrient reduction, changes in pH and accumulation of growth limiting products. These studies have led to the discovery of 48 specific genes encoded by the dormancy (DosR) regulon that are expressed during non-replicating persistence (NRP), a state in which the bacteria slows its metabolic activity and stops replicating (Wayne & Hayes, 1996; Boon & Dick, 2002; Voskuil *et al.,* 2003; Murphy & Brown, 2007). It has been suggested that conditions that are favourable to the growth of the mycobacteria lead to the expression of a family of 5 genes called Resuscitation Promotion Factors (RPFs) (Mukalomova et al., 2002; Downing *et al.,* 2005; Hett *et al.,* 2007; Kana *et al.,* 2008). Studies of the immunogenicity of the proteins expressed by *M.tb* during different stages of growth have ushered an era that could lead to the discovery of improved vaccine, diagnostic, and anti-mycobacterial products.

1.9 Cytokine bio-signatures – Luminex technology

Luminex is a fluorescent bead based time-saving technique that operates on the sandwich principle similar to that of the ELISA assay. This tool presents an opportunity to study and to quantify immune responses by simultaneously measuring up to 100 cytokines in theory in small samples which in most cases are precious. Although Luminex is a new technology, there are several research studies focusing on TB that have been published using this platform. Recently it has been shown that TST conversion is associated with early increases in IFN-γ and IL-10 responses and that this phenomenon preceded latency by several months post exposure (Hussain *et al.,* 2009). Chegou *et al.,* (2009) have recently shown that the combinations of (Epidermal growth factor) EGF, Soluble CD 40 Ligand (sCD40L), Vascular

endothelial growth factor (VEGF), Transforming growth factor alpha (TGFα) and IL-1α differentiate between latent and active infection. In the Gambia, IL-13, IL-17 and IL-18 have also been shown to discriminate between latent and active TB (Sutherland *et al.,* 2009). In a study done in Guinea Bissau Monocyte chemotactic protein-1(MCP-1), MCP-2, MCP-3, Interferon inducible protein 10 (IP10) and Interleukin-1 receptor antigen (IL-1RA), all could discriminate between unexposed healthy controls and active TB cases (Ruhwald *et al.,* 2009).

1.10 The current study

The whole blood assay (WBA) is an *in vitro* technique that enables circulating T cells to respond to antigenic stimulation by producing cytokines which can be quantified (Weir *et al.,* 1994). In the current study, novel *M.tb* antigens were used to stimulate diluted whole blood in culture for 7 days in order to measure cytokine production in harvested supernatants. All participants included in the current studies were HIV-1 antibody negative. The first aim of the study was to cross-sectionally compare the IFN-γ response to classical and novel *M.tb* antigens in TST negative household contacts, TST positive household contacts and newly diagnosed active TB index cases prior to treatment. This was done in order to identify antigens that could significantly differentiate between active TB and latent *M.tb* infection (as measured by the TST). As IFN-γ is a central cytokine associated with a protective immune response to *M.tb* infection, an IFN-γ ELISA was used for this purpose. The results of this work are described in Chapter 2. The second aim was to longitudinally assess immune responses in the group that was TST negative at recruitment; multiple cytokine responses were measured using Luminex technology at recruitment and 6 months later, by which time a proportion of the TST negative subjects had converted to TST positive. This was done in order to ascertain whether there is a multi-cytokine profile that is associated with fast TST conversion versus slow TST conversion assuming the TST negative subjects would convert at

some point. Understanding the cytokine signature that is associated with recent *M.tb* infection may facilitate the future discovery of improved biomarkers for disease progression.

CHAPTER 2: Interferon gamma responses to novel *M.tb* antigens in active TB cases and TB contacts with different tuberculin skin test reactions

2.1 Introduction

It is well established that close contacts of active tuberculosis (TB) patients are most vulnerable to infection (Guwatudde *et al.,* 2003; Hussain *et al.,* 2007). After inhalation, *M.tb* and its human host engages in a continuous struggle, which may lead to sub-clinical disease and, in some instances, may continue to symptomatic clinical disease (Young *et al.,* 2009). The initial response to the *M.tb* challenge involves the host's innate immune cells including alveolar macrophages, dendritic cells and monocytes, resulting in the induction of phagocytosis and the production of cytokines as well as chemokines (van Crevel *et al.,* 2002). Whereas in some cases the innate immune system can eliminate *M.tb* effectively, in others the involvement of the adaptive immune system may become necessary to kill or to control the invading mycobacteria (Bhatt & Salgame, 2007). Should asymptomatic sub-clinical disease become established, it is said that individuals have latent TB infection (LTBI, which is currently accepted as a continuum or dynamic state (Young *et al.*, 2009; Barry *et al.*, 2009). For a long time LTBI has been defined according to the results of a tuberculin skin test (TST), also known as the Mantoux skin test, and is diagnosed using Tuberculin, a purified protein derivative (PPD) which is injected intradermally and causes a delayed type IV hypersensitivity reaction in individuals that are infected with mycobacteria (Mack *et al.*, 2009).

The centuries of co-existence between *M.tb* and humans have led the mycobacteria to evolve and to devise mechanisms that enable it to evade the host's immune assault (Flynn & Chan, 2005). While not completely understood, it is widely accepted that, upon being challenged by adverse conditions, which are part of the immune response, including oxygen depletion,

nutrient reduction, changes in pH, as well as accumulation of growth limiting products, *M.tb* may progressively reduce its metabolic activity, stop replicating and enter a phase known as Non Replicating Persistence (NRP) (Wayne & Sohaskey, 2001; Monack *et al.*, 2004). This stage is preceded by an up-regulation of 48 specific genes encoded by the DosR /Rv3133c regulon (Wayne & Hayes, 1996) (Boon & Dick, 2002; Voskuil *et al.*, 2003; Murphy & Brown, 2007).

NRP creates a reservoir of bacilli that could be reactivated when conditions become favorable for growth (Monack *et al.*, 2004). Prior to reactivation, bacilli express genes that are associated with chromosomal division (Bacon & Marsh, 2007). Reactivation is also associated with granuloma enlargement (Kaufmann, 2006). Progression from NRP to a state of increased metabolic activity and replication is also preceded by the expression of a family of 5 genes called Resuscitation Promotion Factors (RPFs), (Mukalomova *et al.*, 2002; Downing *et al.,* 2005; Hett *et al.*, 2007; Kana *et al.*, 2008) which are regulated independently of one another (Kana & Mizhari, 2010). These RPF genes, namely *rpfA* (Rv0867c), *rpfB* (Rv1009), *rpfC* (Rv1884c), *rpfD* (Rv2389c), and *rpfE* (Rv2450c) are also thought to be important for bacterial virulence (Kana *et al*., 2008), and have been detected in bacilli during acute infection as well as throughout the stationary growth phase (Tufariello *et al.*, 2004), even though they are not critical for bacterial growth (Downing *et al.*, 2005; Hett *et al.*, 2007; Kana *et al*, 2008). Kana and Mizhari (2010) posit that the main function of RPFs is to enable the bacilli to recover from the host's immune damage by re-modelling the bacterial cell wall. The significance of RPFs in *in vivo* processes has been enhanced by the recent detection of RPF-dependent cells in human clinical sputum which illustrates the presence of non-replicating bacilli in these samples (Mukalomova *et al.,* 2010).

In spite of recent advances in this field, the exact mechanisms of reactivation remain unknown. The expression of Rv3407, Rv1131 and Rv1471 by *M.tb* is thought to be

associated with reactivation. A recent study by Schuck *et al.* (2009) has shown that Rv3407 could discriminate between LTBI and active TB cases. Rv3407 encodes an antitoxin (Golby *et al.*, 2007) and is influenced by RPFs which interact with RipA (Hett *et al.,* 2007). Rv1131 encodes a citrate synthase which is an enzyme of the TCA (Krebs) cycle (Cole *et al.*, 1998). Rv1131 protein has a role in the methylcitrate pathway (Munoz-Elias *et al.*, 2006), and is also implicated in the pathogenicity of *M.tb* (Fontán *et al.*, 2009). Rv1471 belongs to the thioredoxin family and functions as a disulfide reductase, maintaining the redox state of cytosol during oxidative stress (Akif *et al.*, 2008).

Stellenbosch University is one of seven African field sites involved in an international collaborative effort to identify biomarkers of protection against TB in the context of HIV. This collective venture, known as GC6, is the 6th in a series of Grand Challenges in Global Health, an initiative launched in 2003 by the Bill & Melinda Gates Foundation (http://www.gcgh.org). One of the goals of the GC6 project is to determine the natural immune profile of latently infected individuals by investigating their IFN-γ responses to a panel of novel *M.tb* antigens. IFN-γ production is widely accepted to be one of the most significant cytokines produced in a protective immune response against TB (Flynn & Chan, 2001; Raja, 2004; Monack *et al.*, 2004). To achieve this objective, 86 *M.tb* antigens including proteins associated with latency, starvation, resuscitation and reactivation, were screened for their ability to induce IFN-γ responses in TST positive HIV negative adults across the African sites (Black *et al.*, 2009). A final panel of the 22 most promising antigens was selected for inclusion in the longitudinal study.

The present M.Sc. project was conducted as a sub-study of GC6. Using the 7-day diluted WBA that was developed by Weir and colleagues (1994) we have examined the *in-vitro* IFN-γ response elicited by all 22 *M.tb* antigens routinely tested in the GC6 study. This has been

done in TST negative and TST positive household contacts (HHC) as well as in untreated active TB patients, at the time of recruitment into the GC6 study. This study was done in order to 1) expand the body of knowledge that seeks to identify *M.tb* derived candidate proteins for inclusion in post-exposure vaccines which may offer protection against progression to active TB disease 2) provide additional information about putative immune correlates of protection against tuberculosis.

2.2 Study Design and Methods

2.2.1 Setting

The study participants were recruited from the suburbs of Uitsig, Ravensmead, Elsiesriver and Adriaanse in Tygerberg District, Western Cape Province, South Africa. This is an area with low HIV prevalence but a high incidence of TB of 764/100 000 (Kritzinger *et al.*, 2009).

2.2.2 The tuberculin skin test (TST)

The TST was done on HHC immediately after collection of blood. Two tuberculin units of *M.tb* PPD RT23 for *in vivo* use (Statens Serum Institute, Denmark) were administered intradermally on the distal forearm and read 48 -72 hours later by trained study nurses. The TST was considered positive if the induration was $\geq$ 10mm, and negative if there was no visible induration (0mm). The skin test was repeated only in HHC that were TST negative at recruitment; at each subsequent study time point (6 and 18 months, see 2.2.3 below) until conversion was observed.

2.2.3 Study population

HHC were recruited from the 4 suburbs mentioned above. For inclusion in the study, contacts had to be between 10 and 60 years old; have been residing with a sputum positive adult TB case diagnosed within the past 2 months; agree to the tuberculin skin test procedure and; to be

tested for HIV. HHC were actively followed up at months 6, 18 and 24. An assisted sputum sample for microscopy and culture was obtained from the contacts by physiotherapy trained nursing assistants using percussion. Exclusion criteria for the study were presence of cancer, diabetes mellitus, chronic emphysema/ bronchitis/ asthma requiring steroid therapy or any steroid therapy within the past 6 months; previous or current treatment of TB or HIV; current or recent (within 6 months) participation in a vaccine/clinical trial; pregnancy and; absence of a permanent physical address or if the individual had not been living in the study area for at least 3 months. All participants gave informed consent and the study was approved by the ethics committee of Stellenbosch University.

By May 2009, 893 HIV negative HHC had been recruited into the GC6 study. At study enrollment, 829 participants (92.8%) tested TST positive (induration $\geq$ 10mm) and 64 (7.2%) were TST negative (no visible reaction). At month-6 follow up, of the 64 participants who had tested TST negative at recruitment, 29 (45.4%) were not skin tested again. Consequently, their TST status at month 6 is not known. Twenty six originally TST negative participants had converted to TST positive by month 6 (40.6%) while 9 (14%) remained TST negative. These 9 participants were skin tested again at month 18 by which time 8 (88.9%) had converted to TST positive with only 1 individual remaining TST negative (Figure 2.1). This participant was not tested again at month 24.

New active TB cases between the ages of 10 and 60 years were identified through the Department of Health community clinics in the 4 suburbs mentioned above, and introduced into the GC6 study by research nurses. Eligibility as a TB index case was based on pre-defined case definitions (Table 2.1). Sputum ZN smears, liquid culture and chest x-rays were done and the likelihood of the disease recorded by a study clinician. After enrolment into the study, all patients diagnosed with TB received chemotherapy as per national guidelines.

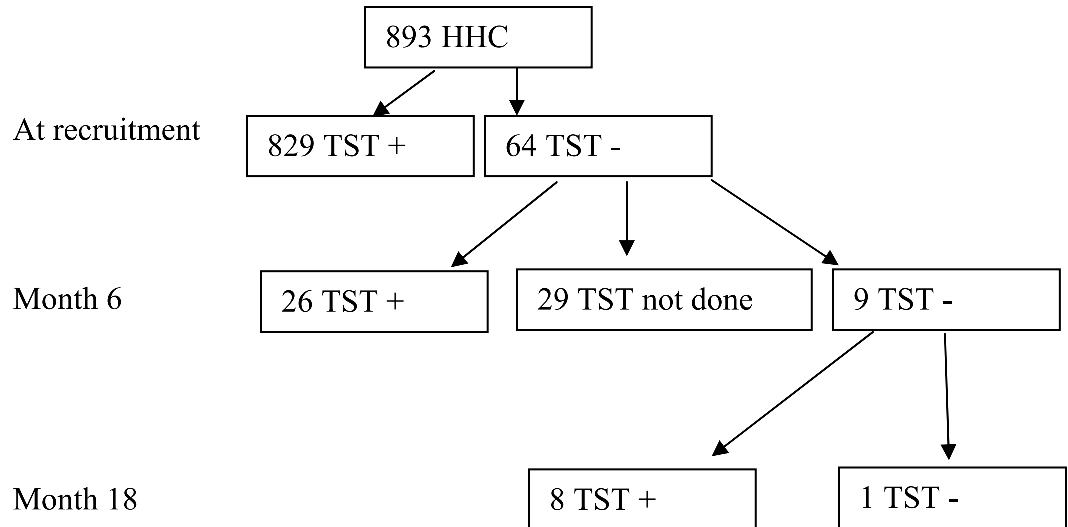


Figure 2.1 TST status at recruitment, 6 months and 18 months in study participants enrolled into the GC6 household contact study. All household contacts recruited into the GC6 study were skin tested with *M.tb* PPD at enrollment. In TST negative subjects the TST was repeated at months 6 and again at month 18 if there was no reaction at 6 months.

Table 2.1 GC6-74 Clinical Case Definition For TB diagnosis. Table 2.1: Definite TB had to have one of (a) ≥ two positive culture sputum results with acid fast bacilli in liquid culture; or (b) ≥ one positive sputum culture with a chest X-ray suggestive of TB. Probable TB had to have one of (a) ≥ one culture positive, with ≥ one symptom compatible with TB and a chest X-ray not suggestive of TB; or (b) negative cultures and a chest x-ray suggestive of TB; or (c) negative cultures and 2 positive sputum smears. Possible TB had to have one of (a) ≥one positive culture and chest X-ray not suggestive of TB; or (b) negative cultures and a chest X-ray suggestive of TB or (c) negative culture and a chest X-ray not suggestive of TB, but ≥2 positive or scanty smear with symptoms compatible with TB. An unlikely TB case had to have negative cultures and a chest X-ray not suggestive of TB.

Culture 1	Culture 2	AFB 1	AFB 2	CXR	Symptoms	Treatment response	Class
+	+	Irrelevant	Irrelevant	Irrelevant	Irrelevant	Irrelevant	Definite
+	Negative	Irrelevant	Irrelevant	+	Irrelevant	Irrelevant	Definite
+	Negative	+	Irrelevant	Negative	+	Irrelevant	Probable
+	Negative	Negative	Negative	Negative	+	+	Probable
Negative	Negative	+	Negative	+	+	Irrelevant	Probable
Negative	Negative	+	Negative	+	Irrelevant	+	Probable
Negative	Negative	Negative	Negative	+	+	+	Probable
Negative	Negative	+	+	+	Irrelevant	Irrelevant	Probable
Negative	Negative	+	+	Negative	+	+	Probable
+	Negative	+	Irrelevant	Negative	Negative	Negative	Possible
+	Negative	Negative	Negative	Negative	+	Negative	Possible
+	Negative	Negative	Negative	Negative	Negative	Negative	Possible
Negative	Negative	+	Negative	+	Negative	Negative	Possible
Negative	Negative	Negative	Negative	+	+	Negative	Possible
Negative	Negative	Negative	Negative	+	Negative	+	Possible
Negative	Negative	+	Negative	Negative	+	+	Possible
Negative	Negative	+	Negative	Negative	+	Negative	Unlikely
Negative	Negative	Negative	Negative	Negative	+	+	Unlikely
Negative	Negative	Negative	Negative	Negative	+	Negative	Unlikely
Negative	Negative	Negative	Negative	Negative	Negative	Negative	Unlikely

Of the 26 participants that were TST negative at recruitment and converted to TST positive 6 months later, whole blood assay supernatants were available for 17 (see 2.2.5 below). The same number of age and gender matched recruitment TST positive contacts and active TB cases that also had whole blood supernatants available were identified using the project database. The clinical and demographic data of the study population is shown in Table 2.2. The median ages of the study participants were 25 years for the TST negative group, 30 years for the TST positive group and 23.5 years for the TB index cases. Males comprised 36.8% of participants in both the TST negative and the TB index case groups while they made up 30.7% of the TST positive group. TST positive participants had skin test indurations ranging between 10mm and 33mm (median 17mm) at recruitment. A visible BCG scar was recorded in 41.1% of the TST negative group, 52.9% of the TST positive group and 56.3% of the TB index cases.

The degree of exposure of the HHC to their respective index cases was determined using a contact score (Table 2.4). The total contact score was calculated by adding the weights for infectivity of the index case (based on smear grade), duration of exposure to the index case, proximity of exposure, and relationship of the contact to the index case (Hesseling et al., 2009).

Table 2.2 Clinical and demographic information of study population. Characteristics of the three study groups at study recruitment, with details on BCG scar status, TST induration and, for HHC, the gradient of exposure to the TB index case. For those study participants that were TST negative at recruitment, the median TST size (and range) as measured at the 6 month time point is shown.

	TST Negative at study recruitment	**TST positive at study recruitment**	**TB index case**
Number of participants	17	17	16
Age median in years, (range)	25 (10 - 52)	30 (14 - 51)	23.5 (17 - 49)
Male/female ratio	7 /10	7/10	5/11
BCG vaccine documented or scar present, n(%)	7(41.1)	9 (52.9)	9(56.3)
Median TST induration at recruitment, (range, mm)	0	17(10 - 33)	Not done
Median TST induration at month 6, (range, mm)	17 (10 - 24)	Not done	Not done
Median *M.tb* contact score	12 (10 - 12)	12 (8 -12)	
BCG vaccine documented or scar present, n(%)	7 (41.1)	9 (52.9)	9(56.3)

2.2.4 Blood Samples and HIV testing

Peripheral blood was obtained in 10ml heparinised tubes at recruitment from each study participant (Becton Dickinson (BD), San Diago, USA). Blood was transported to the laboratory and processed under sterile conditions within 2 hours of collection in a laminar flow hood. Each participant was tested for HIV using a rapid test after pre- and post-test counseling (First Response HIV Card 1–2.0, PMC Medical India Pty Ltd, Daman, India).

2.2.5 Whole blood assay

The WBA followed a previously described method (Weir *et al.,* 1994). Diluted whole blood (1:10 with RPMI 1640 1% (Sigma) + L-glutamine) was incubated in triplicate with recombinant protein antigens (10µg/ml final) in 96-well tissue culture plates (Nunc) (37^0 C, 5% CO_2) in a volume of 200µl per well. Supernatants were harvested on day 7 and stored at

-80^0 C. The negative control was RPMI alone. Phytohemagglutinin (PHA) (Sigma) (5µg/ml) and SEB (1µg/ml) were used as positive controls.

2.2.6 Antigens

All antigens were produced and quality controlled by Leiden University Medical Center (LUMC) as described previously (Leyten *et al.*, 2006). Purity and size were checked by gel electrophoresis and Western blotting with anti-His antibodies and anti-*E.coli* antibodies. Residual endotoxin levels were determined with a Limulus amebocyte lysate assay (Cambrex) and found to be below 50 IU/mg recombinant protein. A total of 17 recombinant antigens were tested; 11 DosR regulon encoded proteins, 3 antigens associated with the reactivation of *M.tb* and 3 RPFs. All recombinant antigens were tested at 10µg/ml. Five 'classical' TB antigens were also included. All antigens and controls and their final dilution in blood was 1 in 10 and are listed in Table 2.3.1 - Table 2.3.4.

Table 2.3.1 Classical *M.tb* antigens

ANTIGEN NAME	*M.tb* GENE	PROTEIN SIZE (a.a)	DESCRIPTION
M.tb PPD			173 proteins*
Esat6/CFP10	fusion product	-	ESAT-6 and CFP-10 fusion protein
Rv3019	*esxR*	96	TB10.3; Secreted ESAT-6-like protein
TB10.4 (Rv0288)	*esxH*	96	Low molecular weight protein Antigen 7
Ag85A (Rv3804c)	*fbpA*	338	Secreted Antigen 85A

Table 2.3.1 List of classical *M.tb* antigens tested. Rv-numbers denote the names of the protein products.

Mehta *et al,* 2007
Zvi *et al,* 2008
Rogerson *et al,* 2006
Black *et al*, 2009
Lin *et al,* 2009
*Borsuk *et al*, 2009

Table 2.3.2 Dos-R regulon antigens

ANTIGEN NAME	***M.tb* GENE OR PROTEIN FUNCTION**	**PROTEIN SIZE (a.a.)**
Rv1737c	*narK2*	395
Rv2029c	*pfkB*	339
Rv1733c	Possible transmembrane protein	210
Rv1735c	CHP	165
Rv0081	Transcriptional regulator	114
Rv0569	CHP	88
Rv2028c	CHP	279
Rv2031c	*acr* (HSPX)	144
Rv3131	Two component response regulator	332
Rv2659c	phiRV2 integrase	375
Rv2660 peptide pool	HP	75

Table 2.3.2: List of DosR regulon Latency Antigens tested. 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

Wayne & Hayes, 1996
Wayne & Sohaskey, 2001
Boon & Dick, 2002
Voskuil *et al*., 2003
Monack *et al*, 2004
Voskuil *et al*., 2004
Leyten *et al,* 2006
Murphy & Brown, 2007
Roupie, et al, 2007
Black *et al*, 2009
Lin *et al,* 2009

Table 2.3.3 Resuscitation Promotion Factors

ANTIGEN NAME	*M.tb* GENE	PROTEIN SIZE (a.a.)	DESCRIPTION
Rv2450	*rpfE*	172	Probable Resuscitation-promoting factor RPFE
Rv0867c	*rpfA*	407	Possible Resuscitation-promoting factor RPFA
Rv1009	*rpfB*	362	Possible Resuscitation-promoting factor RPFB

Table 2.3.3 List of all the Resuscitation-promoting Factors tested. Rv-numbers denote the names of the protein products. Italicised names designate the gene region and capitals the protein function.

Mukalomova *et al*, 2002
Tufariello *et al*, 2004
Downing *et al*, 2005
Hett *et al*, 2007
Kana *et al*, 2008
Kana and Mizhari 2010
Mukalomova *et al*, 2010

Table 2.3.4 Reactivation Antigens

ANTIGEN NAME	*M.tb* GENE or PROTEIN FUNCTION	PROTEIN SIZE (a.a.)
Rv1131	*gltA1*	393
Rv1471	*trxB*	123
Rv3407 *	CHP	99

Table 2.3.4: List of all Reactivation Antigens, produced by the Wayne model, tested. 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. NB*: This antigen was provided by the Max Planck Institute for Infection Biology, Berlin, Germany.

Cole *et al*, 1998
Downing *et al,* 2004
Munoz-Elias *et al*, 2006
Golby *et al*, 2007
Akif *et al*, 2008
Fontán *et al*, 2009
Schuck *et al*,2009

2.2.7 IFN-γ ELISA response cut-off

The IFN-γ ELISA followed a previously described method (Black *et al.*, 2001). Briefly, antibody pairs from Becton Dickinson were used for cytokine (IFN-γ) assessment by ELISA. Supernatants were analyzed in duplicate in 96 well plates, which had been coated overnight at 4 ^{0}C with a primary antibody. Ag-Ab complex reacted with avidin peroxidase and enzymatic activity was measured with OPD at 490nm using a 4 parameter log curve (4PL). The lower detection limit of the standard curve was 31pg/ml and the upper detection limit 4000pg/ml. Low undetectable responses were assigned a value of 15pg/ml. The negative control value for each study participant was subtracted from the antigen-induced IFN-γ values so that all response values could be considered over and above the background response.

A standard method that has been used by others (Lewinsohn *et al.*, 2008) to determine the cut-off for a positive IFN-γ response is to add the average of the negative control values of all participants included in the analysis to two times their standard deviation. However, all negative control values in our data set were read off the standard curve as 0pg/ml, which was converted to 15pg/ml (as described above). A value of 15pg/ml could not be considered to discriminate between responders and non-responders to antigenic stimulation because it was out of range of the standard curve. Therefore, following subtraction of the negative control, a positive response was defined as an IFN-γ concentration greater than 62 pg/ml, which represents twice the lowest detection limit of the ELISA (lowest point on the standard curve is 31pg/ml). This method of defining a cut-off has been published by ourselves and others previously (Black *et al.,* 2003; Lin *et al.,* 2007).

2.2.8 Statistics

The Mann-Whitney test (two tailed) was used to investigate differences in the median responses between study groups. Correlations between antigen responses were assessed with the Spearman correlation coefficient. Fisher's exact test was used to establish differences between groups. A p value of < 0.05 was considered significant. Power calculations were not done.

2.2.9 Research Questions

- In the initially TST positive group:

Is TST induration size associated with either gender, age or BCG scar at recruitment?

- In the group that convert from TST negative to TST positive at 6 months:

Is the 6 month TST induration size associated with gender, age or BCG scar?

- Are there differences in the TB exposure gradients between HHC who are TST negative at recruitment and those who are TST positive at recruitment?
- Is there a relationship between the TST status of the HHC and the smear grade of their respective TB index case?
- Can antigen induced whole blood cytokine responses distinguish between the 3 distinct groups of HIV negative adults included in this study: TST negative HHC (0mm), TST positive ($\geq$ 10mm) HHC and new active TB disease prior to initiation of therapy.

2.3 Results

2.3.1 Comparison of the clinical and demographic information

In the group that were TST positive at recruitment, no association was observed between TST induration size and either age or gender. All study participants that were TST negative at recruitment had converted their skin test by month 6 and, again, TST induration was not associated with gender or age in this group. BCG scar status (presence or absence) was not associated with TST induration size in the TST positive group at recruitment or the TST negative group following conversion at 6 months. Furthermore, the BCG status did not have an influence on TST grouping of contacts (Fisher's Exact Test, p=0.11).

No differences were observed in total contact scores between the TST positive and TST negative participants at recruitment.

Table 2.4 TB Contact Score of Household Contacts To TB Index Cases. The degree of exposure of the HHC to the TB index case was determined by adding the weight of each of the variables. From Hesseling *et al.,* 2009.

Variable	**Weight**
Relationship to index case (relation)	
• No known TB contact	0
• Non-household TB contact	1
• Other relative/friend in household with TB	2
• If a child: secondary caregiver (care provider during day) in household with TB	3
• If a child: primary caregiver in household with TB	4
Infectivity of index case (infectivity)	
• No known TB contact	0
• Sputum acid-fast negative	2
• Sputum acid-fast positive	4
Type of exposure to index case (proximity)	
• No known exposure	0
• Lives and sleeps in different house (e.g. different structure on same plot or completely different plot and house)	1
• Lives and sleeps in same house	2
• Sleeps in same room	3
Duration (total hours) contact per day with TB index case (duration)	
• No known contact	0
• 0-3 h	1
• 4-7 h	2
• 8-11 h	3
• ≥ 12 h	4
Total contact score (maximum score = 15)	

As Shown in Table 2.4 the contact score considers positive sputum as a single factor but does not consider the grading of the smear. In our setting, sputum smears are graded according to the World Health Organization recommended grading system, which has five (5) groups. A negative smear grade indicates that no acid fast bacilli were seen in at least 100 fields; scanty indicates between 1 and 9 bacilli were observed in 100 fields; 1+ indicates between 10 and 99 bacilli were detected in 100 fields; 2+ indicates 1 to 10 bacilli per high power field and 3+ indicates more than 10 bacilli per higher power field (Rieder *et al.,* 2007). We investigated whether there was an association between the TST status of the HHC at recruitment (positive vs negative) and the smear grade (scanty, 1+, 2+ and 3+) of their respective TB index cases. Although two sputum samples were obtained per TB case, only the highest smear grade was considered in the analysis. Sputum smear information for 3 TB index cases (for 1 TST negative and 2 TST positive contacts) was not available. A sputum smear of 2+ or higher was documented for 83% of the TB index cases in contact with TST positive participants compared to 72% of cases in contact with TST negative subjects (Figure 2.2). These differences were not statistically significant.

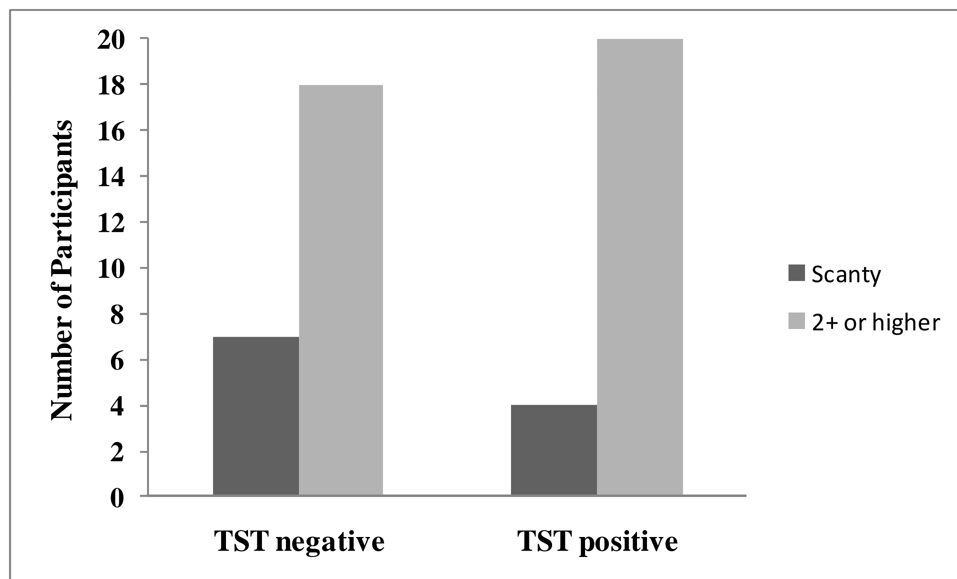


Figure 2. 2: Acid-fast bacilli sputum smear grade of TB index cases in contact with TST negative and TST positive study participants.83% of the TST positive contacts were exposed to TB cases with higher grade sputum smears compared to 72% of TST negative contacts. Scanty means that between 1 and 9 bacilli were observed in 100 fields; 1+ represents 10 -99 bacilli observed in 100 fields; 2+ stands for 1 to 10 bacilli per high power field and 3+ greater than 10 bacilli per higher power field (Rieder *et al,* 2007).

2.3.2 Reproducibility control

IFN- γ ELISAs were done on a total of 62 ELISA plates. Of the 62, 23 plates were assayed with a positive control supernatant obtained from the PHA stimulated blood of a healthy donor. The supernatant was generated using the whole blood assay protocol described above and PHA was used at a final concentration of 5ug/ml. The resulting positive control supernatant (hereafter called positive control 1) was tested in duplicate on the 23 plates. Figure 2.3a shows the IFN-γ concentrations detected in positive control 1 on each plate. For positive control 1, the average IFN-γ concentration was 1503.8pg/ml (range 847pg/ml – 2285pg/ml). The inter-plate and inter-well variability were determined using the coefficient of variation (CV). The average inter-well variation was 3.6% (range 0.15% - 18.9%) whereas the average inter-plate variation was calculated to be 27.2%.

Positive control 2, a recombinant human IFN-γ standard was obtained from Pharmingen (cat 554616, lot 33306) and diluted to 750pg/ml following the manufacturer's recommendations. This second positive control was tested in duplicate on the remaining 39 plates. Figure 2.3b shows the IFN-γ concentrations detected in positive control 2 on each plate. The average IFN-γ concentration detected in positive control 2 was 1381pg/ml (range: 790pg/ml – 3098.5pg/ml). The inter-plate and the inter-well variability were determined using the coefficient of variation (CV). The average inter-well variation was 3% (range: 0.14% - 16.6%) whereas the inter-plate variation was calculated to be 32.8%.

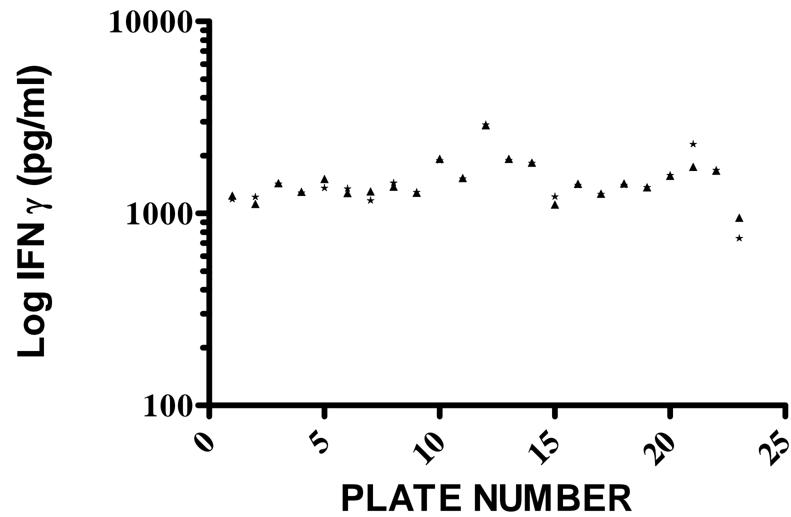
2.3.3 Standardization and normalization of the IFN-γ ELISA results

While the inter-well variation was within acceptable levels, the inter-plate variation was high for both positive control 1 (PC1, 27.2%) and positive control 2 (PC2, 32.8%). The results from each of the ELISA plates were corrected as follows: For the 23 plates tested with PC1, a reference curve (reference curve 1) was created from the average optical densities (ODs) of

four curves with the closest fit to the required four-parameter logistic (4PL) shape. The 4PL model is recommended for immunoassay calibrations (Findlay, 2006). Four (4) plates that were chosen had their standard curves showing constantly decreasing trends with respect to concentration levels and OD signal responses. The mean of the internal positive control 1 value on the 4 selected plates (mean = 1423pg/ml) was plotted onto reference curve 1, and the emerging reference OD of 2.64 was used to determine a correction factor for all the test samples that were assayed on these 23 plates. Each plate assayed with PC1 had its own correction factor. For example, the average optical density of the duplicate positive control supernatants tested on plate number X was 2.83; this value was divided by the reference OD of 2.64, giving a correction factor for of 0.93 for plate X. The test supernatant derived from seven day whole blood stimulation with Rv3019c was assayed on plate X and had an OD of 2.43 was obtained. When read with the original plate X curve, this OD translated to a concentration of 2052pg/ml. However, when the test OD of 2.43 was multiplied by the correction factor (0.93) this resulted in an OD of 2.27 which, when read of the reference curve, gave rise to a corrected concentration of 1175.5pg/ml. In a similar process, reference curve 2 was created from the average ODs of seven curves with the closest fit to the required 4PL shape on plates with internal positive control results closest to the known value of 750pg/ml (790-850pg/ml). The mean of the internal positive control 2 value of the seven selected plates (819pg/ml) was plotted on reference curve 2, and the emerging reference OD of 1.80 was used to determine a correction factor for the samples that were assayed on all 39 plates containing positive control 2. As for PC1, each PC2 plate had its own correction factor. Graph Pad prism (version 5) was used to produce the corrected values for both sets of plates.

It was not possible to run to both PC1 and PC2 in the same plate because when the study commenced, a PHA stimulated positive control was used (PC1) which was later changed to a diluted standard curve positive control (PC2).

(a)



(b)

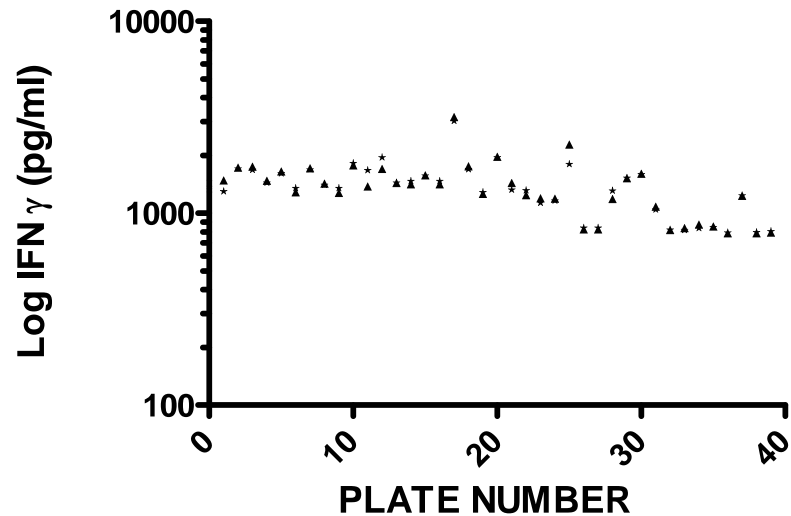


Figure 2.3 a –b : Interferon gamma level in internal positive controls 1 and 2, per plate. Log_{10} interferon gamma level detected in internal positive control supernatants, per plate.. Control supernatants were tested in duplicate on each of the 62 ELISA plates. (a) In 23 plates the positive control was obtained from PHA stimulated supernatant from a healthy control. (b) In 39 plates the positive control was from commercial interferon gamma which was diluted to a final concentration of 750pg/ml. The triangle denotes well 1 and the star denotes well 2.

2.3.4 Responses to negative and positive controls

Figure 2.4a illustrates that none of the study participants in any of the groups produced an IFN-γ response to the negative control (RPMI). All participants across all three study groups responded to whole blood stimulation with the positive control, SEB, at the recruitment time point, with the exception of 2 subjects in the TB index case group (Figure 2.4b), which may be due to anergy or lymphopenia in the blood. Stimulation with SEB resulted in positive responses (>62pg/ml) in 100% of TST negative (n=17) and TST positive (n=17) contacts respectively as well as in 86.7% (n=13/15) of TB cases. The median response to SEB in the TST negative subjects was significantly higher than that observed in the TB index cases (p=0.04, Mann-Whitney test).

All subjects except for 1 in both the TST negative (n=15/16) and TST positive groups (n=16/17) responded to PHA at recruitment (Figure 2.4c), compared to 69% (11/16) of the TB cases. Median responses to PHA were significantly higher in both the TST negative and TST positive groups when compared to the TB index cases (p=0.01; Mann-Whitney test). SEB is a superantigen and PHA a mitogen, and that is why both of them are used as positive controls.

2.3.5 Comparing response frequencies to classical M.tb antigens between study groups at recruitment

M.tb PPD

Figure 2.5a illustrates that 100% of the TST positive contacts responded to *M.tb* PPD stimulation at recruitment, whereas 87.5% (n=13) and 60% (n=9) of TB index cases and TST negative HHC respectively responded to this antigen. The median response of the TST positive contacts was significantly higher than that of both the TST negative contacts and TB

index cases (p=0.0002 and p=0.04 respectively), and the median response of the TB cases was significantly higher than that of the TST negative HHC group (p=0.01). This indicates that ELISA is more sensitive than the TST.

ESAT6/CFP10 fusion protein

ESAT6/CFP10 fusion protein induced responses in 100% of TST positive HHC and 87.5 % (n=13) of the TB cases (Figure 2.5b), whereas there were responses in only 26.7% (n=4) of the TST negative participants. In the TST negative group 3 of the 4 subjects that responded to ESAT6/CFP10 also responded to *M.tb* PPD. Median responses in both the TST positive group and the TB cases were markedly higher than those of the TST negative group (p<0.0001 and p< 0.0001, respectively).

Rv3019c (TB10.3)

Figure 2.5c shows that in the TST positive group the median IFN-γ response to TB10.3 (343pg/ml) was significantly lower than that induced by *M.tb* PPD (1625pg/ml, p<0.0001) or ESAT6/CFP10 fusion protein (1973pg/ml, p<0.0001) indicating that this recombinant antigen was less immunogenic than PPD or the fusion protein. Both the proportion of responders (47%, n=8) and the median response to this antigen was greater in the TST positive participants compared to the TB index cases (6.25%, n=1; median 15pg/ml; p=0.008), and TST negative participants (5.9%, n=1; median 15pg/ml; p = 0.01).

TB10.4

In the TST positive group the median IFN-γ response to TB10.4 (43.7%, n=7) was similar to that observed for TB10.3 (47%, n=8). In the TST positive group, 6 out of the 7 participants that responded to TB10.4 also responded to TB10.3. In TB cases 37.5% (n=6), and in TST negative subjects, 11.7% (n=2), responded to TB10.4 stimulation (Figure 2.5d). TST positive

subjects had a significantly higher median response compared to TST negative contacts, ($p=0.03$).

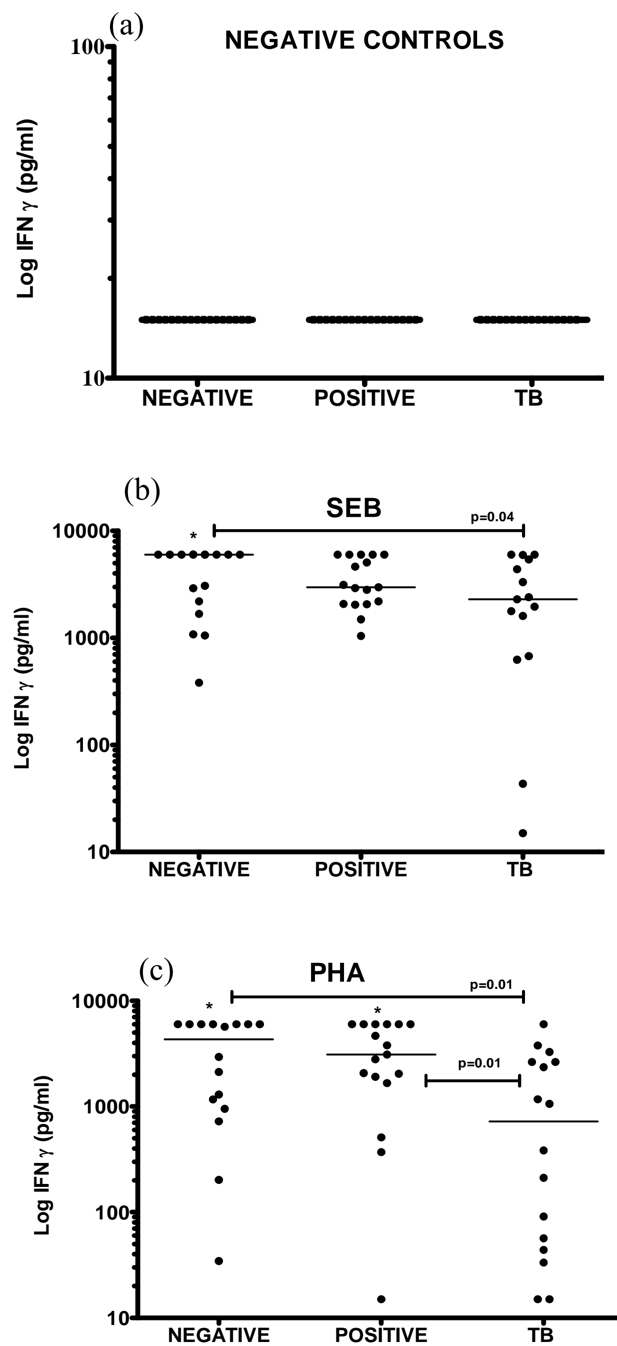


Figure 2.4a-c: Negative control and positive controls used in the interferon-γ ELISA. The ELISA was done on 7-day whole blood culture supernatants of HIV negative participants who were TST negative (n=17), TST positive (n=17) and TB index cases (n=16). The negative control was RPMI alone (a). SEB (1mg/ml), (b) and PHA (5mg/ml), (c) were used as positive controls. The line denotes the median.

Ag85A

Ag85A (Figure 2.5e) elicited responses in 20% (n=3) of the TST positive contacts, 2 of which also responded to TB10.3, while all 3 also responded to TB10.4. Neither the TST negative subjects nor the TB index cases responded to this antigen.

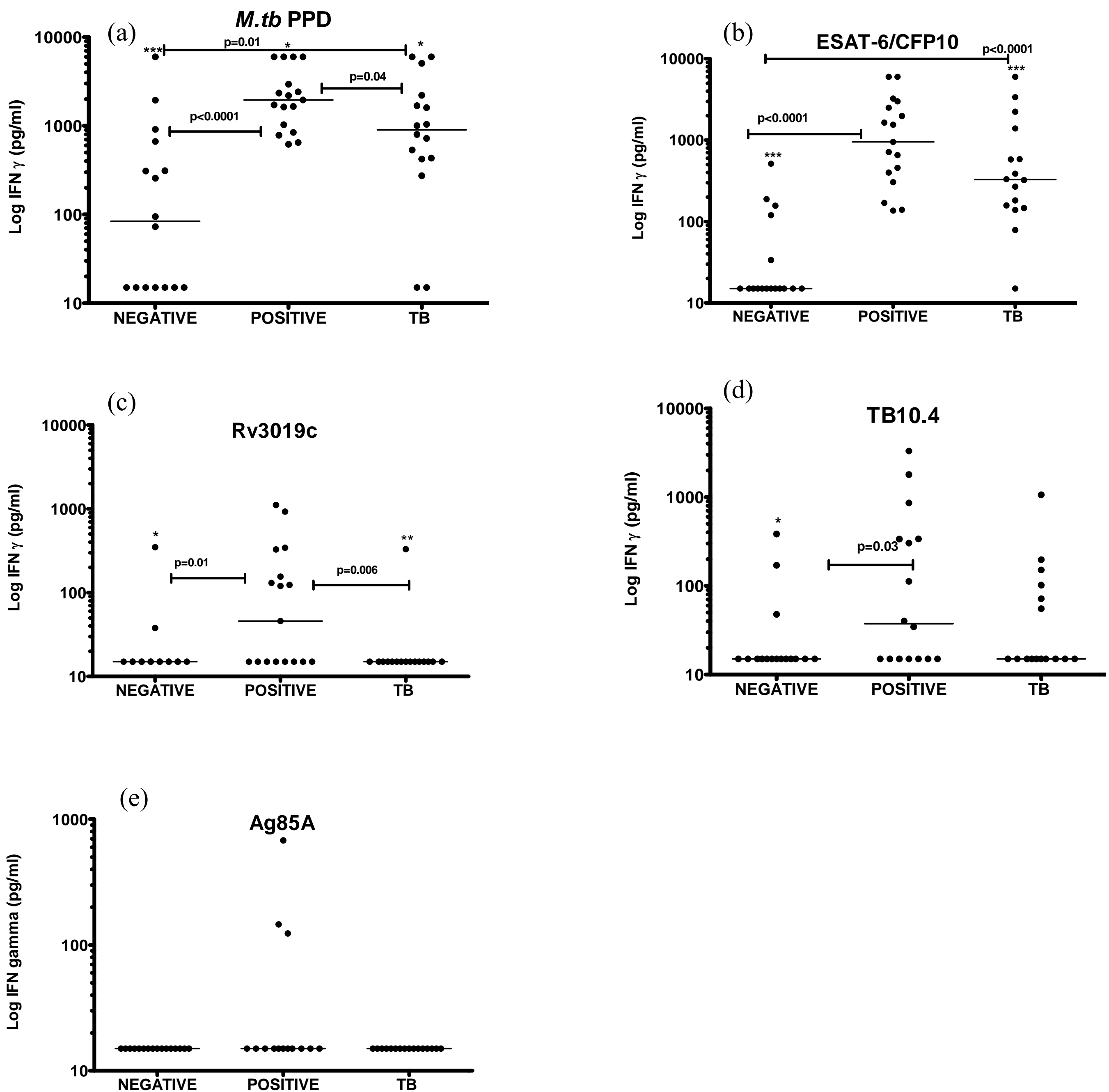


Figure 2.5 a-e: Interferon gamma responses to classical *M.tb* antigens: *M.tb* PPD, ESAT6/CFP10 fusion protein, Rv3019c, TB10.4 and Ag85A. IFN-γ ELISA was performed on 7-day whole blood culture supernatants of TST negative HHC (n=17), TST positive HHC (n=17) and TB index cases (n=16).

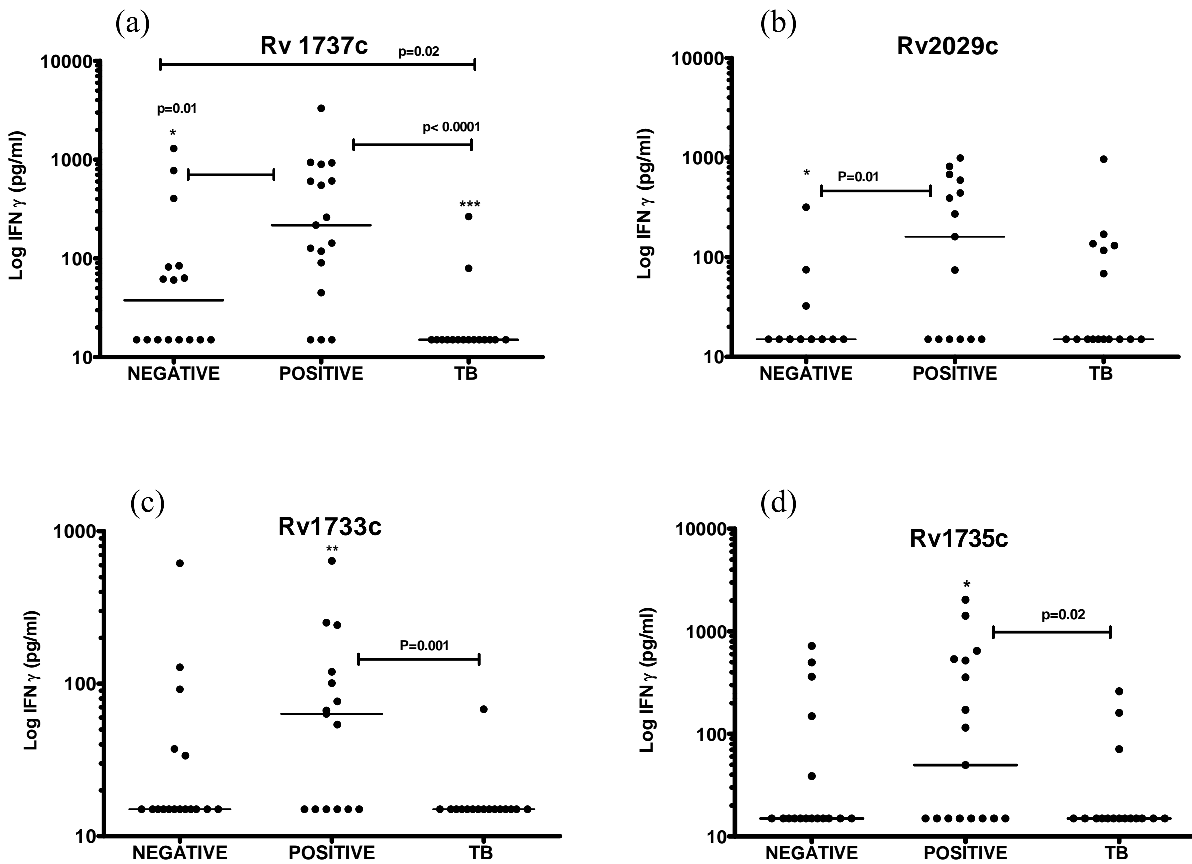


Figure 2.6 a-d: Comparison of IFN-γ responses to DosR regulon-encoded latency antigens in TST negative household contacts, TST positive household contacts and TB cases. IFN-γ levels were measured by ELISA in culture supernatants following 7 day whole blood stimulation with the DosR regulon-encoded antigens. The data shown is for antigens Rv1737c, Rv2029c, Rv1733c and Rv1735c which are antigens that displayed discriminating abilities between disease states. Data of 7 other DosR regulon-encoded antigens tested including Rv0081, Rv0569c, Rv2028c, Rv2031c, Rv3131c, Rv2659 pool C and Rv2660 is not shown.

2.3.6 Comparison of responses to DosR regulon encoded antigens within and between study groups at recruitment

In this section, data is only shown for antigens Rv1737c, Rv2029c, Rv1733c and Rv1735c as they were all able to discriminate between infection and disease states. Data from the other 7 DosR regulon-encoded antigens tested i.e. Rv0081, Rv0569c, Rv2028c, Rv2031c, Rv3131; Rv2659 pool C and Rv2660 is not shown.

TST negative HHC

As shown in Figures 2.6a-d, 35.3% (n=6) of the TST negative subjects responded to Rv1737c, 13.3% (n=2) to Rv2029c. 17.6% (n=3) to Rv1733c and 23.5% (n=4) to Rv1735c. All 3 subjects that responded to Rv1733c also responded to both Rv1735c and Rv1737c. One of the 2 subjects responding to Rv2029c also responded to Rv1733c, Rv1735c and Rv1737c. One subject (5.9%), each responded to Rv0081, Rv2660 and HspX/Rv2031c in the TST negative HHC group (data not shown). No responses were observed to Rv0569, Rv2028c, Rv2659 pool C and Rv3131 in this study group (data not shown).

TST positive HHC

The frequencies of responses to Rv1737c, Rv2029c, Rv1733c and Rv1735c in the TST positive group were 76.5% (n=12), 60% (n=9), 53.3% (n=8) and 47% (n=6) respectively (Figures 2.6a-d). Participants that responded to Rv1735c also responded to Rv1737c, Rv2029c, and Rv1733c; subjects responding to Rv1733c also responded to Rv1737c and Rv2029c; and subjects that responded to Rv2029c also responded to Rv1737c. There were 29.4% (n=5) TST positive participants that responded to Rv0569, whereas 2 subjects (11.7%) responded to Rv2028c in this group and only 1 (5.9%) responded to both Rv0081 and to HspX/Rv2031c. One participant (5.9%) also responded to Rv2659poolC and another (5.9%)

to Rv2660 (data not shown). No responses were observed upon stimulation with Rv3131 in this group (data not shown).

TB cases

Two (12.5%) TB cases responded to stimulation with Rv1737c (Figure 2.6a). The same subjects also responded to Rv1735c (Figure 2.6c). Rv2029c elicited responses in 37.5% (n=6) of the subjects in this group (Figure 5b) and only 1 (6.25%) participant responded to Rv1733c (Figure 2.6d). A single positive response (6.25%) to each of Rv2659 pool C and Rv3131was also observed (data not shown) and no IFN-γ was produced in response to Rv0081, Rv0569, Rv2028c, HspX/Rv2031c or Rv2660 (data not shown).

Significantly higher IFN-γ levels were secreted in response to Rv1737c in TST positive contacts compared to both the TB index cases and the TST negative subjects (Figure 2.6a, $p<0.0001$ and $p=0.01$). Responses to Rv2029c (Figure 2.6b) were significantly enhanced in TST positive participants when compared to TST negative subjects only ($p=0.01$). In contrast, Rv1733c and Rv1735c (Figures 2.6c and 2.6d) induced significantly higher IFN-γ concentrations in TST positive subjects when compared to the TB cases only ($p=0.001$ and $p=0.02$).

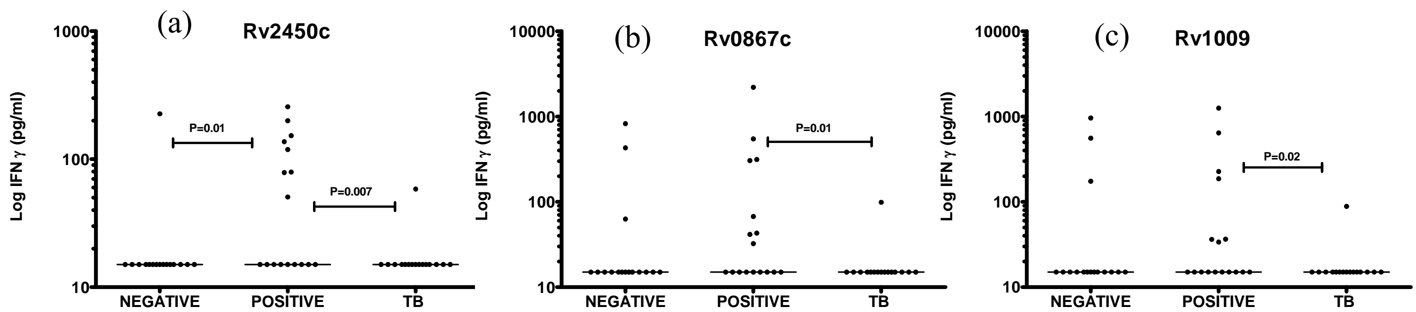


Figure 2.7 a-c: Comparison of TST negative subjects, TST positive subjects and TB index cases in response to stimulation with resuscitation promotion factors. Significant differences in IFN-γ ELISA levels observed between the study groups measured in 7-day whole blood culture supernatants stimulated with (a) Rv2450c, (b) Rv0867c and (c) Rv1009**.**

2.3.7 Comparison of responses to resuscitation promotion factors between study groups

TST negative household contacts

Figures 2.7a shows that 6.25% (n=1) of TST negative participants responded to Rv2450c and 18.75% (n=3) responded to Rv0867c and Rv1009. The individual that responded to Rv2540c also responded to Rv0867c and Rv1009, whereas the 2 (12.5%) others responded to both Rv0867c and Rv1009 in this study group.

TST positive HHC

In Figures 2.7 a-c, 41.1% (n=7) of TST positive subjects responded to Rv2450c; 29.4% (n=5) to Rv0867c and 23.5% (n=4) to Rv1009. Four subjects responded to both Rv2450c and Rv0867c, with 2 (11.25%) of them also responding to Rv1009. Three of the 4 participants that responded to Rv1009 also responded to Rv2450c.

TB cases

Only 1 TB index case (6.25%) responded to both Rv0867c and Rv1009 (Figures 2.7 b and c). The median response to Rv2450c was significantly higher in the TST positive contacts compared to both the TB cases and the TST negative subjects (p=0.007 and p=0.01). Also, TST positive contacts produced significantly enhanced IFN-γ levels to both Rv0867c and Rv1009 when compared to TB index cases (p=0.01 and p=0.02).

Table 2.5: Correlations between *M.tb* antigens in TST negative contacts

	RV1733c	**RV1735c**	**RV1737c**	**RV0569**	**RV1131**	**RV0867c**	**RV1009**	**RV2450c**	**TB10.4**	**RV3019c**
RV1735c	0.78**									
RV1737c		0.63*								
HspX/RV2031				0.64*						
RV1131	0.65*	0.70*	0.61*							
RV0867c	0.82***	0.83***	0.73*		0.84***					
RV1009	0.82***	0.83***	0.73*		0.84***	1.00***				
RV2450c	0.51*	0.51*			0.73*	0.62*	0.62*			
RV3019c	0.65*	0.70*	0.61*		1.00***	0.84***	0.84***	0.73*		
TB10.4	0.49	0.77**	0.56*		0.77**	0.62*	0.62*			0.77**
***M.tb* PPD**	0.62*	0.59*	0.57*			0.62*	0.62*		0.66*	

Table 2.5: Associations between *M.tb* antigens in TST negative HHC were determined using the Spearman Rank Correlations Test. # denotes $p < 0.05$; * denotes $p < 0.01$:** $p < 0.001$;*** $p < 0.0001$

2.3.8 Comparison of responses to reactivation antigens

Two (12.5%) TST negative subjects responded to Rv1131. No responses to Rv3407 or Rv1471 were observed in this group (data not shown). Rv1131, Rv3407 and Rv1471 elicited responses in 17.6% (n=3), 11.8% (n=2) and 5.9% (n=1) respectively of the TST positive contacts (data not shown). One TB index case (6.25%) responded to Rv1131 (data not shown).

2.3.9 Associations between IFN-γ responses to classical and novel M.tb antigens

The Spearman rank correlations test for non parametric samples was used to determine the associations between variables and antigens. Due to the large number of antigens tested (22 in total), only positive ($r>0.5$) and significant ($p<0.05$) correlation co-efficients are shown in all correlation matrices (Tables 2.5 – 2.9).

TST negative HHC

Table 2.5 shows the Spearman correlation co-efficients that were observed between the IFN-γ responses induced by stimulation with all tested *M.tb* antigens in the TST negative HHC. Within the set of 11 Dos-R encoded antigens tested, a very strong and significant association was observed between responses to Rv1733c and Rv1735c ($r = 0.78$, $p<0.01$). Rv1735c was also significantly associated to Rv1737c ($r = 0.63$, $p<0.05$). In addition responses to Rv0569 were significantly correlated with responses to Rv2031c ($r=0.64$, $p<0.05$). Pair-wise responses to each of the 3 tested RPFs were significantly correlated with a particularly strong association observed between Rv0867c and Rv1009 ($r=1$, $p<0.0001$). Responses to Rv2450c were significantly correlated with responses to both Rv0867c and Rv1009 ($r=0.62$, $p<0.05$ respectively). Responses to the classical TB antigen TB10.4 were strongly and significantly associated with responses to Rv3019c (TB10.3; $r=0.77$, $p<0.01$), while also significant to those stimulated by *M.tb* PPD ($r=0.66$, $p<0.05$). Classical *M.tb* antigen Rv3019c was strongly

and significantly associated with all 3RPFs Rv0867c, Rv1009 and Rv2450c (r=0.84, p<0.0001; r=0.84, p<0.0001 and r=0.73, p<0.05 respectively). TB10.4 and *M.tb* PPD, on the other hand, were each significantly associated with both Rv0867c and Rv1009 (both r=0.62, p<0.05). All 3RPFs: Rv2450c, Rv0867c and Rv1009 in this study group were strongly and significantly associated with the reactivation antigen Rv1131 (r=0.73, p<0.05; r=0.84, p<0.0001 and r=0.84, p<0.0001 respectively). Furthermore, Rv1131 was strongly and significantly associated with classical TB antigens Rv3019c and TB10.4 (r=1, p<0.0001 and r=0.77, p<0.01). A strong and significant relationship was also observed between Rv1131 and Rv1735c, while significant associations between this antigen and both Rv1733c and Rv1737c were observed too (r=0.65, p<0.05 and r=0.61, p<0.05). The Dos-R encoded antigens Rv1733c, Rv1735c and Rv1737c, each had strong and significant associations with both Rv0867c and Rv1009 (r=0.82, p<0.0001 and r=0.82, p<0.0001; r=0.83, p<0.0001 and r=0.83, p<0.0001; and r=0.73, p<0.05 and r=0.73, p<0.05 respectively). Rv1733c and Rv1735c, each was significantly associated with Rv2450c (r=0.51, p<0.05). Rv1733c was also significantly correlated with Rv3019c and *M.tb* PPD (r=0.65, p<0.05 and r=0.62, p<0.05. Rv1735c was strong and significantly correlated with Rv3019c and TB10.4, while significantly correlated with *M.tb* PPD (r=0.70, p<0.05; r=0.77, p<0.01 and r=0.59, p<0.05). Rv1737c was also significantly correlated with Rv3019c, TB10.4 and *M.tb* PPD (r=0.61, p<0.05; r=0.56, p<0.05 and r=0.57, p<0.05).

TST Positive HHC

In Table 2.6 the relationships between the clinical data and *M.tb* antigens in TST positive HHC are shown. IFN-γ responses to Rv2029c and TB10.4 were stronger in TST positive HHC with a higher TB contact score (r=0.56, p<0.05 and r=0.68, p<0.05). The older the subjects the stronger their responses to TB10.4 and *M.tb* PPD responses in this study group

Table 2.6: Correlation between the clinical data and *M.tb* antigens in TST positive contacts

	Contact score	Age	BCG Scar R+
RV2029c	0.56*		
RV0867c			0.63*
TB10.4	0.68*	0.50*	
***M.tb* PPD**	0.77**		

Table 2.6: Associations between age, TB contact score, BCG scar, *M.tb* antigens in TST positive HHC were determined using the Spearman Correlation. # denotes p< 0.05; * denotes p< 0.01:** p<0.001;*** p<0.0001

Table 2.7: Correlation between the *M.tb* antigens in TST positive contacts

	RV1733c	RV1735c	RV1737c	RV0569
RV1735c	0.66*			
RV1737c	0.63*	0.86***		
RV2028c		0.59*		
RV2029c	0.75*			
RV2660	0.51*			0.66*
RV1131	0.52*	0.60*	0.53*	
RV0867c		0.90***	0.73**	
RV1009	0.68*	0.71*	0.61*	
RV2450c	0.77***	0.74**	0.70*	
RV3019c	0.76**	0.56*		
TB10.4			0.63*	

The presence of a BCG scar was significantly associated with higher responses to Rv0867c (r=0.63, p<0.05).

Table 2.7 illustrates the associations observed in the responses between *M.tb* antigens in TST positive HHC. Rv1733c was significantly correlated to both Rv1735c and Rv1737c in TST positive subjects (r=0.66, p<0.05 and r=0.63, p<0.05). A significant and strong correlation was observed between Rv1735c and Rv1737c (r=0.86, p<0.0001). Rv1733c was significantly correlated to Rv2660c (r=0.51, p<0.05); Rv1735c was significantly correlated to Rv2028c (r=0.59, p<0.05); Rv0569 was significantly correlated to Rv2660c (r=66, p<0.05) and Rv2028 was significantly correlated to Rv3131 (r=64, p<0.05). Strong and significant associations were observed between responses to Rv1737c and Rv2029c (r=0.75, p<0.05); Rv2028c and Rv2031c (r=0.73, p<0.01) as well as between Rv2659 peptide pool C and Rv3131 (r=1, p<0.0001) in TST positive HHC. When the relationship between the RPFs were investigated, it was observed that there was a strong and significant association between Rv0867c and Rv1009 (r=0.72, p<0.05); whereas Rv2450 was significantly correlated to both Rv0867 and Rv1009 (r=0.62, p<0.05 and r=0.54, p<0.05 respectively). Correlations were observed between antigen responses elicited by RPFs and those caused by other *M.tb* antigens. Strong and significant correlation were observed between Rv0867c and the following antigens: Rv1735c (r=0.90, p<0.0001); Rv1737c (r=0.73, p<0.001) and; Rv3019c (r=0.73, p<0.01). Significant associations were also observed between Rv0867c and the following antigens: Rv2028c (r=0.56, p<0.05); Rv1131 (r=0.65, p<0.05) and; ESAT6/CFP10 fusion protein (r=0.63, p<0.05). A strong and significant correlation was observed between responses caused by Rv1009 and those elicited by Rv1735c (r=0.71, p<0.01). Significant correlations were also observed between Rv1009 and the following antigens: Rv1733c (r=0.68, p<0.05); Rv1737c (r=0.61, p<0.05); Rv2028c (r=0.62, p<0.05); Rv1131 (r=0.52, p<0.05) and; Rv3019c (r=0.56, p<0.05). Rv2450c was strongly and significantly associated

to Rv1733c ($r=0.77$, $p<0.0001$); Rv1735c ($r=0.74$, $p<0.01$) and Rv1737c ($r=0.70$, $p<0.05$). Rv2450c was found to be significantly correlated to Rv2028c ($r=0.56$, $p<0.05$); Rv2029c ($r=0.51$, $p<0.05$); Rv1131 ($r=0.66$, $p<0.05$) and; Rv3019c ($r=0.63$, $p<0.05$). A significant association was observed between reactivation antigens Rv1131 and Rv1471 in this study group ($r=0.61$, $p<0.05$). While Rv1131 was strongly and significantly associated with Rv2660c ($r=0.72$, $p<0.05$), this antigen was also significantly correlated to Rv1733c ($r=0.52$, $p<0.05$); Rv1735c ($r=0.60$, $p<0.05$); Rv1737c ($r=0.53$, $p<0.05$); Rv2659 peptide pool C ($r=0.61$, $p<0.05$); Rv3131 ($r=0.61$, $p<0.05$) and; Rv3019c ($r=0.60$, $p<0.05$). Rv1471 was strongly and significantly associated with Rv2659 peptide pool C ($r=1$, $p<0.0001$) and Rv3131 ($r=1$, $p<0.0001$). Rv1471 was also significantly correlated to 2028c ($r=0.64$, $p<0.05$). Reactivation antigen Rv3407 was strongly and significantly associated with Rv2031c ($r=0.73$, $p<0.01$) and significantly correlated to Rv2028c ($r=0.50$, $p<0.05$). When the relationship between the responses caused by *M.tb* classical antigens and those produced by both other *M.tb* classical as well as *M.tb* antigens in other categories were investigated, it was observed that *M.tb* PPD responses were significantly correlated to those elicited by TB10.4 ($r=0.5$, $p<0.5$). Rv3019c was strongly and significantly associated with Rv1735c ($r=0.76$, $p<0.01$) and this antigen (Rv3019c/TB10.3) was observed to be significantly correlated to both Rv1737c and Rv2028c ($r=0.56$, $p< 0.05$ and $r=0.5$, $p<0.05$ respectively). TB10.4 was significantly associated with Rv1737c ($r=0.63$, $p<0.05$) and strongly significantly correlated to Rv2029c ($r=0.71$, $p<0.05$).

	RV1131	RV0867c	RV1009	RV2450c	RV3019c	Ag85A	ESAT6/ CFP10	TB10.4
RV1471	0.61*							
RV0867c	0.65*						0.63*	
RV1009	0.52*	0.72*						
RV2450c	0.66*	0.62*	0.54*					
RV3019c	0.60*	0.73**	0.56*	0.63*				
TB10.4					0.48	0.46		
***M.tb* PPD**							0.48	0.50*

	RV2028c	RV2029c	HspX/ RV2031	RV2659 pool C	RV2660	RV3131
HspX/RV2031	0.73**					
RV2659 pool C	0.64*					
RV3131	0.64*			1.00***		
RV1131				0.61*	0.72*	0.61*
RV1471	0.64*			1.00***		1.00***
RV3407	0.50*		0.73**			
RV0867c	0.56*					
RV1009	0.62*					
RV2450c	0.56*	0.51*				
RV3019c	0.50*					
ESAT6/CFP10		0.54*				

Table 2.7: Associations between *M.tb* antigens in TST positive HHC were determined using the Spearman Correlation. # denotes $p < 0.05$; * denotes $p < 0.01$:** $p < 0.001$;*** $p < 0.0001$

Table 2.8: Correlations between demographic data and *M.tb* antigens in TB cases

	Sex	Age
RV2029c	0.5*	
***M.tb* PPD**		0.69*

Table 2.8: Associations between age, sex and *M.tb* antigens in TB index cases were determined using the Spearman Correlation. # denotes p< 0.05; * denotes p< 0.01:** p<0.001;*** p<0.0001

Table 2.9: Correlations between *M.tb* antigens in TB cases

	RV1733c	RV1735c	RV1737c	RV2029c	RV3131	RV1131	RV0867c	RV3019c	Esat6/CFP10
RV1735c	0.53*								
RV1737c	0.73*	0.84							
RV2029c									
RV3131									
RV3407					1.00*				
RV0867c	1.00***	0.53*	0.73*						
RV1009	1.00***	0.53*	0.73*				1.00*		
RV2450c						1.00*			
Esat6/CFP10				0.60*					
TB10.4								0.46	0.71*
***M.tb* PPD**									

Table 2.9: Associations between *M.tb* antigens in TB index cases were determined using the Spearman Correlation. # denotes p< 0.05; * denotes p< 0.01:** p<0.001;*** p<0.0001

TB cases

The correlations between age, gender and *M.tb* antigens are described in Table 2.8. Females were more likely to elicit strong IFN-γ responses to Rv2029c ($r=0.50$, $p<0.05$). Older TB cases elicited stronger responses to *M.tb* PPD ($r=0.69$, $p<0.05$).

Table 2.9 shows the association observed between *M.tb* antigens in TB index cases. Rv1733c and Rv1735c, both displayed strong and significant correlations with Rv1737c ($r=0.73$, $p<0.05$ and $r=0.84$, $p<0.0001$ respectively), and Rv1733c was significantly correlated to Rv1735c ($r=0.53$, $p<0.05$). RPFs Rv0867c and Rv1009 were strongly associated with each other ($r=1$, $p<0.0001$). In this study group, ESAT6/CFP10 fusion protein was strongly and significantly associated with TB10.4 ($r=0.71$, $p<0.05$). Reactivation antigen Rv1131 was strongly and significantly associated with Rv2450c ($r=1$, $p<0.0001$) and Rv3407 was strongly and significantly associated with Rv3131 ($r=1$, $p<0.0001$). When the DosR regulon encoded antigens were investigated for associations with RPFs, strong and significant correlations were observed between Rv1733c and both Rv0867c and Rv1009 ($r=1$, $p<0.0001$ and $r=1$, $p<0.0001$). Furthermore, strong and significant associations between Rv1737c and both Rv0867c and Rv1009 ($r=0.73$, $p<0.05$ and $r=0.73$ $p<0.05$). Rv1735c was also significantly correlated to both Rv0867c and Rv1009 ($r=0.53$, $p<0.05$). A significant correlation was observed between responses to Rv2029c and those resulting from ESAT6/CFP10 fusion protein in this group ($r=0.60$, $p<0.05$).

2.4 Discussion

In this study we wanted to investigate whether there were differential immunologic responses to *M.tb* antigens in TST negative household contacts, TST positive household contacts and active TB cases. We did not perform an IGRA because it was part of the study design. We made several assumptions: Firstly, we assumed that the TST negative subjects were not infected with *M.tb* and that TST positive subjects had LTBI. Secondly, we presupposed that TST negative subjects were more protected than both TST positive subjects and TB index cases, and that TST positive subjects were more protected that the TB cases assuming they had been exposed to *M.tb*. Thirdly, we assumed that in TST positive subjects *M.tb* transmission had occurred within the household and that therefore the strain of *M.tb*would be the same. In this investigation we observed that novel *M.tb* antigens could differentiate between non infected, *M.tb* infected and active TB disease.

In the main GC-6 study, 92.8% (n=829) of the 893 HIV negative HHC of TB index cases had tested TST positive at baseline, suggesting a high rate of TB transmission in these communities. A recent study conducted within the same geographical region by Kritzinger *et al.* (2009) has noted the continued escalation of TB incidence. Similar proportions of TST positive subjects (89%) were also observed in a recent study that was conducted in South African gold mines (Hanifa *et al.*, 2009) even though that study was not designed as a TB contact study and both the both HIV negative and HIV positive participants were included in it. On the other hand, a TB contact study that was conducted in Pakistan has shown high rates (80%) of TST positive contacts (Hussain *et al.*, 2007), while in Brazil the proportion of TST positive HHC observed was 43% (Cailleaux-Cezar *et al.*, 2009) and that of The Gambia 27% (Jackson-Sillah, 2007). Several studies have demonstrated that the proportion of healthy HIV negative household contacts of TB index cases that develop active TB within two years of exposure to respective TB cases is between 4 and 6.7% (Teixeira *et al.*, 2001; Wang & Lin,

2000; Hussain *et al*., 2007). Countries that have successfully minimized TB transmission have used tracing of contacts of active TB patients as a vital prevention strategy which involves provision of treatment to those found to be latently infected (Morrisson *et al.*, 2008). These authors assert that the treatment and elimination of latent TB infection may be paramount in the eradication of global TB. Others, however, note that, for developing countries it is the treatment of active TB cases which urgently requires attention in spite of the understanding of the greater need for treatment of LTBI (Baltussen *et al.,* 2005). However, this is true only if the force of infection would decrease, which is not the case in the communities in our study where we have observed a high incidence of both LTBI and active TB. In our investigation with 893 study participants, the proportion of TST negative subjects at baseline was only 7.2% (n=64). At month 6, of the 64 participants, 29 (45.4%) were not skin tested again, 26 (40.6%) had converted to TST positive, while 9 (14%) remained TST negative. The 26 TST negative subjects under investigation in this study, which had converted their TST status by month 6, suggests that they may have been fighting the bacterial challenge before the establishment of the infection or had not been infected for some reason such as increased ventilation within the household or due to anergy.

In our study groups the gradient of exposure to the TB index case did not play a role in the TST results of contacts. The exposure gradient, however, does not include ventilation, which is important in TB transmission. Several studies have observed a positive association between the risk of being TST positive in TB contacts and the extent of exposure to the TB index case (Lienhardt *et al.*, 2003; Adetifa *et al.*, 2007; Gustafson *et al.*, 2007). In our setting, maybe non household exposure such as in the public transport, sheebens, schools etc. is more important. Age did not affect the TST result in our investigation, but our cohort was comprised of adults. Which may part explain why Gustafson *et al.* (2007) observed that there was a tendency for adult household contacts to test TST positive. In our study, we observed

that older subjects produced stronger IFN-γ responses to TB10.4 and *M.tb* PPD in TST positive contacts. In TB index cases age was positively associated with stronger responses to *M.tb* PPD only. In this study group we also observed that females were more likely to elicit stronger responses to Rv2029c. The BCG scar status (presence or absence) did not have an influence on the TST results in our study. This is in agreement with other studies (Lienhardt *et al.*, 2003; Machado *et al.*, 2009; Adetifa *et al.,* 2010). Further, we did not observe a relationship between BCG status and induration size in the TST positive group at recruitment or the TST negative group following conversion at 6 months**.** Whereas Lewinshon *et al.*, (2008) did not observe any association between BCG status and IFN-γ responses to *M.tb* antigens, we did observe a positive association between the BCG scar and responses to Rv0867c in the TST positive group only. BCG vaccination has also been shown to lead to increased IFN-γ responses to PPD and Ag85A in TST negative subjects (Lin *et al.*, 2007). The differences that we observed in our study compared to those of others may be due to regional differences or genetics or some other variable. Although not significant, 83% of TST positive subjects had been exposed to 2+ or higher graded sputum smear TB cases compared to 72% observed with TST negative contacts. The increased risk of being TST positive in HHC of TB cases with higher smear grades has been observed in other studies (Lienhardt *et al.,* 2003; Okada *et al.*, 2008; Akhtar & Rathi, 2009). Chee *et al.*, (2004) have observed a trend for contacts that were exposed to more infectious TB index cases to have greater TST induration.

We observed that TST induration in TST positive contacts was not associated with antigen stimulated IFN-γ production. Earlier, Arend and colleagues (2001) showed that T cell responses to *M.tb* culture filtrate protein, PPD, ESAT-6, CFP10 and TB10.4 were associated with TST results. Recently, the size of the TST induration in TST positive subjects has been linked to antigenic stimulation including stimulation with *M.tb* culture filtrate protein

(Lewinshon *et al.*, 2008), TB10.3 in Uganda and ESAT6/CFP10 fusion protein in South Africa (Black *et al.*, 2009).

There was no spontaneous IFN-γ production from unstimulated whole blood across all the groups tested in our study. With the exception of 2 TB cases, all participants elicited positive responses to SEB. We observed that stimulation with PHA resulted in higher levels of IFN-γ in both TST negative and TST positive groups compared to TB cases. Previously, it has been demonstrated that responses to PHA by TB patients was significantly lower than those produced by household contacts (Vekemans *et al.*, 2001). Our results also show significantly lower levels of IFN-γ production in response to PHA in active TB cases.

We investigated IFN-γ responses in TST negative contacts, TST positive contacts and TB index cases and observed that *M.tb* PPD stimulation elicited higher IFN-γ responses in TST positive contacts when compared to both TST negative contacts and TB index cases. In concordance, it has been shown that IFN-γ responses to *M.tb* PPD were elevated in TST positive subjects compared to TST negative subjects (Arend *et al.*, 2001; Ordway *et al.,* 2004). It has also been shown that latently infected individuals had high IFN-γ responses to PPD compared to TB cases (Demissie *et al.*, 2006). TST positive subjects have also been shown to elicit stronger IFN-γ responses compared to active TB cases when stimulated with *M.tb* antigens (Leyten *et al.,* 2006; Schuck *et al.,* 2009). While Ribeiro-Rodrigues and co-workers (2006) have observed that *M.tb* specific T cell responses were suppressed in TB cases when compared to TST positive subjects, they observed higher CD4+ cells in TB cases than in TST positive subjects. Others have shown that δγ IFN-γ producing T cells were elevated in TST positive subjects compared to TST negative subjects when stimulated with heat killed *M.tb* (Ordway *et al.*, 2004). Recently, Sutherland *et al.*, (2009) did not find significant differences in IFN-γ $CD4^+$ producing cells between TST positive and TST

negative contacts, nor between TB index cases and TST positive contacts, due to stimulation with PPD. The source of increased IFN-γ that we observed in our study may be due to the δγ T cells. In TST positive subjects, δγ T cells are thought play an important anti-bacterical role in *M.tb* (Dagna *et al.*, 2002). In our study, we noted that the TB cases produced higher IFN-γ responses to *M.tb* PPD when compared to TST negative contacts. Higher IFN-γ $CD4^{+}$cells have been observed in active TB cases compared to TST negative HHC in response to PPD (Sutherland *et al.*, 2009). We observed that 60% (9/15) of the TST negative household contacts responded to *M.tb* PPD. While responses to *M.tb* PPD have previously been observed in *M.tb* unexposed, non-BCG vaccinated TST negative controls (Lin *et al.*, 2007), in our study, we cannot rule out the possibility of responses being as a result of BCG vaccination as 4 participants that responded to *M.tb* PPD had evidence of prior vaccination, although it is not known how long ago they had been vaccinated. Of the 9 participants that responded to *M.tb* PPD, 2 also responded to ESAT6/CFP10 suggesting that they may have become infected with *M.tb* without mounting a DTH response at that time point because 6 months into the study TST indurations were observed in those subjects. It has been hypothesized that BCG vaccination or possible exposure to environmental mycobacteria, many of which share *M.tb* PPD antigens, may be responsible for some of the responses to *M.tb* PPD (Lin *et al.*, 2009). The observation of a stronger *in vitro* response to *M.tb* PPD in the TST positive group in this investigation is not surprising because the tuberculin skin test also uses *M.tb* PPD. However there was no correlation between TST induration and the IFN-γ response to PPD in the TST positive group.

ESAT6/CFP10 fusion protein

As expected all TST positive HHC responded to ESAT6/CFP10 fusion protein, 87.5 % of the TB cases, and just 26.7% (n=4) of the TST negative participants. Both TST positive HHC

and TB cases exhibited markedly higher responses to the fusion protein compared to the TST negative group. ESAT6 is an immuno-dominant *M.tb* antigen (Rogerson *et al.*, 2005). IFN-γ producing $CD4^{+}$ cells have been shown to increase in response to ESAT6/CFP10 fusion protein and *M.tb* PPD in latently infected individuals (Schuck *et al.,* 2009). It is interesting to note that in the TST negative group 3 of the 4 subjects that responded to ESAT6/CFP10 fusion protein also responded to *M.tb* PPD. While we did not use the IGRA to test the participants for LTBI, ESAT6 and CFP10, a combination of antigens that was used in this study are used to diagnose LTBI in IGRA (Ruhwald & Ravn, 2009). A positive IGRA result is thought to be indicative of recent exposure to *M.tb* (Nakaoka *et al.*, 2006; Arend *et al.*, 2007). In this investigation, the TST negative subjects that produced IFN-γ in response to ESAT6/CFP10 fusion protein may be an indication of an early infection which could not be picked up by a TST, a phenomenon that may be partly explained by the current opinion that suggests the existence of the presence of a spectrum of events from inhalation to infection which may or may not lead to active disease (Young *et al.*, 2009; Barry *et al.*, 2009). All TST negative subjects in this cohort converted their TST status to positive by month 6, indicating that they may have become latently infected by the follow up time point, 6 months later.

In the TST positive group the median IFN-γ response to TB10.3 (343pg/ml) was significantly lower than that induced by *M.tb* PPD (1625pg/ml), or ESAT6/CFP10 fusion protein (1973pg/ml) indicating that this recombinant antigen was less immunogenic than PPD or the fusion protein. Significantly higher IFN-γ levels were observed in response to TB10.3 between the TST positive participants and both TB index cases and TST negative participants. *M.tb* PPD is a mixture of 173 proteins (Borsuk *et al.*, 2009), which may explain the strength of its immunogenicity compared to other classical *M.tb* antigens.

TB10.3 and TB10.4 are part of a family of ESAT proteins that are highly expressed during *M.tb* infection (Rogerson, 2006). The percentage of IFN-γ responders to TB10.4 (41.2%) in TST positive HHC was similar to that observed for TB10.3 (47%). In this group 6 out of the 7 participants that responded to TB10.4 also responded to TB10.3. TB10.4 has been observed to be more inmmunogenic than TB10.3 in TB patients (Skjøt *et al.*, 2002). Also, a recent mouse model has demonstrated the immunogenicity of TB10.4 with the evidence of highly expressed CD8 and CD4 T cells during *M.tb* infection (Hoang *et al.*, 2009). While that study did not compare the immunogenicity of TB10.3 to that of TB10.4, and TB10.4 we observe in our study that TB10.3 was more immunogenic than TB10.4 in TST positive household contacts, whereas it was similar in TST negative HHC and in TB cases. These differences may be as a result of population differences or *M.tb* strain differences.

Of the classical *M.tb* antigens, Ag85A was the least immunogenic in this study, producing responses in 20% of the TST positive contacts only. Positive responses to Ag85A have been observed in TST negative, *M.tb* unexposed BCG vaccinated adults (Lin *et al.,* 2007). The differences in the responses to this antigen may be population specific.

We investigated the differential responses to 11 DosR regulon antigens in our study groups. Rv1737c elicited higher IFN-γ levels in TST positive contacts compared to both TB index cases and TST negative subjects. Furthermore, we observed higher responses to this antigen in TST negative subjects compared to TB cases. Both Rv1733c and Rv1735c induced significantly higher IFN-γ concentrations in TST positive subjects when compared to the TB cases only. Rv2029c resulted in enhanced IFN-γ production in TST positive participants when compared to TST negative subjects only. Rv2029c has been shown to discriminate between ESAT6/CFP10 responders and non-responders (Lin *et al.,* 2007). Also, it has previously been demonstrated that IFN-γ responses to both Rv1733c, Rv2029c were

enhanced in TST positive subjects compared to TB cases (Leyten *et al.*, 2006). Schuck *et al.,* (2009) have also observed higher IFN-γ responses to Rv1733c in TST positive subjects compared to TB cases. With respect to Rv1733c, our results are in agreement with those seen by others. However, in this study Rv2029c could discriminate between TST positive and TST negative participants only. The other 7 DosR regulon-encoded antigens tested including Rv0081, Rv0569c, Rv2028c, Rv2031c, Rv3131, Rv2659 pool C and Rv2660 did not display abilities to discriminate between the study groups in our study.

IFN-γ responses to resuscitation promotion factors Rv2450c, Rv0867c and Rv1009 were compared between TST positive and TST negative contacts as well TB cases. We observed higher IFN-γ concentration in response to Rv2450c in TST positive contacts compared to both the TB cases and the TST negative subjects. Also, both Rv0867c and Rv1009 elicited higher IFN-γ levels to TST positive contacts compared to TB index cases. While this is in line with what has been shown previously with respect to Rv1009 and Rv2450c and their abilities to elicit higher IFN-γ levels in TST positive subjects than in TB cases (Schuck *et al.*, 2009), in our investigation, we also observed higher responses to Rv2450c in TST positive subjects than those of TST negative subjects. Perhaps, Rv2450c may offer a possibility for further investigations in our setting.

We also investigated immunologic responses to reactivation antigens Rv1131, Rv1471 and Rv3407 in our study groups. Schuck *et al.* (2009) have shown Rv3407 elicited IFN-γ production in latently infected subjects but not in TB cases, but we did not observe these properties in our investigation. The differences in the results may be explained by the population differences or *M.tb* strains.

We investigated the associations between variables and between antigens in all the study groups. As expected Rv1733c, Rv1735c and Rv1737c were positively associated in all study

groups. The same pattern was observed with all RPFs Rv2450c, Rv0867c and Rv1009. In the TST negative group, the classical *M.tb* antigen TB10.4 was positively associated with both TB10.3 (Rv3019c) and *M.tb* PPD, whereas ESAT6/CFP10 fusion protein did not correlate with any of the novel antigens tested in this study.

When investigating correlations between antigens in TST positive contacts, we observed a positive association between TB10.4 and *M.tb* PPD**.** It has been recently demonstrated in TB contacts that strong responses to both *M.tb* PPD and ESAT6/CFP10 were associated with inferior mycobacterial killing abilities, compared to ESAT6/CFP10 non-responders that responded to *M.tb* PPD (Kang *et al.*, 2010). These authors suggest that the capacity to kill mycobacteria could be indicative of the strength of adaptive immunity against *M.tb*. While all TST positive subjects responded to both *M.tb* PPD and ESAT6/CFP10 fusion protein, no significant correlation was observed between these antigens in this study group. This suggests that subjects that elicited strong responses to *M.tb* PPD did not produce equally strong responses to ESAT6/CFP10 and may be falling outside of the group with purported inferior mycobacterial killing capabilities. In TST negative participants we observed that 3 of the 4 subjects that responded to ESAT6/CFP10 fusion protein also responded to *M.tb* PPD. Although none of the participants in both contact groups in our study had progressed to active disease in the two year follow up period, their susceptibility to active future disease cannot be ruled out. We did not observe any significant correlation between Rv3019c (TB10.3) and TB10.4 in the TST positive group in this study even though we noted that there was a similar proportion of responders to these classical *M.tb* antigens (TB10.3, 47%, n=8; TB10.4, 43.7%, n=7); moreover 6 out of the 7 participants that responded to TB10.4 also responded to TB10.3. In TB index cases we observed a positive correlation between ESAT6/CFP10 fusion protein and both TB10.4 and Rv2029c.

In our investigation we discovered that there were some antigens that displayed discriminating abilities between non-infection, infection and TB disease which were also significantly correlated. In both TST positive subjects and TB cases, ESAT6/CFP10 was significantly correlated to Rv2029c. The work of Lin and others (2007) also demonstrated that 81% of TST positive subjects that responded to ESAT6/CFP10 fusion protein also responded to Rv2029c. Both Rv2029c and the fusion protein could distinguish between TST negative and TST positive subjects. It is important to note that none of the ESAT6/CFP10 responders in the TST negative group responded to Rv2029c which is a DosR-regulon encoded antigen. As Rv2029c is a Dos R encoded antigen, this could mean that these subjects had been recently infected and the bacteria were still actively replicating and had not yet entered dormancy. In addition, Rv2029c was positively correlated to Rv2450c in the TST positive group. Within the TST negative study group there was 1 participant that responded to Rv2029c and another 1 responded to Rv2450c. While the positive responder to R2029c in the TST negative group did not respond to ESAT6/CFP10 fusion protein, the responder to Rv2450c also responded to the fusion protein, suggesting the latter responder may have been latently infected. Our results suggest that a combination of Rv2029 to ESAT6/CFP10 fusion protein may be useful in distinguishing between latently infected and non infected individuals.

In conclusion, 11 antigens could discriminate between the not infected, *M.tb* infection and active TB including *M.tb* PPD, Rv3019c, ESAT6/CFP10 fusion protein, TB10.4, Rv1737c, Rv1733c, Rv1735c, Rv2029c, Rv2450c, Rv0867c and Rv1009. The capability of both Rv2029c and Rv2450c antigens to distinguish between infection and disease states and their positive association in the TST positive group only may be important in the identification of possible diagnostic or vaccine candidates. It is important to be able to distinguish between

LTBI and active TB. In our investigation, however, we did not find any promising candidates for distinguishing between LTBI and active TB.

CHAPTER 3: MULTI-PLEX CYTOKINE ANALYSIS IN ADULT HIV NEGATIVE FAST AND SLOW TST CONVERTORS

3.1 Introduction

For a long time it was believed that IFN-γ was the single most critical cytokine required for protection against TB (Flynn *et al.*, 1993). There is mounting evidence which indicates that while its importance cannot be disputed, IFN-γ alone cannot confer protection against *M.tb* and that there may be additional cytokines and chemokines that may be protective against this pathogen (Flynn & Chan, 2001).

Cytokines are simple polypeptides that are produced in response to antigens. They act in an autocrine or paracrine manner which may lead to an increase or decrease in the rate of cell proliferation, changes in cell differentiation states and/ or a change in the expression of some functions (Vilcék, 2003).

Studies into the host immune response have been made possible by cell culture systems with recombinant purified proteins used to stimulate the production of cytokines. This study was intended to supplement the findings of chapter 2, where IFN-γ was utilized as a marker of the immune profile of TST negative and TST positive contacts as well as TB cases in response to 11 latency antigens, 3 reactivation associated antigens and 3 RPFs (LUMC) plus 4 classical TB antigens. In chapter 2 we observed that 11 stage-specific antigens of *M.tb* had the ability to discriminate between participants that were TST negative and those who were either TST positive and/ or had active TB and between TST positive and active TB. Those antigens include *M.tb* PPD, ESAT6/CFP10 fusion protein, Rv3019c, TB10.4, Rv1733c, Rv1735c, Rv1737c, Rv2029c, Rv2450c, Rv0867c and Rv1009.

In this investigation we used the Luminex platform to study multi-cytokine immune responses to *M.tb* antigens in the TST negative group described in Chapter 2. Cytokine responses were measured at recruitment and 6 months later, after 17 of the 26 subjects had converted to TST positive. Luminex technology is a fluorescent bead based time-saving technique that operates on the sandwich principle similar to that of the ELISA assay. This tool presents an opportunity to study and to quantify immune responses by simultaneously measuring up to 100 cytokines in theory in small samples, which in most cases are precious. Because this platform is expensive, only the conditions with the strongest discriminating abilities were included in this investigation, namely:- *M.tb* PPD, ESAT6/CFP10 fusion protein, Rv1733c, Rv1737c, Rv2029c and Rv2450c. As stated in the previous chapter at study recruitment *M.tb* PPD and Rv1737c could discriminate between TST negative and TST positive contacts; between TST negative contacts and TB cases as well as between TST positive contacts and TB cases. ESAT6/CFP10 fusion protein could discriminate between TST negative and TST positive contacts as wells as between TST negative contacts and TB cases. Rv2450c was able to differentiate between TST negative and TST positive contacts as well as between TST positive contacts and TB cases. Even though both Rv1733c and Rv1735c could distinguish between TST negative and TST positive contacts, Rv1733c elicited the most significant differences and on that basis was included in this study.

3.2 Materials And Methods

3.2.1 Study population

Table 3. 1 summarizes the demographic data of the participants included in the Luminex study. The two study groups investigated in this chapter, consisting of 17 fast TST convertors and 9 slow TST convertors, are a sub-set of the 64 subjects that tested TST negative at recruitment. All the fast convertors tested in this chapter had also been assayed for IFN-γ production levels in response to *M.tb* antigens in the previous chapter. In the slow TST convertor study group, 6 participants had supernatants available for the recruitment time point, 8 had samples available for the 6 month time point. The one study participant that remained TST negative at month 18 was included in the slow TST convertor study group. The median age of the fast TST convertors (25 years) was significantly greater than that of the slow TST convertors (15 years, p=0.04) with a BCG scar present in 41.1% of fast TST convertors compared to 77.7 % of slow TST convertors**.** Exposure of the study participants to their respective TB index cases measured with the contact score described in the previous chapter was similar (12) between slow and fast convertors.

Table 3.1 Clinical and demographic data of the study population. 26 HIV negative, TST negative subjects were enrolled into the study and followed for 6, 18 and 24 months. 17 participants converted to TST positive by month 6 (fast TST convertors), while 9 remained TST negative, (slow TST convertors). By month 18, all but 1 had converted to TST positive. TST was not done at month 24.

	Fast convertors	**Slow convertors**
Number of participants	17	9
Age median in years, (range) in years	25 (10-52)	15(10-55)
Male/female ratio	6/11	5/4
BCG vaccine documented or scar present, n (%)	17 (41.1)	7 (77.7)
Median TST induration at month 6, mm (range, mm)	16 (10-24)	0
Median *M.tb* contact score	12 (8-14)	12 (10-13)
Number with contact score not available, n (%)	1 (5.8)	0

Table 3.2.1 Cytokines and chemokines included in the 27-plex Luminex kit and secreted by cells of the innate immune system.

Cytokine/ chemokine	Major source	Target cells	Function
VEGF	Endothelial cells	Endothelial & dendritic cells	Proliferation, angiogenesis, increased vascular permeability
	Hematopoietic cells	Hematopoietic cells & Monocytes	Promotes differentiation and chemo-attraction
TGFα	Macrophages& epithelial cells	Macrophages	Mitogenic Stimulates cell adhesion and proliferation
EGF	Macrophages & epithelial cells	Epithelial cells	Cell proliferation, migration, differentiation Up-regulates VEGF induction
Eotaxin	Endothelial cells & fibroblasts	Eosinophils, mast cells, basophils	Proliferation, differentiation, chemo-attractant for fibroblast and epithelial cells
Fractalkine	Endothelial cells Epithelial cells, Dendritic cells	NK cells, lymphocytes, monocytes	Mediates cell adhesion, enhances NK cell cytotoxicity, stimulates cell migration, induces activation of phosphoinositide3 kinase & mitogen activated protein kinase and stimulates calcium mobilisation
G-CSF	Bone marrow stromal cells, fibroblasts, endothelial, macrophages	Bone marrow stromal cells Neutrophils	Proliferation and maturation of granulocyte progenitors and precursors Promotes and survival of neutrophils
IP-10	Macrophages	Neutrophils, Activated T cells Endothelial cells Monocytes	Chemo attractant for neutrophils Enhances neutrophil degranulation Promotes growth and enhances inflammatory properties Promotes chemotaxis
MCP-1	Monocytes, endothelial cells, keratinocytes, fibroblasts & neutrophils	T cells, hematopoietic progenitor cells. Endothelial cells	Proliferation of hematopoietic progenitor cells, chemo-attractant for T cells macrophages & neutrophils Responsible for angiogenesis
MIP-1α/ CCL3	Monocytes, NK cells, macrophages, epithelial and endothelial cells, fibroblasts	Neutrophils, macrophages, NK cells	Chemo attractant for neutrophils, macrophages & NK cells
MIP-1β/ CCL4/LAG-1	Macrophages	Granulocytes & macrophages	Activation of granulocytes, induction of pro-inflammatory cytokines, proliferation of hematopoietic progenitor cells
IL-1RA	Monocytes, macrophages, neutrophils, fibroblasts and epithelial cells	Macrophages T cells, islet cells	Inhibits release of IL-1 and TNF-α from macrophages Inhibits release of IL-2 and expression of IL-2R in T cells Blocks proliferation of T cells
IL-12 (IL-12p70)	Monocytes, macrophages and dendritic cells	T cells NK cells B cells Macrophages and DCs	Promotes differentiation and proliferation of T cells. Stimulates cytotoxic effects Promotes antibody production. Enhances the antigen presenting function.
(IL-12p40)		Hematopoietic stem cells & antigen presenting cells	Activates early hematopoiesis. Determines IL-12 production: Antagonist to IL-12p70

Langrish *et al*., 2004; Xia &Sui, 2009;

Gabrilovich & Dikov, 2003; Mukaida *et al*., 2003

3 2.2 The Luminex Assay

Luminex technology is an immunoassay that uses fluorescent beads to determine the profile of multiple cytokines and chemokines in a single sample. Four 27-plex kits were obtained from Millipore (Cat. No. MPXHCYTO-60K-27, Lot no. 1643604, expiry date 30/09/2010). Tables 3.2.1 – 3.2.3 show the 27 chemokines/ cytokines measured and which cell types have been reported to produce them. A plate layout template was designed for each Luminex plate and the assay was done according to the manufacturer's instructions. The experiments and all incubation steps were done at room temperature. A standard curve ranging from 3.2 pg/ml – 10 000 pg/ml was generated by serial dilution of reconstituted standard.

Each multiplex kit contains two pre-mixed quality control solutions, namely QC-I and QC-II, which are used to validate the results of each assay. These control solutions are included in duplicate on every plate and have pre-determined acceptable measurement ranges for each analyte.

Study samples were supernatants derived from 7-day culture of diluted whole blood, with *M.tb* antigens *M.tb* PPD, ESAT6/CFP10 fusion protein, Rv1733c, Rv1737c, Rv2029c and Rv2450c. As described in the previous chapter, the supernatants were harvested and stored at – 80 ^{0}C. For this investigation culture supernatants were thawed at room temperature, mixed by vortexing and then centrifuged for 10 minutes at 1500rpm in order for cellular debris to settle. Supernatants were tested undiluted and in single wells. For each participant, unstimulated culture supernatant was added in single wells, as per plate layout template. The blank, with RPMI (containing 1% L-glutamine) only was included on each plate in duplicate. The filter of each well was first blocked with 200µl of the assay buffer for 10 minutes on a plate shaker. The buffer was removed by vacuum aspiration and 25µl of the assay diluent was added to wells designated for samples while RPMI was added to those wells selected for

standards. Samples and standards were added as per template, followed by the addition of 25µl antibody coated fluorescent beads, after which plates were incubated for an hour on a plate shaker. After the fluid was removed by vacuum aspiration, a biotinylated secondary antibody and streptavidin-phycoerythrin labeled antibodies were added with alternate incubation and washing steps between the additions of each antibody. The last step was the addition of 100µl of sheath fluid for 5 minutes, followed by reading on the Bio-plex assay reader using a 5 parameter logistic regression (5PL) curve. The channel through which the fluorescent beads flow for measurement into the assay reader is called RP1. Fluorescence detected in samples and controls was read using both low RP1 and high RP1fluorescent channels. It is important to read the results with both channels because while the low RP1 channel measures a broad range of cytokines/chemokines with a large array of concentrations, the high RP1 channel has greater sensitivity and is valuable when measuring cytokines/chemokines present in low concentrations.

The negative control value was subtracted from all antigen induced responses prior to analysis.

Table 3.2.2 Cytokines and chemokines included in the 27-plex Luminex kit and secreted by cells of both the innate and the adaptive immune systems.

Cytokine/ Chemokine	Major source	Target cells	Function
IL-1: IL-1α, IL-1β	Monocytes, macrophages, dendritic cells, T and B cells, NK cells.	Monocytes, eosinophils, dendritic cells & fibroblasts. Vascular endothelial cells. T and B cells	Pro-inflammation: Initiation of COX-2, type2 phospholipase A and iNOS Co-stimulatory for lymphocyte proliferation. Involved in antigen presentation. Increase the expression of ICAM-1 and VCAM-1. Increases T-cell dependent antibody production
IL-4	CD 4+, CD8+ T cells, NK cells, basophils, and mast cells and eosinophils	B cells T cells NK cells Mast cells Endothelial cells	Increases MHC-II antigens and co-stimulatory molecules on B- cells. Induces isotype switching. Enhances antibody production & CD3 expression. Induces CD40 – CD40L dependent proliferation. Growth of T helper cells and cytotoxic T cells Promotes TH1 and TH2 cell proliferation. Promotes histamine release. Up-regulates VCAM1 expression
IL-6	T cells fibroblasts	Hematopoietic stem cells Hepatocytes B cells, T cells Eosinophils	Induces hematopoiesis Stimulates acute phase protein production. Induces proliferation and differentiation. Promotes activation, growth and differentiation
IL-8	Monocytes, T cells, neutrophils, NK cells, endothelial and epithelial cells	Neutrophils T cells Basophils	Promotes chemotaxis, increases intracellular calcium, respiratory burst, endothelial cell adhesion and migration. Promotes degranulation & Histamine release
IL-10	T cells, B cells, monocytes and macrophages, NK cells	B & Tcells, monocytes, macrophages, Dendritic cells, NK cells	Suppress immune responses on T cells, B cells, antigen presenting cells and to skew the immune responses from TH1 to TH2
IL-13	Macrophages, dendritic cells, B and T cells, NK cells, basophils, eosinophils, mast cells	B cells Monocytes/ macrophages DCs, macrophages & Eosinophils Endothelial cells, Epithelial cells.	Enhances antibody production. Up-regulates expression of adhesion molecules. Promotes VCAM-1 expression.
IFNγ	T cells, NK cells, Lymphocytes, dendritic cells, macrophages	Macrophages Phagocytes T cells B cells T and B cells	Stimulates TNF-α & IL-12 production Promotes macrophage activation killing of non specific intracellular and extracellular pathogens. Assists in TH1 development. Regulates B-cell development, proliferation & antibody secretion. Isotype switching, promotes antigen processing and presentation
TNF-α	Macrophages, monocytes, NK cells, B cells, T cells, basophils, mast cells, neutrophils, fibroblasts	Monocytes/ Macrophages Neutrophils NK cells Vascular endothelial cells	Induction of IL-1, IL-8,GM-CSF, IFNγ, TGFβ, EG2, Chemotaxis, Phagocytosis NK cell function Modulates angiogenesis & increased permeability
GM-CSF	T cells, neutrophils, macrophages, NK cells, fibroblasts, epithelial cells.	Neutrophils, Macrophages & dendritic cells	Stimulation of anti-microbial properties. Enhances uptake of opsonized pathogens. Increases phagocytosis Enhances microbial killing.

Enzler &Dranhoff, 2003;
De la Barrera *et al.,* 2004

Table 3.2.3 Cytokines and chemokines included in the 27-plex Luminex kit and secreted by cells of the adaptive immune system

Cytokine/ chemokine	**Major source**	**Target cells**	**Function**
IL-2	CD4+ T cells	T and B cells NK cells Monocytes	Enhances proliferation and differentiation Augments cytolytic activity of NK cells. Stimulates cytolytic activity. Promotes the production of GM-CSF, IL-1β, IL-6.
IL-5	T cells	Eosinophils Basophils B cells	Activates eosinophils. Stimulates histamine Stimulates IgA production.
IL-17	T cells	Bone marrow cells, macrophages, NK cells fibroblasts T cells,	Proliferation and differentiation of T cells, activation of NK cells; induces hematopoiesis Augments the production of pro-inflammatory chemokines and cytokines

Kok *et al.,* 2003; Boyman *et al.,* 2007.

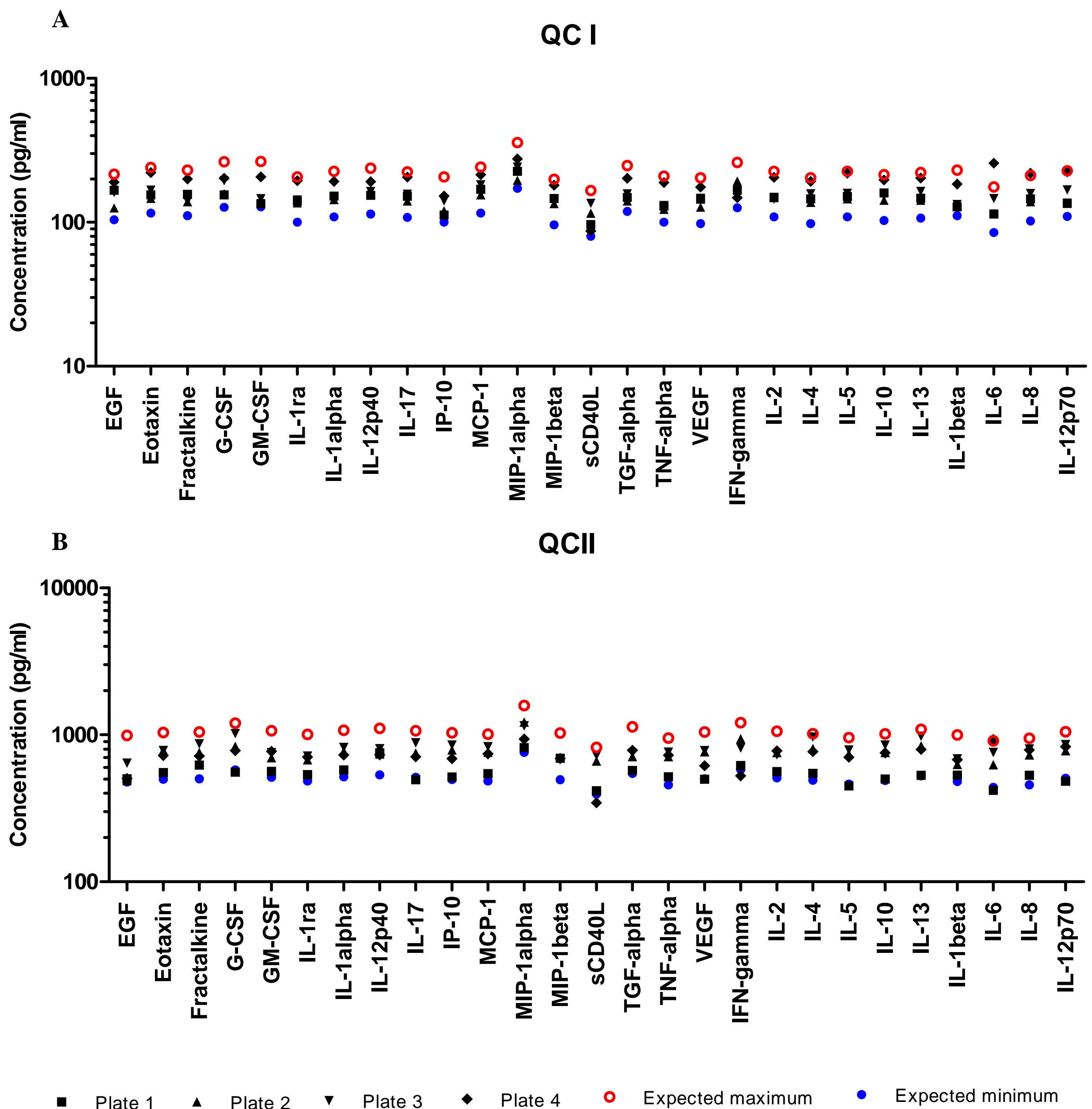


Figure 3.1A - B: Luminex assay quality control assessment _QCI (A) and QCII (B). Four 27-plex kits were used. Quality control evaluation was measured in duplicate for QCI and QCII for each analyte of the 27 cytokines/chemokines. Expected minimum and maximum values for each analyte are shown with observed per plate in (A) QCI and (B) QCII.

3. 2.3 Results of Kit Validation and Quality Control (QC)

The quality control validation results indicated that the 4 kits used in this study had performed optimally (Figure 3.1 A and B). The recoveries of the 27 chemokines/ cytokines were all within the desired 70% - 130% range. In both QC-I and QC-II the observed values were within the expected ranges for all analytes in all plates with the exception of plate 4 where IL-6 levels were higher (257pg/ml and 928pg/ml) than the expected maximum values of 176pg/ml and 918pg/ml respectively. For QC-II in plate 4, the observed sCD40L level (396pg/ml) was found to be slightly lower than the minimum expected concentration (416pg/ml).

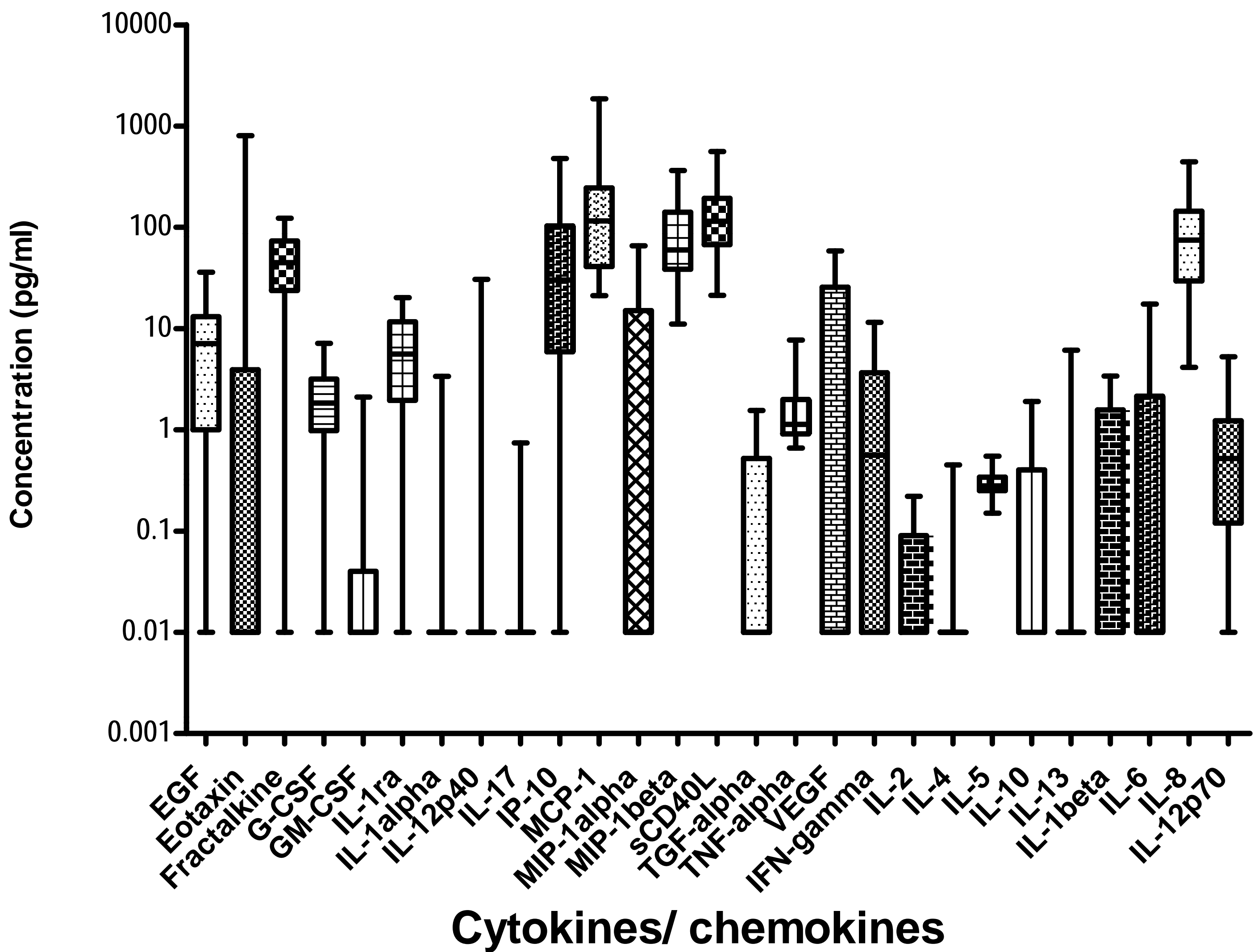


Figure 3.2: Cytokine profile in supernatant from unstimulated 7-day whole blood at study recruitment Box plot of the negative control condition using the 27-plex kit. Supernatant from 17 fast TST convertors and 6 TST convertors was tested with a 27-plex cytokine assay kit at recruitment. Minimum and maximum values, error bars and median concentrations are shown. The highest unstimulated median cytokine levels were seen for MCP-1, MIP-beta, sCD40L and IL-8at baseline .

3. 2.4 Statistical Analysis

The Mann-Whitney U test was used to measure differences between medians. The mixed model of the analysis of variance (MANOVA) was used to determine differences between fast and slow TST convertors at recruitment and over time. A p value <0.05 was considered statistically significant. The Spearman correlation test was used to investigate the association between variables.

3.3 Research Questions

The main research questions that were addressed in this chapter were:

1. At study recruitment, is there a difference in the multi-cytokine response to *M.tb* antigens in HIV negative household contacts with a prolonged negative skin test reaction compared to those that TST convert earlier?
2. Can the antigen-induced cytokine expression profile at recruitment predict the rate of TST conversion?
3. Do cytokine levels change in fast and/or slow TST convertors over 6 months?

3.4 Results

3.4.1 Magnitude of response in fast versus slow TST convertors at recruitment

3.4.1.1 Negative control at recruitment

Of the 26 TST negative participants, supernatants from 3 slow TST convertors were not available at study recruitment. Unstimulated culture supernatants from the recruitment time point of 23 subjects included in the study were tested with the 27-plex cytokine assay (Figure 3.2). At this time point all subjects were TST negative. The values of 19 of the 27 analytes were below detectable levels (<3.2pg/ml). The concentrations of the remaining 8 analytes were as follows: EGF, 7.7pg/ml (0.01pg/ml – 36pg/ml); IP10, 32.8pg/ml (0.01- 477pg/ml); Fractalkine, 41.9pg/ml (0.01 – 123pg/ml); IL-1ra , 5.6pg/ml (0.01pg/ml – 20.2pg/ml); MIP-1beta, 60.7pg/ml (11pg/ml – 363.8pg/ml); IL-8, 81.6pg/ml (4pg/ml - 442.8pg/ml); MCP-1, 104.8pg/ml (21pg/ml - 1857pg/ml) and sCD40L, 112.5pg/ml (21pg/ml - 560pg/ml).

3.4.1.2 Fast versus slow TST convertors at recruitment

Fast TST convertors produced higher levels of GM-CSF, IP-10, IL-8, IL-2, IL-5 and IL-13 than slow TST convertors when diluted whole blood was stimulated with *M.tb* PPD at recruitment (Figure 3.3 A-F; p=0.0003, p=0.003, p=0.01, p=0.02, p=0.0007 and p<0.0001 respectively). Slow TST convertors produced markedly enhanced median levels of IL-12p70 (Figure 3.4: A) in response to both of the DosR regulon-encoded antigens Rv1737c and Rv2029c (p=0.02 and p=0.01). These antigens produced a similar response with VEGF (p=0.03 and p=0.03; data not shown). Figure 3.4: B illustrates that MCP-1 levels were significantly enhanced in slow convertors when compared to fast convertors in response to Rv1733c, Rv1737c, Rv2029c and ESAT6/CFP10 fusion protein (p=0.009, p=0.001, p=0.002 and p=0.004). IL-10 production (Figure 3.5) was also significantly higher in slow TST convertors following stimulation with Rv2029c (p=0.01).

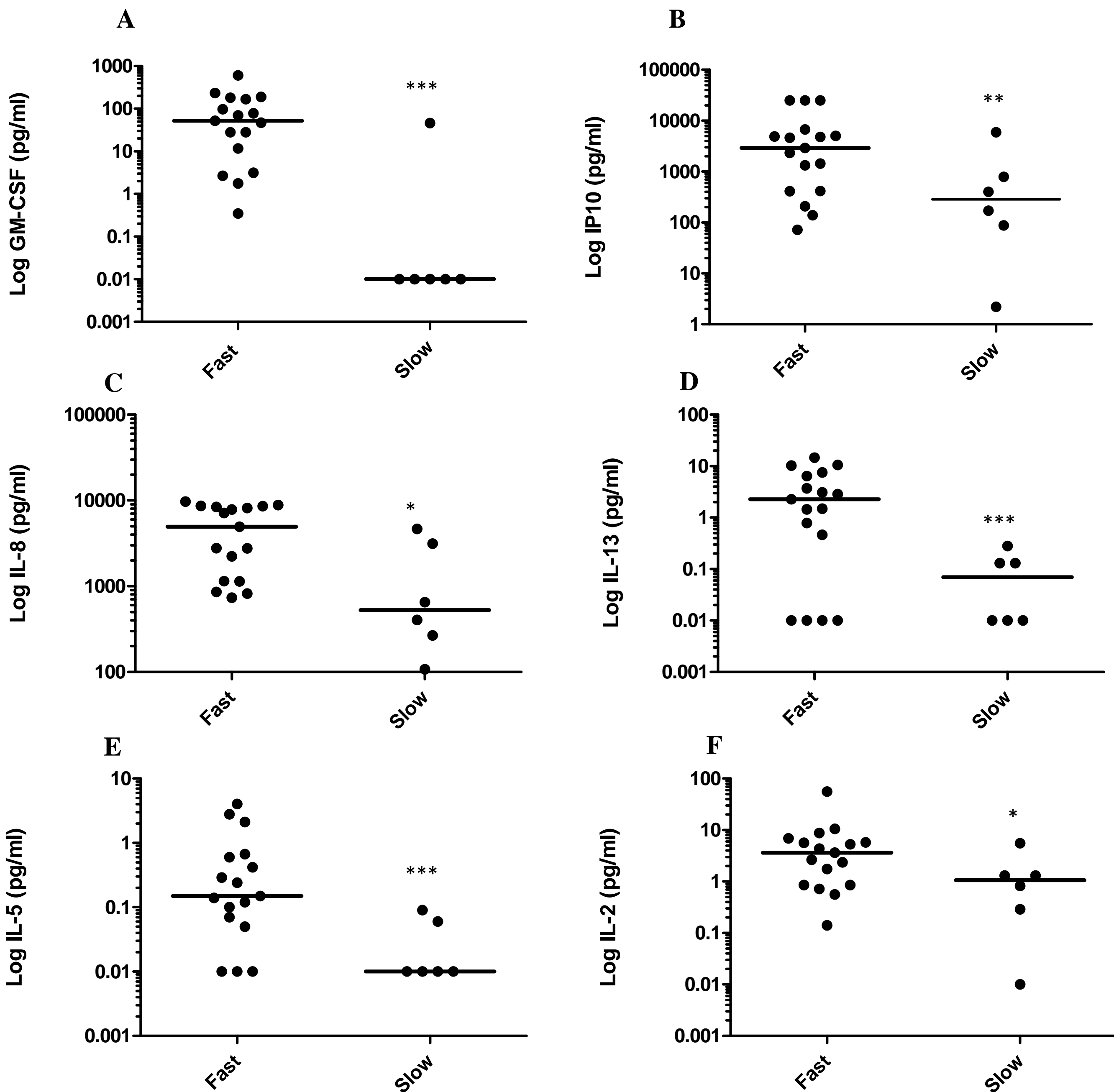


Figure 3.3 A - F: Cytokine responses to *M.tb* PPD at recruitment: fast versus slow TST convertors. Diluted whole blood from 17 fast TST convertors and 6 slow TST convertors was stimulated with *M.tb* PPD for 7 days at recruitment..Significantly higher median levels of (A) GM-CSF were observed in fast convertors when compared to slow convertors (p=0.0003). A similar observation was made for IP-10 (B) and IL-8 (C) which are pro-inflammatory chemokines (p=0.003, p=0.01). TH2 cytokines IL-13 (D), IL-5 (E) and IL-2 (E) produced the same pattern (p<0.0001, p=0.0007 and p=0.02 respectively).

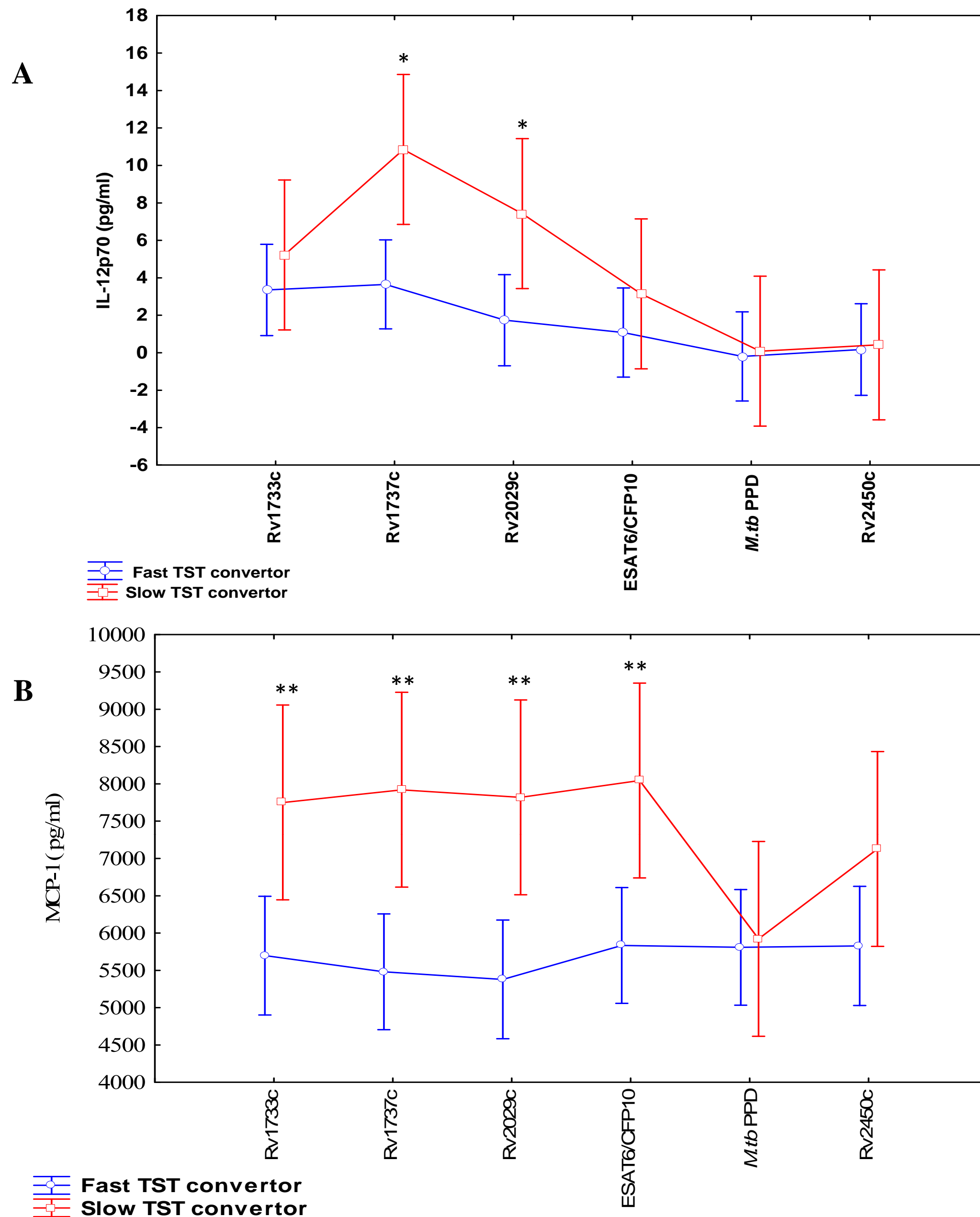


Figure 3.4A - B: Cytokine responses to *M.tb* derived antigens at recruitment: fast versus slow TST convertors. Diluted whole blood from 17 fast TST convertors and 6 slow TST convertors was stimulated with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein, *M.tb* PPD and Rv2450c for 7 days at recruitment. Slow convertors produced significantly higher median levels of (A) IL-12p70 in responses to Rv1737c and Rv2029c when compared to fast convertors, and the same pattern was observed for VEGF(p=0.02, p=0.01 and p=0.03, p=0.03). (B) MCP-1 response to Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein Rv2029c when compared to fast convertors (p=0.009, p=0.001, p=0.002, p=0.004).

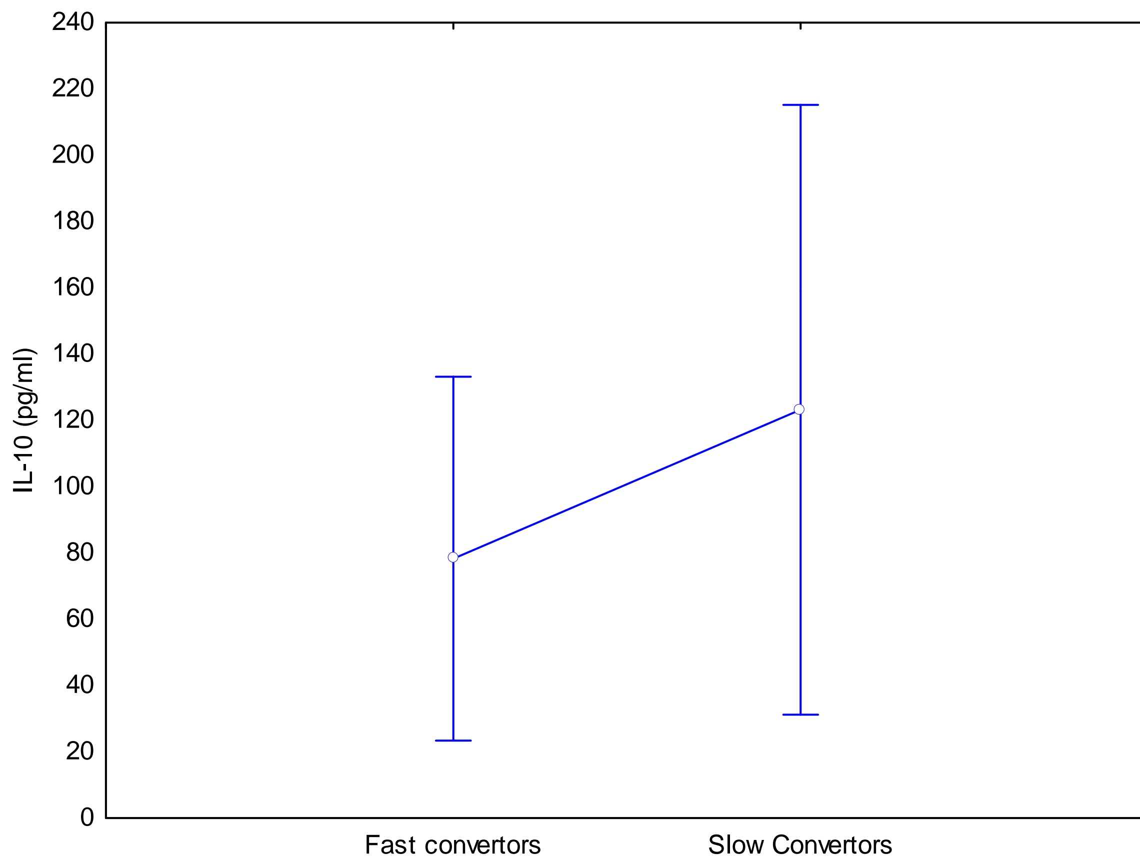


Figure 3.5: IL-10 responses to Rv2029c at recruitment: fast versus slow TST convertors. Diluted whole blood from 17 fast TST convertors and 6 slow TST convertors was stimulated with Rv2029c for 7 days at recruitment. Higher IL-10 production was observed in slow convertors compared to fast convertors (p=0.01).

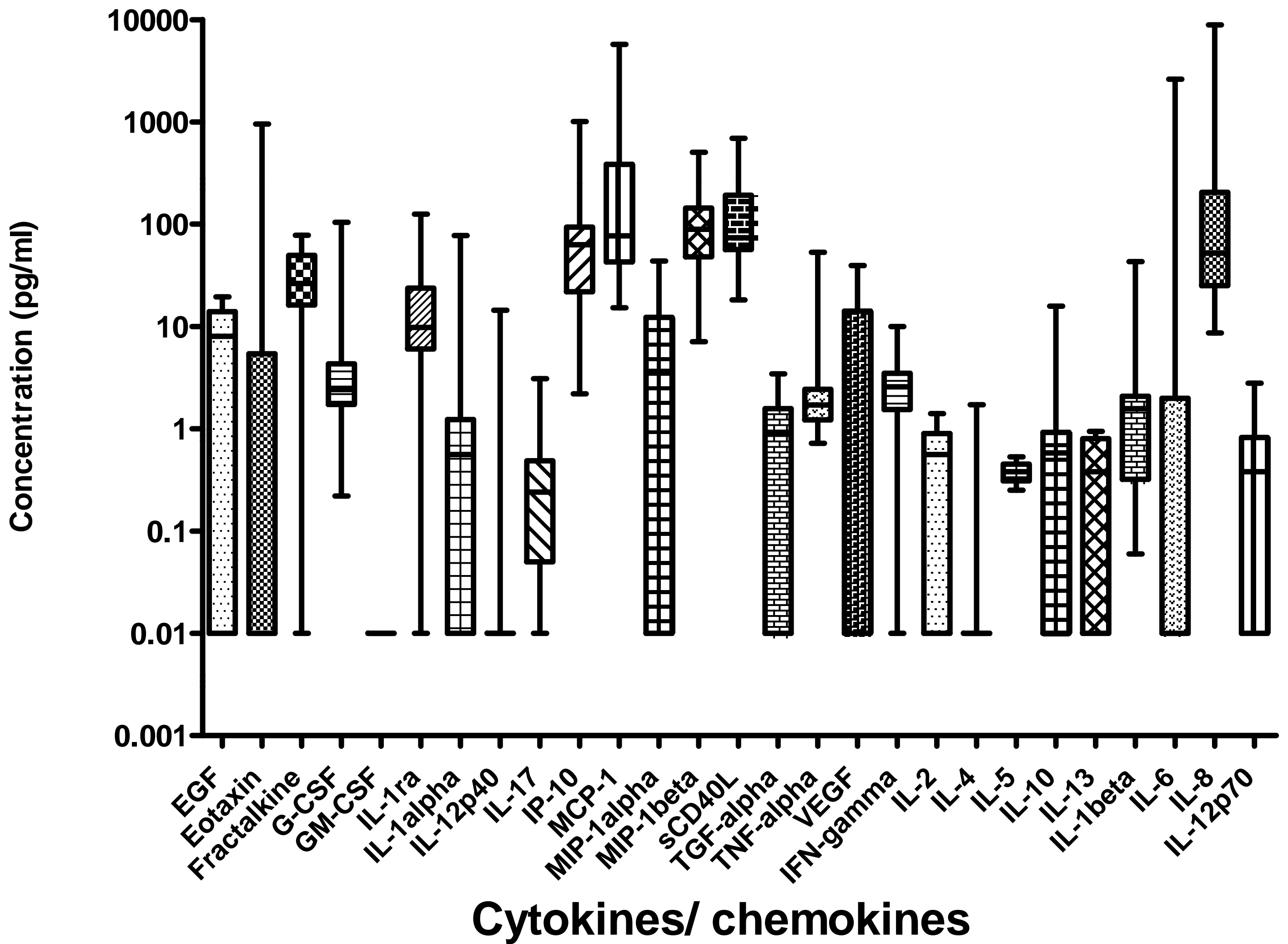


Figure 3.6: Cytokine profile in supernatant from unstimulated 7-day whole blood at month 6. Box plot of the negative control condition using the 27-plex kit. 6 months into the study supernatant from 17 fast TST convertors and 8 slow TST convertors was tested on a 27-plex cytokine assay. The box plot shows minimum, median and maximum values with error bars. The highest unstimulated median cytokine levels were seen for IP10, MCP-1, MIP-1 beta, sCD40L and IL-8 at 6 months.

3.4.2 Magnitude of response in fast and slow TST convertors over time

Differential cytokine production over time was investigated by measuring the same 27 cytokines/ chemokines described above in whole blood assay supernatants collected at the month 6 time point in fast (n=17) and slow convertors (n=8). The emerging 6 month cytokine/chemokine profile was compared to the recruitment cytokine/ chemokine profile. The results are described according to cytokines that changed over time in fast convertors only, in slow convertors only, or in both fast and slow convertors.

3.4.2.1 Negative control at 6 months

Figure 3.6 shows the production levels of the 27 cytokines measured in unstimulated culture supernatants at month 6. Seventeen subjects had converted to TST positive and 9 remained TST negative. The values of 18 of the 27 analytes were below detectable levels (<3.2pg/ml). The concentrations of the remaining 9 analytes were as follows: MIP-1alpha, 3.2pg/ml (0.01pg/ml – 43.7pg/ml); EGF, 7.6pg/ml (0.01pg/ml – 19.4pg/ml); IL-1ra, 9.4pg/ml (0.01pg/ml – 125.2pg/ml); Fractalkine, 26.4pg/ml (0.01pg/ml – 77.8pg/ml); IL-8, 50.9pg/ml (8.6pg/ml – 8890pg/ml); IP10, 58.7pg/ml (2.1- 1003pg/ml); sCD40L, 73.7pg/ml (18.1pg/ml – 690.6pg/ml); MCP-1, 77.1pg/ml (15.2pg/ml – 5719pg/ml); and MIP-1beta, 87.9pg/ml (7.1pg/ml – 505pg/ml).

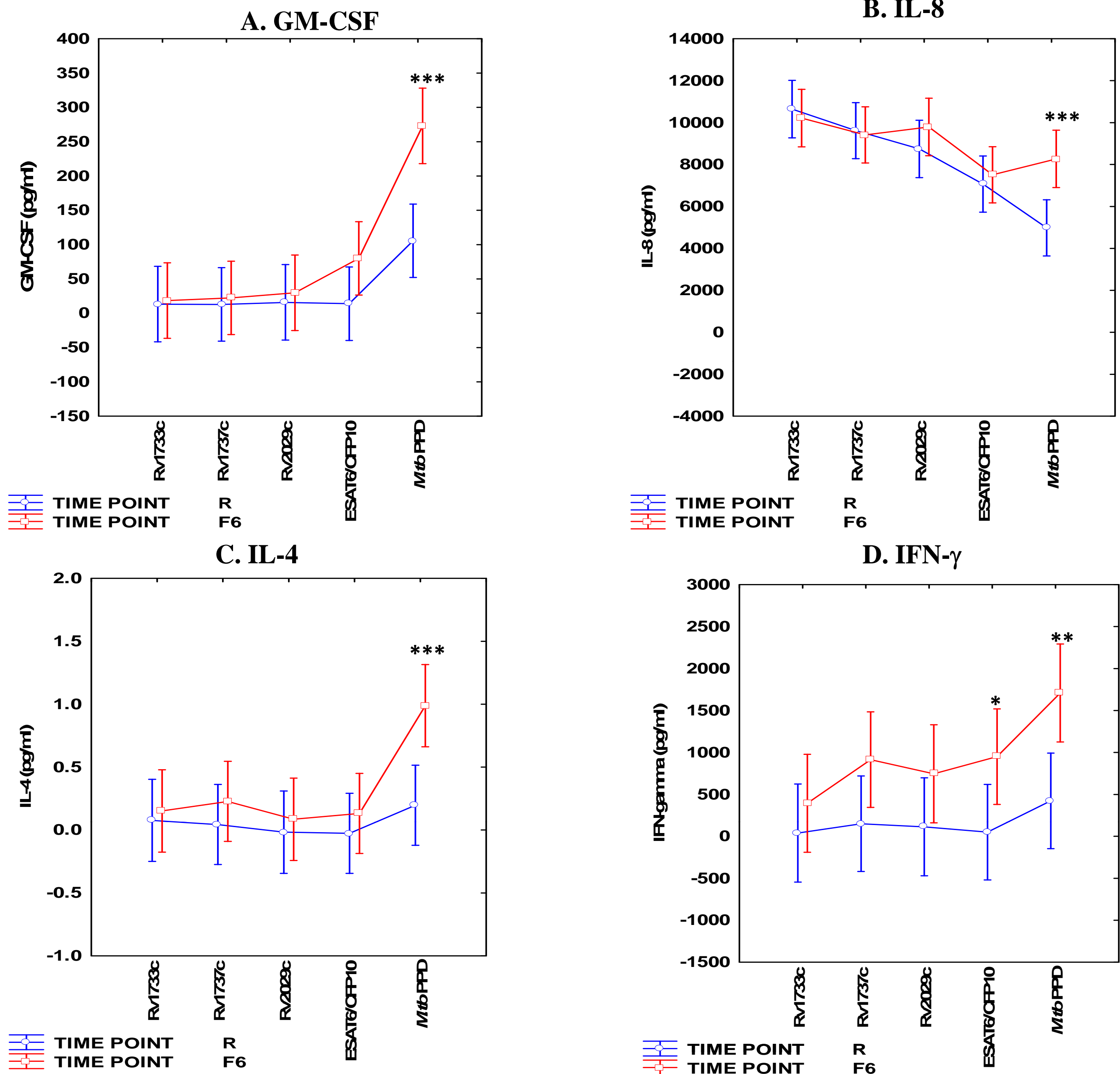


Figure 3.7A - D: Cytokines that increase over time in response to classical *Mtb* antigens only and in fast convertors only. Whole blood from 17 fast TST convertors was stimulated in fast convertors with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein and *M.tb* PPD for 7 days at recruitment and at 6 months. GM-CSF (A) was induced only by *M.tb* PPD at recruitment and the response was enhanced at 6 months ($p<0.0001$). A similar pattern was observed for IL-2 ($p=0.001$, data not shown). (B) IL-8 was strongly expressed in response to all antigens at both time points. At 6 months IL-8 responses to *M.tb* PPD were significantly increased when compared to recruitment ($p=0.0008$). EGF displayed the same pattern ($p=0.004$, data not shown). (C) IL-4 levels in response to all antigens were very low with the exception of *M.tb* PPD at month 6 ($p=0.0008$). The same pattern was seen for sCD40 ligand, IL-5, IL-13, fractalkine and IL-17 ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p=0.003$ and $p=0.005$ respectively, data not shown). (D) IFNγ production had significantly increased by 6 months upon stimulation with both ESAT-6/CFP10 fusion protein and *M.tb* PPD ($p=0.02$, $p= 0.002$). This presentation was also observed for IP-10 ($p= 0.003$, $p<0.0001$, data not shown).

3.4.2.2 Cytokines that change over time in fast convertors only

Only *M.tb* PPD elicited both pro-inflammatory chemokines and adaptive immune system cytokines at recruitment in fast TST convertors, which were enhanced at 6 months (Figure 3.7A–D). GM-CSF was induced in fast convertors at recruitment and the response was enhanced at 6 months (Figure 3.7A, $p<0.0001$). A similar pattern was observed for IL-2 ($p=0.001$, data not shown). Figure 3.7B demonstrates that IL-8 was strongly expressed in response to all antigens at both time points. At 6 months IL-8 responses to *M.tb* PPD were significantly increased when compared to recruitment ($p=0.0008$). EGF displayed the same pattern ($p=0.004$, data not shown). IL-4 levels in response to all antigens were very low with the exception of *M.tb* PPD at month 6 (Figure 3.7C, $p=0.0008$). The same pattern was seen for sCD40 ligand, IL-5, IL-13, Fractalkine and IL-17 ($p <0.0001$, $p<0.0001$, $p<0.0001$, $p=0.003$ and $p=0.005$ respectively, data not shown). Stimulation with all antigens except for *M.tb* PPD resulted in very low IFN-γ production at recruitment. Stimulation with ESAT-6/CFP10 fusion protein and *M.tb* PPD resulted in significantly increased IFN-γ production 6 months (Figure 3.7D, $p=0.02$, $p= 0.002$). This presentation was also observed for IP-10 ($p= 0.003$, $p<0.0001$, data not shown).

Figure 3.8: A-B shows that both MIP-1α and IL-1 α were markedly elevated in response to Rv1737c at 6 months in fast convertors only when compared to recruitment ($p=0.02$ and $p=0.02$). Stimulation with DosR regulon-encoded antigens Rv1733c, Rv1737c and Rv2029c resulted significantly higher IL-1ra responses only in fast convertors at 6 months compared to recruitment (Figure 3.8C, $p=0.006$, $p=0.03$ and $p=0.02$).

At 6 months, stimulation with Rv2029c and ESAT-6/CFP10 fusion protein elicited significantly higher MCP-1 levels when compared to recruitment (Figure 3.9, $p=0.006$ and $p=0.04$).

Figure 3.8

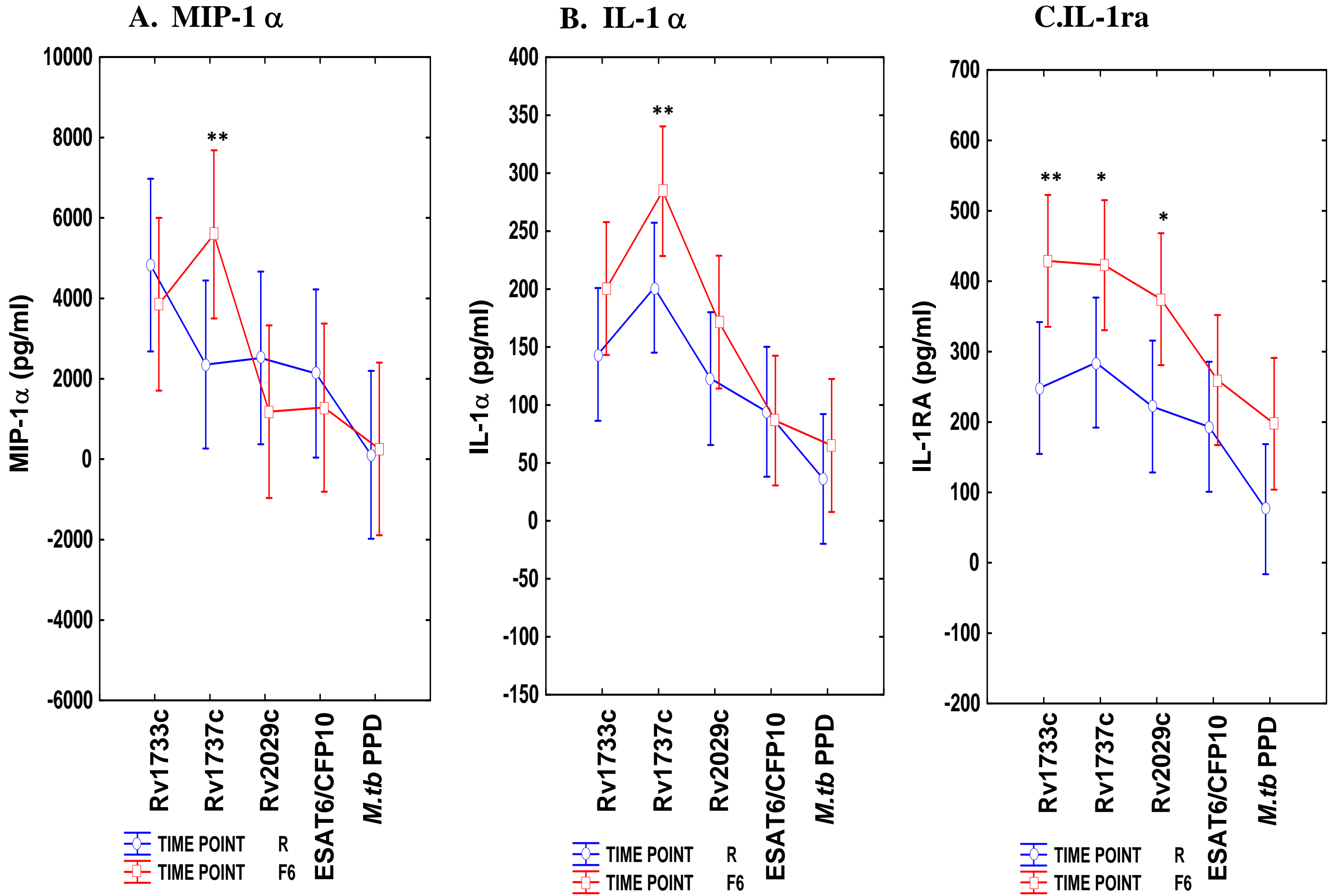


Figure 3.8A - C: Cytokines that increase over time in response to DosR regulon-encoded antigens only and in fast convertors only. Whole blood from 17 fast TST convertors was stimulated with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein and *M.tb* PPD for 7 days at recruitment and at 6 months. (A-B) MIP-1α and IL-1α were markedly elevated in response to Rv1737c at 6 months in fast convertors only when compared to recruitment (p=0.02 and p=0.02). (C) Significantly higher IL-1ra responses to Rv1733c, Rv1737c and Rv2029c were observed only in fast convertors at 6 months compared to recruitment (p=0.006, p=0.03 and p=0.02).

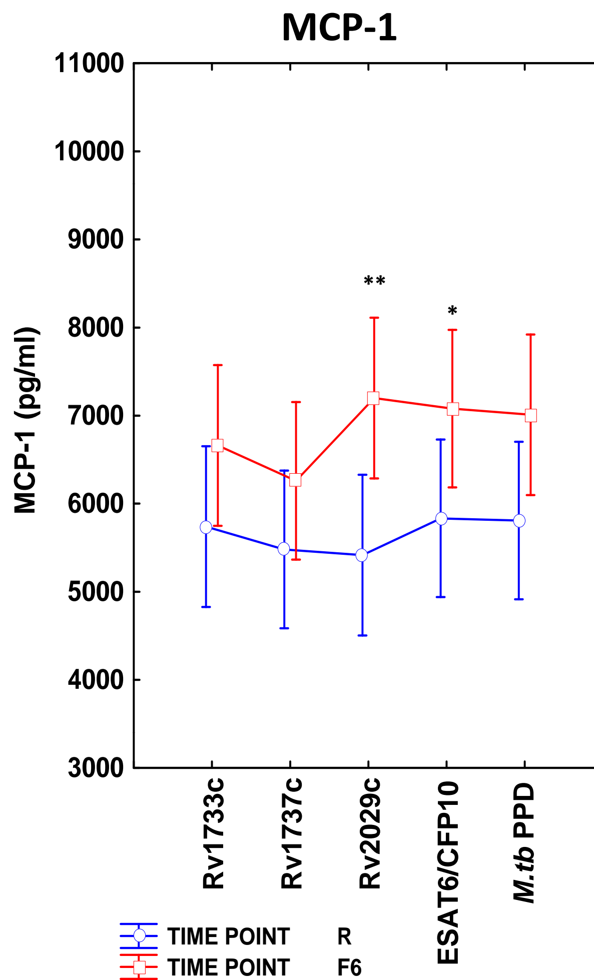


Figure 3.9: Cytokines that increase over time in response to classical *Mtb* antigens and DosR regulon-encoded antigens in fast convertors only. Whole blood from 17 fast TST convertors was stimulated with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein and *M.tb* PPD for 7 days at recruitment and at 6 months. Significantly higher MCP-1 levels in response to Rv2029c and ESAT-6/CFP10 fusion protein at 6 months when compared to recruitment (p=0.006 and p=0.04).

3.4.2.3 Cytokines that change over time in slow convertors only

Rv2029c elicited significantly lower IL-10 and G-CSF at month 6 when compared to recruitment (Figure 3.10: A- B, p=0.002 and p=0.01). In this study group, lower VEGF production was observed in response to Rv1737c at month 6 when compared to recruitment (Figure 3.10C, p=0.04).

3.4.2.4 Cytokines that change over time in both fast and slow convertors

In Figure 3.11A, significantly higher IL-12p40 responses were observed at month 6 compared to recruitment when both fast and slow convertors were stimulated with Rv1733c (p=0.01, 0.02) and Rv1737c (p=0.003, p=0.006). However, stimulation with Rv2029c elicited higher IL-12p40 concentrations at 6 months in fast TST convertors only (p=0.02). Significantly higher IL-12p70 responses to Rv1737c were seen at month 6 compared to recruitment in both fast and slow convertors (Figure 3.11B, p=0.005 and p= 0.006).

TGFα production displayed a distinct pattern in fast and slow TST convertors due to stimulation with *M.tb* antigens. Figure 3.12 illustrates that fast convertors produced enhanced TGFα levels to *M.tb* PPD stimulation at 6 months (p=0.005) whereas slow convertors produced significantly lower cytokine responses to Rv2029c at 6 months (p=0.04).

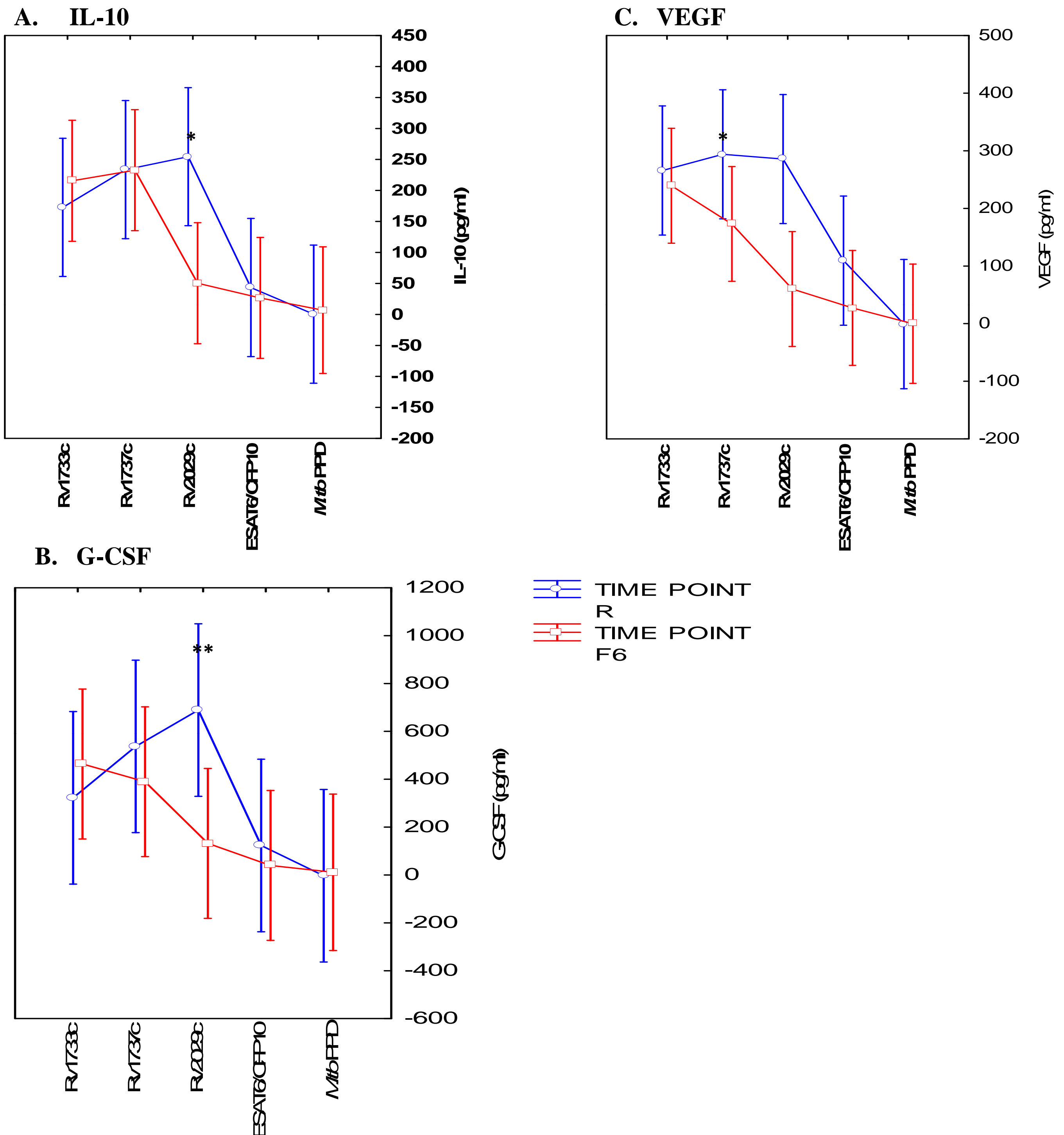


Figure 3.10A - C: Cytokines that decrease over time in response to DosR regulon-encoded antigens only and in slow convertors only Whole blood from 6 slow TST convertors was stimulated with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein and *M.tb* PPD for 7 days at recruitment and in subjects at 6 months. Slow convertors produced significantly lower (A) IL-10 and (B) G-CSF upon stimulation with Rv2029c at month 6 when compared to recruitment (p=0.002 and p=0.01). (C) Lower VEGF production was observed in response to Rv1737c at month 6 when compared to recruitment (p=0.04).

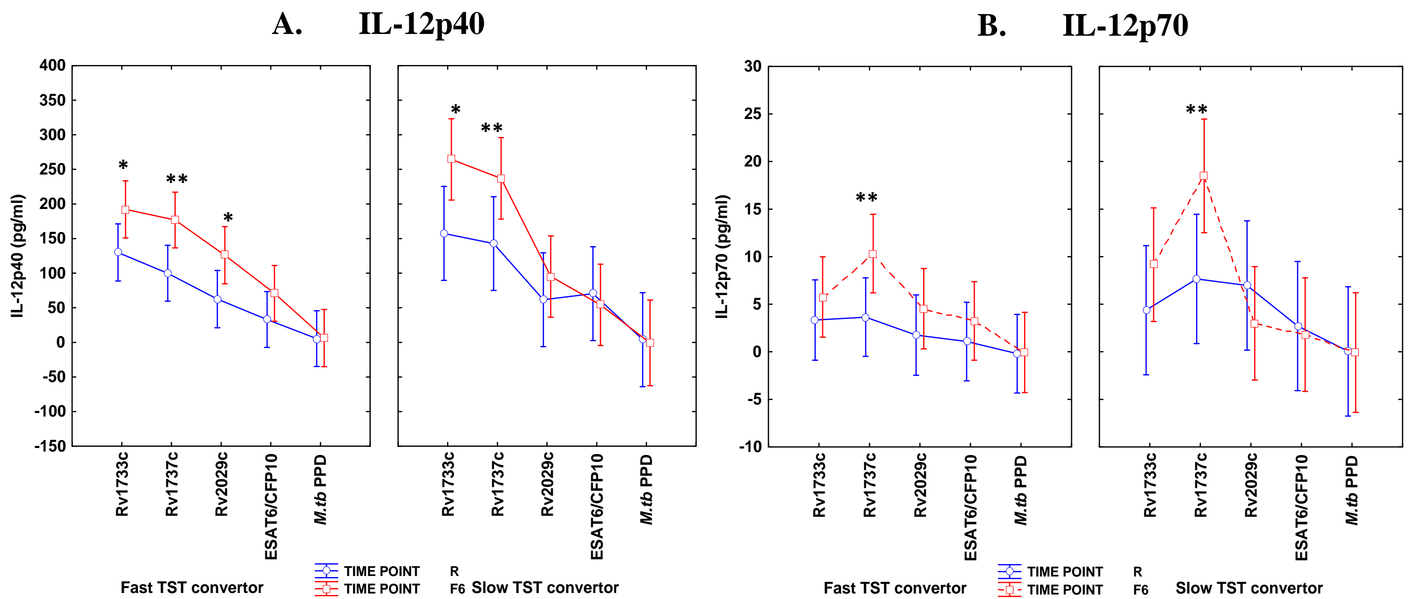


Figure 3.11A-B: Cytokines that increase over time in response to DosR regulon-encoded antigens only in fast and slow convertors Whole blood from 17 fast TST convertors and 6 and 8 slow TST convertors (at recruitment and at month 6 respectively) was stimulated with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein and *M.tb* PPD for 7 days at recruitment and at 6 months. (A) Significantly higher IL-12p40 responses were observed at month 6 compared to recruitment when (i) both fast and slow convertors were stimulated with Rv1733c (p=0.01, 0.02) and Rv1737c (p=0.003, p=0.006) (ii) fast convertors were stimulated with Rv2029c (p=0.02). (B) Significantly higher IL-12p70 responses were observed at month 6 compared to recruitment when both fast and slow convertors were stimulated with Rv1737c (p=0.005 and p= 0.006).

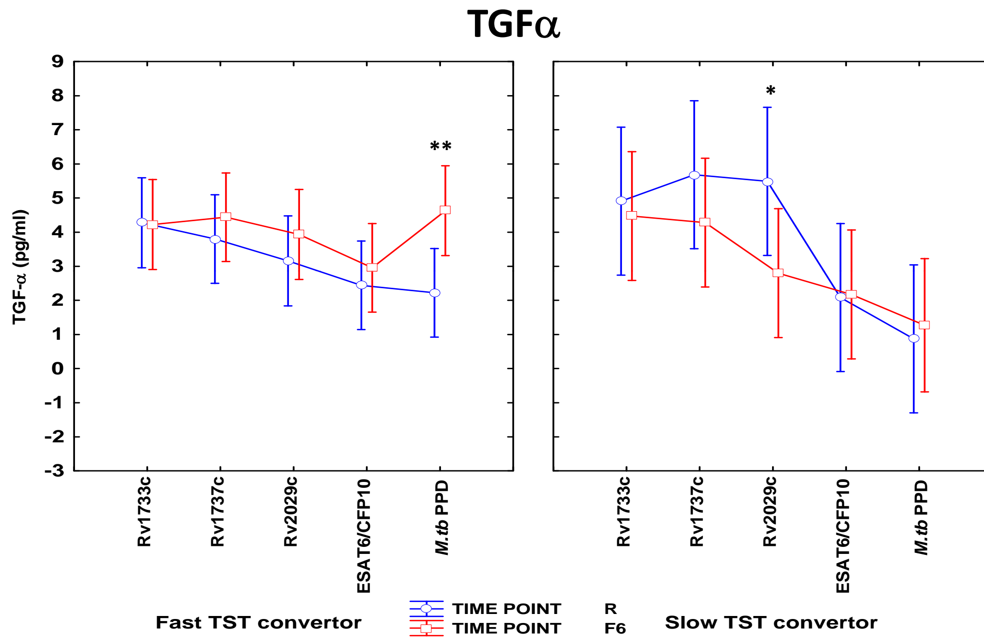


Figure 3.12: Cytokines that change over time in response to classical or DosR regulon-encoded *M.tb* antigens in fast and slow convertors. Whole blood from 17 fast TST and 8 slow convertors was stimulated with *M.tb* antigens Rv1733c, Rv1737c, Rv2029c, ESAT6/CFP10 fusion protein and *M.tb* PPD for 7 days at recruitment and at 6 months (6 slow TST convertors at month 6). Fast convertors produced significantly higher cytokine responses to *M.tb* PPD stimulation at 6 months (p=0.005) whereas slow convertors produced significantly lower cytokine responses to Rv2029c at 6 months (p=0.04).

3.4.3 Percent responders: fast TST convertors versus slow TST convertors

A response was considered positive for an analyte if the antigen-induced concentration was greater than the mean of the negative control value plus 2 standard deviations. If this cut-off value was lower than the lowest point on the standard curve (3.2pg/ml), the cut-off point for the analyte was set to 3.2pg/ml. Table 3.3 shows the response cut-off values for each analyte. Table 3.4 A - E shows the frequency of responses to *M.tb* antigens in fast and slow convertors at recruitment and after 6 months for each of the 27 cytokines tested.

3.4.3.1 Classical TB antigens: M.tb PPD

In fast convertors at recruitment, *M.tb* PPD induced positive responses for 22 of the 27 cytokines, and for 10 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study this antigen induced positive responses for 23 of the 27 cytokines and for 12 cytokines this antigen induced responses in over 50% of fast convertors (Table 3.4 A).

In slow convertors at recruitment, *M.tb* PPD induced positive responses for 11 of the 27 cytokines, and for 2 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study, this antigen also induced positive responses for 11 of the 27 cytokines, and for the same 2 cytokines this antigen induced responses in over 50% of the study group. The positive cytokine responses at the different time points were not from the same cytokines. While there were no IL-10, IL-2 and IL-17 produced in response to *M.tb* PPD in slow convertors at recruitment, these cytokines were produced at month 6 in response to this antigen. On the other hand whereas there were IL-12p40, MIP-1β and VEGF responses to *M.tb* PPD at recruitment in this study group these cytokines were not produced at month 6.

Table 3.3 Cut-off point per cytokine/chemokine in the 27-plex Luminex kit. A distinct cut-off value for each cytokine in the 27-plex Luminex kit was determined as the mean of the negative control value plus 2 standard deviations. Where this cut-off fell below the lowest detection limit of the assay (3.2pg/ml), the value was changed to 3.2pg/ml. Cytokines are kinetic and therefore measuring these 27 cytokines/chemokines at 7 days only is a biased picture.

Cytokine/Chemokine	Cut-off values (pg/ml)
EGF	24.9
Eotaxin	394.8
Fractalkine	96.0
G-CSF	34.3
GM-CSF	3.2
IL-1ra	53.5
IL-1alpha	24.5
IL-12p40	15.3
IL-17	3.2
IP-10	433.0
MCP-1	2192.4
MIP-1alpha	34.3
MIP-1beta	273.8
sCD40L	388.0
TGF-alpha	3.2
TNF-alpha	18.0
VEGF	36.1
IFN-gamma	8.0
IL-2	3.2
IL-4	3.2
IL-5	3.2
IL-10	5.3
IL-13	3.2
IL-1beta	14.2
IL-6	810.6
IL-8	2830.0
IL-12p70	3.2

Table 3.4 A. Percentage of responders to classical *M.tb* antigen *M.tb* PPD per cytokine/ chemokine in the 27-plex Luminex kit.at recruitment and at month 6. Diluted whole blood of fast TST convertors (n=17 at recruitment, n=16 at month 6) and slow TST convertors (n=6 at recruitment, n=8 at month 6) was stimulated with *M.tb* PPD for 7 days and cytokines were measured in harvested supernatants using the 27-plex Luminex kit. The percentage of responders was determined using pre-determined, cytokine specific response cut-off values.

Cytokine /Chemokine	Fast TST convertors At Recruitment	Slow TST convertors At Recruitment	Fast TST convertors At Month 6	Slow TST convertors At Month 6
Innate cytokines				
EGF	0	0	5.8	0
Eotaxin	0	0	0	0
Fractalkine	0	0	5.8	0
G-CSF	5.9	0	5.8	0
IL-1ra	70.5	50	94.1	57.1
IL-12p40	17.6	16.7	23.5	0
IL-12p70	0	0	0	0
IP10	70.5	16.7	94.1	42.8
MCP-1	100	83.3	100	85.7
MIP-1alpha	76.4	16.7	82.3	14.2
MIP-1beta	29.4	16.7	47	0
TGF alpha	52.9	0	0	0
VEGF	5.9	16.7	0	0
Innate and adaptive cytokines/chemokines				
GM-CSF	64.7	16.7	94.1	42.8
IFN gamma	64.7	16.7	94.1	42.8
IL-1alpha	29.4	0	41.1	0
IL-1 beta	17.6	0	41.1	0
IL-6	29.4	0	23.5	0
sCD40L	0	0	5.8	0
IL-4	5.9	0	11.7	0
IL-8	52.9	33.3	94.1	14.2
IL-10	35.2	0	64.7	28.5
IL-13	29.4	0	76.4	0
TNF alpha	70.5	16.7	88.2	14.2
Adaptive cytokines				
IL-2	52.9	0	82.3	14.2
IL-5	5.9	0	35.2	0
IL-17	17.6	0	64.7	14.2

Table 3.4 B. Percentage of responders to classical *M.tb* antigen ESAT6/CFP10 fusion protein per cytokine/ chemokine in the 27-plex Luminex kit at recruitment and at month 6. Diluted whole blood of fast TST convertors (n=16 at recruitment, n=17 at month 6) and slow TST convertors (n=6 at recruitment, n=8 at month 6) was stimulated with ESAT6/CFP10 fusion protein for 7 days and a cytokine/chemokine profile of the percentage of responders determined using a 27-plex kit.

Cytokine /Chemokine	**Fast TST convertors at Recruitment**	**Slow TST convertors at Recruitment**	**Fast convertors At Month 6**	**Slow convertors At Month 6**
Innate cytokines				
EGF	17.6	0	17.6	0
Eotaxin	0	0	0	0
Fractalkine	0	0	0	0
G-CSF	41.2	16.7	35.3	37.5
IL-1ra	88.2	100	100	87.5
IL-12p40	47	66.7	52.9	62.5
IL-12p70	0	33.3	17.6	25
IP10	58.8	66.7	82.3	62.5
MCP-1	100	100	92.1	100
MIP-1alpha	58.8	66.7	64.7	37.5
MIP-1beta	58.8	66.7	64.7	75
TGF alpha	17.6	16.7	41.1	25
VEGF	35.2	33.3	23.5	37.5
Innate and adaptive cytokines/chemokines				
GM-CSF	56.3	75	70.5	50
IL-1alpha	52.9	83.3	70.5	62.5
IL-1 beta	76.4	83.3	82.3	87.5
IL-4	0	0	0	0
IL-6	58.8	66.7	64.7	62.5
IL-8	82.3	83.3	94.1	87.5
IL-10	58.8	66.7	64.7	75
IL-13	0	0	11.7	0
IFN gamma	70.5	50	88.2	75
sCD40L	0	0	0	0
TNF alpha	94.1	83.3	94.1	87.5
Adaptive cytokines				
IL-2	0	0	47	25
IL-5	0	0	11.7	12.5
IL-17	12.5	0	29.4	12.5

3.4.3.2 Classical TB antigens: ESAT6/CFP10 Fusion Protein

Table 3.4B shows that in fast convertors at recruitment ESAT6/CFP10 fusion protein induced positive responses for 19 of the 27 cytokines, and for 13 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study this antigen induced positive responses for 23 of the 27 cytokines and for 14 cytokines this antigen induced responses in over 50% of fast convertors.

In slow convertors at recruitment ESAT6/CFP10 fusion protein induced positive responses for 18 of the 27 cytokines, and for 14 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study this antigen induced positive responses for 21 of the 27 cytokines and for 18 cytokines this antigen induced responses in over 50% of fast convertors.

3.4.3.3 DosR regulon encoded antigens: Rv1733c

Table 3.4C shows that in fast convertors at recruitment Rv1733c induced positive responses for 20 of the 27 cytokines, and for 16 cytokines this antigen induced responses in over 50% of the study group. All 20 cytokines that responded at recruitment also responded to this antigen 6 months into the study, and for 18 cytokines this antigen induced responses in over 50% of the study group.

In slow convertors at recruitment Rv1733c induced positive responses for 20 of the 27 cytokines, and for 18 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study Rv1733c induced positive responses for 19 of the 27 cytokines and for 18 of the same cytokines that this antigen induced responses in over 50% at recruitment, were also induced at month 6 in slow convertors.

Table 3.4C. Percentage of responders to Dos-R regulon encoded antigen Rv1733c per cytokine/ chemokine in the 27-plex Luminex kit at recruitment and at month 6. Diluted whole blood of fast TST convertors (n=16) and slow TST convertors (n=6 at recruitment, n=8 at month 6) was stimulated with Rv1733c for 7 days and a cytokine/chemokine profile of the percentage of responders determined using a 27-plex kit.

Cytokine /Chemokine	Fast TST convertors at Recruitment	Slow TST convertors at Recruitment	Fast TST convertors At Month 6	Slow TST convertors At Month 6
Innate cytokines/chemokines				
EGF	12.5	33.3	43.7	37.5
Eotaxin	0	0	0	0
Fractalkine	0	0	0	0
G-CSF	100	100	87.5	87.5
IL-1ra	100	100	100	100
IL-12p40	100	100	87.5	100
IL-12p70	12.5	50	62.5	75
IP10	36.3	50	81.2	75
MCP-1	100	100	87.5	100
MIP-1alpha	100	100	93.7	100
MIP-1beta	100	100	100	100
TGF alpha	56.3	50	75	62.5
VEGF	68.8	100	75	87.5
Innate and adaptive cytokine/chemokines				
GM-CSF	87.5	100	93.7	100
IFN gamma	62.5	66.7	75	100
IL-1alpha	100	100	100	100
IL-1 beta	100	100	100	100
IL-4	0	0	0	0
IL-6	100	100	100	100
IL-8	100	100	93.7	100
IL-10	100	100	100	100
IL-13	0	0	0	0
sCD40L	0	0	0	0
TNF alpha	100	100	100	100
Adaptive cytokines				
IL-2	0	0	0	0
IL-5	0	0	0	0
IL-17	6.25	16.7	12.5	0

3.4.3.4 DosR regulon encoded antigens: Rv1737c

Table 3.4D shows that in fast convertors at recruitment Rv1737c induced positive responses for 20 of the 27 cytokines, and for 15 cytokines this antigen induced responses in over 50% of the study group. 6 months into the study, 21 cytokines responded to Rv1737c including all 20 that had responded at recruitment to this antigen; and for 18 cytokines this antigen induced responses in over 50% of the study group.

In slow convertors at recruitment Rv1737c induced positive responses for 20 of the 27 cytokines, and for 17 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study Rv1737c induced positive responses for the same 20 of the 27 cytokines and for 18 cytokines this antigen induced responses in over 50% were induced at month 6 in slow convertors.

3.4.3.5 DosR regulon encoded antigens: Rv2029c

Table 3.4E shows that in fast convertors at recruitment Rv2029c induced positive responses for 20 of the 27 cytokines, and for 16 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study, 23 cytokines responded to Rv2029c including all 20 that had responded at recruitment this antigen, and for 17 cytokines this antigen induced responses in over 50% of the study group.

In slow convertors at recruitment Rv2029c induced positive responses for 19 of the 27 cytokines, and for 17 cytokines this antigen induced responses in over 50% of the study group. 6 Months into the study Rv2029c induced positive responses in 20 of the 27 cytokines and for 17 cytokines this antigen induced responses in over 50% that were induced at month 6 in slow convertors.

Table 3.4 D. Percentage of responders to Dos-R regulon encoded antigen Rv1737c at recruitment and month 6, per cytokine/ chemokine in the 27-plex Luminex kit. Diluted whole blood of fast TST convertors (n=17) and slow TST convertors (n=6 at recruitment, n=8 at month 6) was stimulated with Rv1737c for 7 days and a cytokine/chemokine profile of the percentage of responders determined using a 27-plex kit.

Cytokine /Chemokine	Fast TST convertors at Recruitment	Slow TST convertors at Recruitment	Fast TST convertors At Month 6	Slow TST convertors At Month 6
Innate cytokines/chemokines				
EGF	29.4	33.3	29.4	50
Eotaxin	0	0	0	0
Fractalkine	0	0	0	0
G-CSF	88.2	83.3	88.2	87.5
IL-1ra	94.1	100	94.1	100
IL-12p40	94.1	100	88.2	100
IL-12p70	47	50	58.8	50
IP10	47.8	50	88.2	87.5
MCP-1	94.1	100	88.2	100
MIP-1alpha	82.3	100	88.2	100
MIP-1beta	94.1	100	94.1	100
TGF alpha	47.3	33.3	82.3	62.5
VEGF	88.2	83.3	82.3	62.5
Innate and adaptive cytokines/chemokines				
GM-CSF	76.4	100	88.2	100
IFN gamma	82.3	100	82.3	100
IL-1alpha	94.1	100	94.1	100
IL-1 beta	94.1	100	94.1	100
IL-4	0	0	5.8	0
IL-6	94.1	100	94.1	100
IL-8	94.1	100	88.2	100
IL-10	94.1	100	94.1	100
IL-13	0	0	0	0
sCD40L	0	0	0	0
TNF alpha	94.1	100	94.1	100
Adaptive cytokines				
IL-2	0	0	0	0
IL-5	0	0	0	0
IL-17	17.6	16.7	11.7	12.5

Table 3.4E. Percentage of responders to DosR regulon-encoded antigen Rv2029c per cytokine/ chemokine in the 27-plex Luminex kit: at recruitment and month 6. Diluted whole blood of fast TST convertors (n=17 at recruitment, n=16 at month 6) and slow TST convertors (n=6 at recruitment, n=8 at month 6) was stimulated with Rv2029c for 7 days and a cytokine/chemokine profile of the percentage of responders determined using a 27-plex kit.

Cytokine /Chemokine	Fast TST convertors at Recruitment	Slow TST convertors at Recruitment	Fast TST convertors At Month 6	Slow TST convertors At Month 6
Innate cytokines/chemokines				
EGF	12.5	16.7	25	18.7
Eotaxin	0	0	0	0
Fractalkine	0	0	0	0
G-CSF	75	75	62.5	50
IL-1ra	93.8	100	100	100
IL-12p40	68.8	100	87.5	87.5
IL-12p70	25	33.3	37.5	50
IP10	50	50	87.5	50
MCP-1	93.8	100	93.7	100
MIP-1alpha	68.8	83.3	81.2	62.5
MIP-1beta	75	83.3	93.7	87.5
TGF alpha	37.5	50	18.7	12.5
VEGF	56.3	66.7	56.2	50
Innate and adaptive cytokines/chemokines				
GM-CSF	87.5	83.3	93.7	87.5
IFN gamma	75	66.7	87.5	87.5
IL-1alpha	93.8	100	100	75
IL-1 beta	93.8	100	100	100
IL-4	0	0	0	0
IL-6	81.3	83.3	100	87.5
IL-8	93.8	100	93.7	100
IL-10	75	66.7	100	87.5
IL-13	0	0	6.2	0
sCD40L	0	0	0	0
TNF alpha	93.8	100	100	100
Adaptive cytokines				
IL-2	0	0	25	0
IL-5	0	0	6.2	0
IL-17	18.7	0	25	25

3.4.3.6 Resuscitation promotion factors: Rv2450c

Table 3.4 F shows the frequency of responses to resuscitation promotion factor Rv2450c in fast and slow convertors at recruitment for each of the 27 cytokines tested. In fast convertors at recruitment Rv2450c induced positive responses for 18 of the 27 cytokines, and for 1 cytokine this antigen induced responses in over 50% of the study group. In slow convertors at recruitment Rv2450c induced positive responses for 16 of the 27 cytokines, and for 4 cytokines this antigen induced responses in over 50% of the study group.

Table 3.4F Percentage of responders RPF antigen Rv2450c at recruitment only: per cytokine/ chemokine in the 27-plex Luminex kit. Diluted whole blood of fast TST convertors (n=17) and slow TST convertors (n=6) was stimulated with Rv2450c for 7 days and a cytokine/chemokine profile of the percentage of responders determined using a 27-plex kit.

Percentage of responders to Cytokine /Chemokine	Fast TST convertors at Recruitment	Slow TST convertors at Recruitment
Innate cytokines		
EGF	31.3	16.7
Eotaxin	0	0
Fractalkine	0	0
G-CSF	5.9	0
IL-1ra	58.8	100
IL-12p40	5.9	50
IL-12p70	0	0
IP10	17.6	75
MCP-1	5.9	16.7
MIP-1alpha	23.5	0
MIP-1beta	23.5	33.3
TGF alpha	0	0
VEGF	5.9	16.7
Innate and adaptive cytokines/chemokines		
GM-CSF	25	16.7
IL-1alpha	23.5	16.7
IL-1 beta	41.2	33.3
IL-4	0	0
IL-6	17.6	16.7
IL-8	29	33.3
IL-10	11.6	16.7
IL-13	0	0
IFN gamma	35.2	50
sCD40L	0	0
TNF alpha	35.3	33.3
Adaptive cytokines		
IL-2	0	0
IL-5	0	0
IL-17	25	33.3

3.4.4 Correlations between cytokines

3.4.4.1 Correlations between cytokines in fast TST convertors at recruitment

Cytokines that were significantly associated with each other are illustrated in Appendix 1. In this section only strong and highly significant correlations which are defined as association with $r > .80$ and $p \leq 0.0001$ are reported.

Upon stimulation of fast TST convertors with *M.tb* PPD, IL-1β strongly associated with G-CSF, IL-6 and IL-1α. IL-17 was highly associated with IP10 and GM-CSF.

Following stimulation with ESAT6/CFP10 fusion protein, G-CSF and IL-1α were strongly associated with each other and with MIP-1β, IL-1β, IL-6, IL-8, TNF-α and IL-10. Apart from being highly correlated to each other, TNF-α and IL-10 were also strongly associated with MIP-1α and MIP-1β. TNF-α was also strongly associated with IL-1β, IL-6, IL-8 and IL-12p40. IL-10 was also highly correlated with IL-6. IL-1β was highly correlated with both IL-6 and IL-8, while IL-6 was also highly correlated with IL-8. IL-1ra strongly correlated with EGF.

Rv1733c resulted in strong associations between MIP-1α and MIP-1β, as well as between TNF-α and IL-6 only.

Rv1737c stimulation produced strong association between G-CSF and IL-1α. These two cytokines were also correlated with IL-6. IL-10 was strongly correlated with G-CSF, IL-1β and IL-6. IL-12p70 was strongly associated with both TNF-α and IP10.

Following stimulation with Rv2029c, TNF-α was strongly correlated with MIP-1α, IL-6, IL-10, IL-12p40 and IL-12p70. IL-10 also strongly associated with both IL-1β and IL-6. In addition, a strong association between MIP-1α and MIP-1β was observed.

Both IL-1α and TNF-α were strongly associated with IL-1β and IL-6 when fast TST convertors were stimulated with Rv2450c at recruitment. IL-1β was also highly correlated with IL-6, whereas IL-1ra was strongly associated with both IL-6 and IL-1α. A strong association between MIP-1α and MIP-1β was also observed as a result of this antigen.

3.4.4.2 Correlations between cytokines in slow TST convertors at recruitment

At recruitment, none of the antigens tested elicited strong correlations between cytokines measured in slow TST convertors.

3.4.4.3 Correlations between cytokines in fast TST convertors at month 6

Six months into the study, the fast TST convertors were stimulated with *M.tb* PPD resulting in a cytokine correlation profile that demonstrated strong association between GM-CSF and with IL-5, IL-8 and IL-13. IL-1β was strongly correlated with G-CSF, IL-5 and IL-8. Strong associations were also observed between MIP-1α and MIP-1β as well as between IL-1α and IL-12p40.

At this time point, stimulation with ESAT6/CFP10 fusion protein resulted in G-CSF and IL-1α both being strongly associated with MIP-1β, IL-1β, and IL-6. TNFα was also strongly correlated with IL-1α, IL-6, IL-10 and MIP-1β. Whereas IL-1β was also strongly correlated with IL-6, IL-10, strong association also observed between GM-CSF and IL-2 as wells as between MIP-1α and MIP-1β.

Rv1733c responses led to strong associations between G-CSF and IL-1α with IL-1β. Rv1737c at month 6 in fast TST convertors resulted in strong association observed between TGFα and IL-6, IL-1β and IL-10, TNFα and IP10 as well as with both IL-10 and IL-1β with G-CSF.

Stimulation with Rv2029c at month 6 resulted in strong associations being observed between MIP-1α and MIP-1β, IL-1α and IL-1β as well as between TNFα and both IL-12p40 and IL-12p70.

3.4.4.4 Correlations between cytokines in slow TST convertors at month 6

M.tb PPD stimulated 7-day diluted whole blood from slow TST convertors at month 6 did not result in a strongly correlation between cytokines. On the other hand, stimulation with ESAT6/CFP10 fusion protein resulted in a highly significant correlation between IFN-γ and IP10.

Stimulation with latency antigens, Rv1733c, Rv1737c and Rv2029c did not yield strong cytokine associations in the slow TST convertor group 6 months into the study.

3.4.5 Comparison of the ELISA and Luminex methods

We wished to validate the results emanating from both assays by comparing the IFN-γ production that was determined using the ELISA and the Luminex upon stimulation of the fast TST convertors by 5 conditions at recruitment, when they were still TST negative. In Table 3.5 significant correlations in the interferon gamma production measured with both assays was observed as a result of stimulation with 4 out of 6 conditions tested.

Table 3.5 Correlation co-efficient between the ELISA and the Luminex Assay. The Spearman correlation test was performed in on order determined whether there was agreement between the 2 cytokine assays. P<0.05 was considered significant.

	ELISA Median (range in pg/ml)	**LUMINEX Median (range in pg/ml)**	**r**	**P**
M.tb PPD	**174 (15 – 1957pg/ml)**	**33 (0.01 – 5260pg/ml)**	**.54**	**0.02**
ESAT6/CFP10 fusion protein	**52 (15 – 1574pg/ml)**	**43pg/ml (0.01 – 226pg/ml)**	**0.56**	**0.02**
Rv1733c	**15 (15 – 1029pg/ml)**	**27 (0.01 – 116pg/ml)**	**.72**	**0.002**
Rv1737c	**49.2 (15 - 1801pg/ml)**	**109.6 (0.01 – 1052pg/ml)**	**.64**	**0.005**
Rv2029c	**32.9 (15 - 1360pg/ml)**	**33 (0.01 - 550pg/ml)**	**-.09**	**0.74**
Rv2450c	**15 (15 – 226pg/ml)**	**5 (0.01 – 92pg/ml)**	**.14**	**0.60**

3.5 Discussion

In this chapter we used the luminex assay to investigate multiple cytokine expression profiles of 26 HHC of TB index cases that were TST negative at study recruitment, followed them for 6 months, when 17 (65.4%) of them had converted their status to positive, while 9 (34.6%) remained negative. The luminex platform has been recommended as a screening tool in order to determine markers that may be followed up using more senitive instruments (Djoba Siawaya, 2008). This report demonstrates that the cytokine expression patterns due to antigenic stimulation in fast convertors were significantly different to those of the slow convertors. Both the fast and the slow convertors had been equally exposed to their respective TB index cases. Others have observed that TST conversion in contacts was associated to the gradient of exposure to TB index cases (Whalen *et al.,* 2006). The main variables that were in the contact gradient of that study were whether the contact was a spouse to the index case or a relative or a caregiver. These authors also observed that 27% of initially TST negative HHC converted to TST positive within a year. In their study, del Corral *et al.,* (2009) observed a TST conversion rate of 44.5% within a year in contacts that had tested negative at baseline. In the main GC-6 study we observed that 92.8% (829) of the HHC were already TST positive at baseline. We do not have the month 6 TST results of 29 (45.4%) of the 64 participants who had tested TST negative at recruitment and as a result, we cannot be sure about the TST conversion rate in the main study. We know, however, that of the available TST results at month 18, only 1 subject had remained TST negative, whom we had included in the slow convertor group. While a study that has been conducted in the same community that we conducted our own study in has previously shown that only 19% of TB had occurred within the household (Verver *et al.,* 2004), in our study we assumed that transmission had occurred within the household. It is possible therefore that TB infection of as many as 81% (722) of the participants in our main population had taken place outside of the household. In our

investigation we observed that participants that converted faster were significantly older than those that converted later and that the BCG scar was present in 41.1% of fast TST convertors compared to 77.7 % of slow TST convertors**.**

At the point of study recruitment, both pro-inflammatory and Th2 cytokines were significantly elevated in fast TST convertors in response to *M.tb* PPD. These included IP-10, IL-8, GM-CSF, IL-2, IL-5 and IL-13. IP10 is a chemokine of the innate immune system, is mainly produced by the macrophages and attracts NK cells, neutrophils, monocytes and $CD4^+$ memory T cells as well as promoting the growth of endothelial cells (Mukaida *et al.,* 2003). IL-8, GM-CSF AND IL-13 are cytokines of both the innate and the adaptive immune systems. IL-8 is a potent neutrophil attractant (Mukaida *et al.,* 2003). GM-CSF stimulates neutrophil antimicrobial activities, enhances phagocytosis in macrophages and stimulates the production of other pro-inflammatory cytokines (Enzler & Dranoff, 2003); and IL-13 down-regulates and modulates inflammation and also induces TGFβ (Grüning *et al.,* 2003). IL-2 and IL-5, on the other hand, are produced by the cells of the adaptive immune system. IL-2 plays a unique role in the expansion of Foxp3- suppressor cells (Zheng *et al.,* 2007) and is essential for optimizing CD8+ T-cell functions (Boyman *et al.,* 2010). IL-5 is responsible for eosinophil localization, enhances the histamine releasing abilities of basophils and stimulates antibody production (Kok *et al.,* 2003). The involvement of both the innate and the adaptive immune systems in response to *M.tb* PPD in fast convertors when they were still TST negative may be indicative of an innate immune system that is fighting the bacterial challenge and leading to the mobilization of the adaptive immune system. It is well established that while in some cases innate immunity may be able to effectively eliminate *M.tb* in other instances adaptive immunity may be required to fight and to contain the infection (Bhatt & Salgame, 2007).

In their review, Doherty and co-workers (2009) assert that cytokine expression profiling presents an important instrument in biomarker studies. The cytokine expression pattern that was revealed in response to *M.tb* PPD in TST negative HHC in our study demonstrates that it may be possible to predict whether TST negative HHC would convert their TST faster or lower. When fast convertors had become TST positive at month 6, all cytokines that had been produced in response to *M.tb* PPD at recruitment in this study group were enhanced. In addition, responses to *M.tb* PPD in fast convertors at this time point resulted in increased concentrations of innate immune system cytokines including TGFα, EGF and MCP-1, those of both the innate and adaptive immune system such as IL-4, sCD40L and IFN-γ, as well as IL-17, which is a cytokine of the adaptive immune system. When we investigated the relationship between cytokines in response to *M.tb* PPD in fast convertors, we observed that GM-CSF was associated with IP-10, IL-2, IL-5, IL-8 and IL-13 at both time points. While responses to ESAT6/CFP10 fusion protein did not result in significant differences between the study groups at recruitment, we observed that 6 months into the study the fusion protein resulted in significantly higher MCP-1, IP10 and IFN-γ concentrations in fast TST convertors only. This is in concordance with a previous study which showed that there was an association between TST conversion and increased IP10 levels as a result of stimulation with ESAT6/CFP10 fusion protein (Lighter, 2009b). Six months into our study there were no changes in IP10 levels due to ESAT6/CFP10 fusion protein in subjects that did not convert their TST status. Further, no changes were observed in MCP-1, GM-CSF and IL-8 over time due to *M.tb* PPD in this study group.

We have shown that, at recruitment, slow convertors produced significantly higher VEGF, IL-12p70 and IL-10 in response to Dos-R antigens only. VEGF is responsible for proliferation of dendritic cells, angiogenesis and increased vascular permeability of endothelial cells, differentiation of hematopoietic cells as well as chemo-attraction of

monocytes (Gabrilovich & Dikov 2003). IL-12p70 is produced by macrophages and dendritic cells within hours after the encounter with pathogens and acts on NK cells as well as on macrophages and dendritic cells (Langrish *et al.,* 2004). IL-10 suppresses the immune responses on B cells, T cells as well as on antigen presenting cells (De la Barrera *et al.,* 2004), it seems that at the point of study enrolment slow convertors may have mounted a potent innate immune response through enhanced NK cytotoxic effects and may have been able to fight the *M.tb* onslaught**.** ESAT6/CFP10 fusion protein and all 3 Dos-R antigens tested in this chapter induced high levels of MCP-1 in both fast and slow convertors, and these responses were significantly higher in slow convertors. MCP-1 is one of the most important chemokines that is produced in response to *M.tb* and attracts monocytes, dendritic cells, NK cells and memory T cells (Xia & Sui, 2009). At the recruitment time point when all the HHC were TST negative, Dos-R antigens incited a potent innate immune response that was stronger in slow convertors than in fast convertors. 6 Months into the study, MCP-1 levels, in response to Rv2029c and ESAT6/CFP10, had increased significantly in fast convertors only indicating that, not only were these subjects mounting adaptive immune responses, but they were also engaging vigorous innate immune responses in their endeavour to contain the infection. Both IL-12p40 and IL-12p70 were collectively expressed at higher levels at the 6 month time point in both fast and slow TST convertors but this was seen only in response to Dos-R regulon-encoded antigens indicating that in both study groups there was an on-going attempt to fight the enduring *M.tb* challenge as illustrated by the up-regulated adaptive responses in fast convertors and the sustained innate responses in slow convertors. With regards to other cytokine responses to Dos-R antigens over time, increased expression of MIP-1α, IL-1α and IL-1ra in fast convertors were observed, whereas these antigens resulted in decreased production of G-CSF, IL-10 and VEGF in slow TST convertors. These responses further demonstrate the differential cytokine responses of these study groups. We

did not perform another TST after this time point after fast convertors had become TST positive. All, but 1 of the slow convertors converted their TST to positive by month 18 and no other TST was performed on them as well. Therefore we do not know the TST status of either group at later time points. However, by month 24, none in either group had developed TB.

CHAPTER 4: MAIN FINDINGS AND CONCLUSIONS

The present study demonstrates that, in our setting, there is a high rate of transmission of tuberculosis infection in household contacts of active TB cases. At the point of study recruitment, 92.8% of the household contacts were tuberculin skin test (TST) positive, and all but 1 out 893 contacts had converted to TST positive by month 18. The transmission rates of TB infection to household contacts vary widely from 27% in The Gambia, (Jackson-Sillah, et *al.,* 2007), to 43% in Brazil (Cailleaux-Cezar *et al.,* 2009) to 80% in Pakistan (Hussain *et al.,* 2007). It is possible that some of the TST positive individuals in our cohort had been exposed to environmental mycobacteria although in a highly TB endemic area it is likely to be *M.tb*. Antigens that are present in PPD are also found in BCG and in other mycobacteria, rendering the TST non-specific (Lalvani & Pareek, 2009). There is a possibility that the participants in our study had been infected outside of the household, in public transport such as taxis, which have no ventilation and that is used daily for travel. However, since all were exposed to recently diagnosed sputum positive TB case in the household, it is also likely that transmission had occurred within the household setting. Whereas IGRAs are said to have greater abilities to detect recent exposure, the TST has been shown to have greater abilities for detection of remote exposure (MWWR, 2010). IGRA testing was not done in this study; however we assumed that the 26 study participants that converted their TST status by month 18 had been recently infected by *M.tb*. While both the TST and the IGRAs cannot distinguish between active and latent tuberculosis (Mack *et al.,* 2009), during the period under investigation in this study active TB disease in HHC was excluded at all time points and none of the contacts had progressed to active TB after 24 months. After *M.tb* infection, it is not known who is most vulnerable to progression to disease. Yet, future active disease cannot be excluded because the incidence of TB in this community has continued to rise due to increased transmission (Kritzinger, *et al.,* 2009; Shanaube *et al.,* 2009). This could be

attributed to the poverty levels that prevail within this setting as there is a causal link between the risk of developing TB and social capital (van Rie *et al.,*1999; Holtgrave and Crosby, 2004; Baker *et al.,*2008; Harling *et al.,* 2008). Several longitudinal studies involving healthy HIV negative household contacts of TB index cases have established that between 4 and 6.7% of contacts developed active TB within 2 years of the initial contact (Teixeira *et al., 2001*; Wang & Lin, 2000; Hussain *et al.,* 2007). It is not surprising that the transmission of TB was 99.9% in our study setting because *M.tb* is transmitted in aerosols from person to person and those living in close contact to active TB cases are more vulnerable to infection (Guwatudde *et al.,* 2003; Hussain *et al.,* 2007). It has been shown that children living in close contact with a TB patient under conditions of overcrowding are more vulnerable to TB disease (Tornee *et al.,* 2005). Active case finding is not implemented in South Africa. It is possible therefore that there could be more active TB cases that remain undetected within our setting, which could be fueling the high rate of LTBI and active TB in that community. Pronyk *et al* (2001) have shown in their study that there were 2 undetected active TB cases for every 9 that were detected.

No differences were observed in the contact gradient to the TB index case of household contacts that were already TST positive at recruitment compared to those that were TST negative. It is important to note that ventilation is not included in calculating the contact score. Furthermore, of the 26 participants who were TST negative at recruitment and were successfully followed up, with 17 converting to TST positive by month 6 and 9 remaining TST negative, there were no differences between their gradients of exposure to respective TB index cases. Although our study has not investigated the risk of progression to active disease among the household contacts, others have observed that the duration of contact was associated with risk of disease and that the presence of cavitary disease in TB cases was

associated with bacillary burden as well as with the clinical characteristics of the index case (Guwatudde *et al.,* 2003).

Wood *et al.,* (2010) found LTBI infection of 28% in the 5-10 year age group which increased to 88% in the 31-35 years age group. While our cohort was HIV negative, there are 5.7 million South Africans living with HIV (UNAIDS, 2008). It is known that the risk of developing active TB is higher in HIV infected individuals (Narain, 1992). This means that should those contacts acquire HIV infection, they could be most vulnerable to active TB disease. TB infection levels are also high among South African miners (Hanifa *et al.,* 2009).

In this study we have observed that BCG scar status (presence or absence) did not have an influence on the TST results in our study because the force of infection is so high in our setting. This is in agreement with other studies (Marks *et al*., 2000; Lienhardt *et al.*, 2003). Further, we did not observe a relationship between BCG scar status and induration size in the TST positive group at recruitment or the TST negative group following conversion at 6 months. When all household contacts at recruitment were considered, we observed a trend for an association between TST status (positive or negative) at recruitment and the smear grade of the known TB index case because the bacillary burden is important for transmission to be successful. Is has previously been observed that there was higher risk of being TST positive in household contacts of TB cases with higher smear grades (Lienhardt *et al.*, 2003; Okada *et al.,*2008).

We measured interferon gamma production in response to a panel of classical and novel *M.tb* antigens to identify whether they could discriminate between TB infection states (as measured by the TST) and active TB cases. With regards to the classical TB antigens, as expected, all TST positive HHC responded to ESAT6/CFP10 fusion protein. In active TB cases, we observed an 87.5 % response rate to the fusion protein. Of the 17 TST negative

subjects, 4 (26.7%) responded to ESAT6/CFP10 fusion protein. All TST positive subjects also produced a positive IFN-γ response to *M.tb* PPD, even though there was no correlation between TST induration size at recruitment and IFN-γ responses to *M.tb* PPD (r=0.06, p=0.81). Three of the 4 TST negative subjects that responded to ESAT-6/CF10 fusion protein also produced a positive IFN-γ response to *M.tb* PPD. This may indicate that even though the DTH could not pick up the infection at the point of the study recruitment, it is possible that those participants were already infected. Both ESAT6 and CFP10 are found in IGRAs (Mack *et al.,* 2009; Ruhwald & Ravn, 2009) and *M.tb* PPD has also previously been included as an antigen in these cell culture based tests for infection.

The ability of a number of antigens across the growth phases of *M.tb* to differentiate infection and disease states as we have shown with Rv1737c, Rv2450c, Rv2029c, TB10.3, *M.tb* PPD may offer a possibility for the development of diagnostic or vaccine instruments using a combination of those antigens. Recently a combination of classical antigens, RPFs and Dos-R antigens has been used to generate a vaccine (Zvi et al., 2008). Because *M.tb* changes gene expression in response to its microenvironment, a combination of antigens expressed at different growth phases which can differentiate between diseases states may offer possibilities for the development of diagnostic tools that may be able to discriminate between latent infection and active disease. Also, there is a need for vaccines that can be used after individuals have been latently infected in order to minimise the risk of reactivation and reinfection (Kauffman *et al.,* 2010). The combination of these antigens with discriminatory abilities may also offer an opportunity for the development of post-exposure vaccines.

A prolonged ability to fight off infection may represent a marker of a protective phenotype against TB. In the household contacts that were TST negative at recruitment, we were interested in finding out whether we could identify differences in the cytokine responses to

M.tb antigens between those contacts that converted their TST status by month 6 to those that only converted later. In this study we observed that, at the point of study recruitment, *M.tb* PPD elicited higher pro-inflammatory and Th2 cytokines in fast TST convertors. The cytokines that were higher in fast convertors were GM-CSF, IP-10, IL-8, IL-13, IL-5 and IL-2. In addition, IL-12p70 and VEGF produced in response to Rv1737c and Rv2029c respectively were also significantly higher in the fast TST convertors. This suggests that *M.tb* PPD, Rv1737c or Rv2029c may be useful predictors of the rate of infection in TST negative household contacts. We also observed that in contacts that took longer to convert their TST status, Dos-R regulon-encoded proteins Rv1733c, Rv1737c and Rv2029c, all elicited much stronger MCP-1 responses compared to fast convertors at recruitment. To our knowledge this is the first study that investigates the predictive role of multiple cytokines in TST conversion rate using several novel *M.tb* antigens. Hussain *et al.,* (2009) observed that TST conversion is associated with early increases in IFN γ and IL-10. In our study we also observed that IL-10 could discriminate between fast TST and slow convertors at recruitment, in response to Rv2029c.

Over time, IL-8, fractalkine, EGF, IFN-γ, IL-2, IL-4, sCD40 ligand, IL-5, IL-13, and IL-17 were all increased in fast convertors only in response to *M.tb* PPD. IFN-γ levels were also higher in fast convertors in response to ESAT-6/CFP10 fusion protein 6 months into the study.

By the 6 month time point, IL-10 and G-CSF levels had decreased in response to Rv2029c in slow convertors, and VEGF had also decreased in response to Rv1737c. In the slow TST convertors, the immune responses appear to be mostly due to the innate immune system, therefore not specific.

A major limitation of this study is the small number of participants. Also, none of the subjects were tested for IGRA. The WHO has recently recommended that both the TST and an IGRA should be used in diagnosing LTBI (MWWR, 2010). The cost of conducting both tests may be limiting for a developing country, like South Africa. Another limitation of this study is that we did not ascertain the infecting *M.tb* strains of the index cases of HHC. It is possible that diferent strains may determine diferent diseases phenotypes. While we are still a long way from finding the exact immune correlates that may provide protection against *M.tb*, this study shows that the immune responses to *M.tb* in household contacts that differ in their TB infection and disease states are distinct. Also, this study provides an insight into the different immune profiles that are associated with TST conversion over time.

Mechanisms that are involved in determining the rate of infection with *M.tb*, as well as different states of TB infection and disease could be further elucidated through studies investigating phenotypic differences in cells of the innate and adaptive immune system using flow cytometry across different study groups. The immune response of the slow TST convertors by the time they convert to TST positive at month 18 not known. Answering these questions could lead to a better understanding of the protective immune response against *M.tb* and provide information leading towards the design of more effective diagnostic tests and vaccines against tuberculosis. In this exploratory study, we observed correlates that may be used to predict TST negative subjects that would convert their earlier when they were still TST negative. However, it is difficult to predict immune correlates of protection from this study as no household contacts had progressed to active disease 24 months into the study.

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Appendix 1.1: Table of cytokine correlations: Fast TST convertors at recruitment in response to *M.tb* PPD

Cytokine/ Chemokine	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MIP-1α	MIP-1β
IL-1ra	0.56*								
IL-1α	0.61* 0.	54*							
IL-12p40			0.65*	0.55*					
IL-17	0.	85***		0.57*					
IP-10		0.88***				0.86***			
MCP-1	0.53*								
MIP-1α			0.50*						
MIP-1β					0.60*			0.76**	
TGF-α		0.52*						0.59*	
TNF-α	0.	72*		0.77**		0.58*			0.50*
IFN-γ						0.66*	0.51*	0.51*	
IL-2	0.	56*				0.57* 0.	75**		
IL-5		0.63*				0.70*	0.70*		
IL-10	0.	58*		0.74*		0.68* 0.	57*		
IL-13		0.64*		0.54*		0.55*	0.50*		0.57*
IL-1β	0.89***		0.62* 0.	83***					
IL-6	0.80**		0.57*	0.68*					
IL-8	0.64* 0.	60*		0.86***		0.54*			

Cytokine	TNF-α	IFN-γ	IL-2	IL-10	IL-13	IL-1β	IL-6
IL-5			0.65*				
IL-10	0.52* 0.	78**					
IL-13	0.58*			0.54*			
IL-1β	0.70* 0.			56*			
IL-6	0.54*			0.53*		0.83***	
IL-8	0.76** 0.	51* 0.		80** 0.	54* 0.	80** 0.	73*

Appendix 1.1: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and $^{***}p<$ or = 0.0001.

Appendix 1.2: Table of cytokine correlations: Fast TST convertors in response to ESAT6/CFP10 fusion protein at recruitment

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IP-10	MCP-1	MIP-1α	MIP-1β
G-CSF	0.66*									
IL-1ra	0.81*** 0.	72* 0.	57*							
IL-1α	0.67*	0.93***	0.55*	0.75**						
IL-12p40	0.66* 0.	72* 0.		64* 0.	67*					
IP-10			0.63*							
MCP-1							-0.56*			
MIP-1α		0.79** 0.	68* 0.	61* 0.	79** 0.	59*				
MIP-1β		0.86***	0.52*	0.56*	0.79***	0.61*			0.93***	
TGF-α		0.61* 0.		62* 0.	61* 0.			51* 0.	53* 0.	69*
TNF-α	0.56*	0.84***		0.64*	0.85***	0.80***			0.83***	0.76**
VEGF	0.62* 0.	77** 0.		73* 0.	59* 0.	74** 0.			58* 0.	63*
IFN-γ			0.83***				0.54*		0.66*	0.50*
IL-10	0.52* 0.	89*** 0.	57* 0.	64* 0.	89*** 0.	68* 0.			90*** 0.	87***
IL-1β	0.71*	0.95***		0.71*	0.90***	0.76**			0.77**	0.81***
IL-6	0.71* 0.	94*** 0.		75** 0.	92*** 0.	75** 0.			78** 0.	84***
IL-8		0.84***		0.56*	0.87***	0.59*			0.76**	0.75**
IL-12p70	0.53* 0.	63* 0.			60* 0.	70* 0.			60* 0.	55*

Appendix 1.2: Spearman correlation values with * denoting $p<0.05$, ** $p <0.001$ and $^{***}p<$ or = 0.0001.

Cytokine	TGF-α	TNF-α	VEGF	IFN-γ	IL-10	IL-1β	IL-6	IL-8
VEGF	0.55* 0.	68*						
IL-10	0.54* 0.	88*** 0.	64* 0.	52*				
IL-1β	0.54*	0.88***	0.75**		0.84***			
IL-6	0.65* 0.	83*** 0.	71* 0.		81*** 0.	96***		
IL-8	0.52*	0.82***	0.51*		0.79**	0.85***	0.88***	
IL-12p70		0.65*			0.62* 0.	74** 0.	70* 0.	60*

Appendix 1.2: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and $^{***}p<$ or $= 0.0001$.

Appendix 1.3: Table of cytokine correlations: Fast TST convertors in response to Rv1733c at recruitment

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10
IL-1ra		0.67*						
MCP-1						0.60*		
MIP-1α		0.53* 0.	63*					
MIΠ−1β		0.69*						
TGF-α	0.52* 0.	62* 0.		75**				
TNF-α					0.54*			
IFN-γ							0.50* 0.	56*
IL-10		0.56*						
IL-6		0.75**		0.51*	0.66*			
IL-8						0.50*		
IL-12p70						0.52*		0.69*

Appendix 1.3: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***$p\leq 0.0001$ (printed as "p< or = 0.0001").

Cytokine	MCP-1	MIP-1α	MIP-1β	TGF-α	TNF-α	IFN-γ	IL-10
MIP–1β		0.82^{***}					
TGF-α			0.52^{*}				
TNF-α		0.66^{*}					
IL-10	0.56^{*} 0.					58^{*}	
IL-1β						0.51^{*} 0.	64^{*}
IL-6		0.74^{*}	0.62^{*}	0.67^{*}	0.84^{***}		
IL-8							
IL-12p70					0.74^{*}	0.52^{*}	

Appendix 1.3: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and $^{***}p<$ or $= 0.0001$.

Appendix 1.4: Table of cytokine correlations: Fast TST convertors in response to Rv1737c at recruitment

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IP-10	MCP-1
GM-CSF	0.52*	0.53*						
IL-1ra		0.64*						
IL-1α	0.51*	0.80***	0.63*	0.65*				
IL-12p40	0.61* 0.				57*			
IP-10			0.68*		0.63*			
MIP-1α		0.72* 0.	63* 0.		68* 0.		63*	
MIP-1β		0.76**			0.64*			
TGF-α	0.59* 0.	57* 0.		74** 0.	57* 0.			63*
TNF-α		0.59*	0.53*		0.68*	0.76**	0.76**	
VEGF		0.60* 0.		72* 0.	52* 0.			52*
IFN-γ		0.55*	0.71*				0.79**	
IL-10		0.95*** 0.	54* 0.	58* 0.	72* 0.		55*	
IL-1β		0.76** 0.	71* 0.		79** 0.	62* 0.	71*	
IL-6		0.87***	0.53*	0.59*	0.85***	0.66*	0.56*	0.52*
IL-8								
IL-12p70		0.51*	0.57*		0.50*	0.61*	0.80***	

Cytokine	MIP-1α	MIP-1β	TGF-α	TNF-α	VEGF	IFN-γ	IL-10	IL-1β	IL-6	IL-8
MIP-1β	0.80*									
TGF-α		0.52*								
TNF-α	0.73**									
VEGF			0.53*							
IFN-γ	0.72*			0.63*						
IL-10	0.72* 0.	74** 0.	51* 0.	67* 0.	62* 0.	60*				
IL-1β	0.65* 0.			79** 0.	66* 0.	60* 0.	83***			
IL-6	0.77**	0.68*	0.60*	0.79**		0.54*	0.84***	0.77**		
IL-8	0.56*									
IL-12p70	0.80*			0.85***		0.73**	0.62*	0.69*	0.66*	0.51*

Appendix 1.4: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and *** $p<$ or = 0.0001.

Appendix 1.5: Table of cytokine correlations: Fast TST convertors in response to Rv2029c at recruitment

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MCP-1	MIP-1α
G-CSF	0.59*									
GM-CSF		0.51*								
IL-1ra	0.59*	0.54*								
IL-1α		0.70* 0.	64*							
IL-12p40	0.65*			0.61*						
IP-10			0.52*				0.55*			
MIP-1α	0.70*	0.63*			0.55*	0.65*		0.60*		
MIP-1β	0.69* 0.	64* 0.		69* 0.		59* 0.				88***
TNF-α	0.67* 0.	65* 0.	51* 0.	50* 0.	72* 0.	82*** 0.		62* 0.		84***
VEGF				0.58*						
IFN-γ			0.65* 0.				63* 0.	73*		
IL-10	0.74**	0.74*		0.63*		0.73*				0.82**
IL-1β	0.55*	0.62*	0.56*		0.57*	0.69*		0.50*		0.76*
IL-6	0.59* 0.	72* 0.		74* 0.	64* 0.	80** 0.		50* 0.		79*
IL-8	0.57*				0.56*	0.75**		0.59*	0.58*	0.50*
IL-12p70						0.70* 0.		68* 0.		73*

Cytokine	MIP-1β	TGF-α	TNF-α	VEGF	IFN-γ	IL-10	IL-1β	IL-6	IL-8
TGF-α	0.69*								
TNF-α	0.63*								
IFN-γ			0.56*						
IL-10	0.71*	0.53*	0.84***	0.55*	0.53*				
IL-1β	0.63*		0.80**	0.50*	0.57*	0.82***			
IL-6	0.74* 0.	53* 0.	88*** 0.			83*** 0.	71*		
IL-8			0.79**		0.53*	0.66*	0.56*	0.59*	
IL-12p70			0.82*** 0.		59* 0.	66* 0.	57* 0.	61* 0.	79**

Appendix 1.5: Spearman correlation values with * denoting $p<0.05$, ** $p <0.001$ and *** p< or = 0.0001.

Appendix 1.6: Table of cytokine correlations: Fast TST convertors in response to Rv2450c at recruitment

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IP-10	MIP-1α	TNF-α	IL-10	IL-1β	IL-6
GM-CSF	0.58*	0.70*	0.52*								
IL-1ra	0.64*	0.78**	0.63*								
IL-1α			0.57*	0.83***							
IL-12p40			0.52*								
IL-17			0.74*								
MIP-1β							0.82***				
IL-1β	0.57*	0.69*	0.54*	0.88	0.92***	0.58*		0.91***	0.65*		
IL-6		0.64*	0.53*	0.82***	0.93***	0.57*		0.91***	0.71*	0.96***	
IL-8			0.53*								

Appendix 1.6: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***$p\leq 0.0001$ (printed as "p< or = 0.0001").

Appendix 1.7: Table of cytokine correlations: Slow TST convertors at recruitment in response to *M.tb* PPD

Cytokine	IL-12p40	IP-10	MCP-1	MIP-1α	MIP-1β	IFN-γ
MIP-1α	1.00*					
TNF-α					0.88*	
IL-10		0.93*				0.87*
IL-8			0.89* 0.			99*

Appendix 1.7: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***p< or = 0.0001.

Appendix 1.8: Table of cytokine correlations: Slow TST convertors at recruitment in response to ESAT6/CFP10 fusion protein

Cytokine	GM-CSF	IL-1α	IL-12p40	IP-10	MIP-1α	TNF-α	IL-1β	IL-6
IP-10	0.94*	0.89*						
MIP-1β					0.99*			
VEGF		0.88*						
IFN-γ		0.89*						
IL-6			0.94*			0.89*	0.94*	
IL-8			0.89* 0.	89* 0.			89* 0.	94*

Appendix 1.8: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***$p\leq 0.0001$.

Appendix 1.9: Table of cytokine correlations: Slow TST convertors at recruitment in response to Rv1733c

Cytokine	**G-CSF**	**GM-CSF**	**IL-1ra**	**IL-1α**	**IL-17**	**VEGF**	**IL-1β**
IL-1α		-0.99*	0.60*				
IL-12p40			0.54*				
TGF-α			0.90*				
VEGF	0.89* 0.		94*				
IL-10			0.89*			0.94*	
IL-1β		-0.93* 0.		89* -0.90	*		
IL-6		-0.93*		0.89*	-0.90*		1.00*

Appendix 1.9: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***$p \leq 0.0001$.

Appendix 1.10. Table of cytokine correlations: Slow TST convertors at recruitment in response to Rv1737c

Cytokine	G-CSF	GM-CSF	IL-1ra	IL-1α	IP-10	MCP-1	MIP-1α	MIP-1β	TGF-α	TNF-α	VEGF	IL-1β
IL-1α			0.94*									
IP-10		0.89*										
MIP-1α	1.00*											
MIP-1β	1.00* 1.						00*					
TNF-α					0.94*							
VEGF	1.00* 1.						00* 1.	00*				
IFN-γ									0.94*			
IL-10	1.00* 1.						00* 1.	00* 1.			00*	
IL-1β			0.94*	0.89*								
IL-6			1.00* 0.	94* 0.								94*
IL-12p70		0.94			0.94*					0.89*		

Appendix 1.10. Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***$p\leq 0.0001$.

Appendix 1.11. Table of cytokine correlations: Slow TST convertors at recruitment in response to Rv2029c

Cytokine	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IP-10	MIP-1α	TGF-α	TNF-α	VEGF	IFN-γ	IL-10	IL-1β
IL-1ra	0.89*	0.89*											
IL-1α	0.94*												
IL-12p40	0.94*	0.94*	0.94*	0.89*									
IP-10	0.94* 0.	94* 0.	94* 0.	89* 1.	00*								
MIP-1α	0.94*	0.94*	0.94*	0.89*	1.00*	1.00*							
MIP-1β	1.00* 0.		89* 0.	94* 0.	94* 0.	94* 0.	94*						
TNF-α		0.89*	0.89*		0.94*	0.94*	0.94*	0.09*					
VEGF	0.99* 0.		93* 0.	90* 0.	93* 0.	93* 0.	93* 0.	64* 0.	81*				
IFN-γ	0.94*		0.94*		0.89*	0.89*	0.89*	0.66*	0.77*	0.99*			
IL-10	0.94* 0.				83* 0.		83* 0.	77* 0.	66* 0.	93* 0.	89*		
IL-1β	1.00*		0.89*	0.94*	0.94*	0.94*	0.94*	0.60*	0.83*	0.99*	0.94*	0.94*	
IL-6						0.94*							
IL-12p70	0.93*	0.93*	0.99*		0.99*	0.99*	0.99*		0.93*	0.94*	0.93*	0.81*	0.93*

Appendix 1.11. Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and *** $p\leq 0.0001$.

Appendix 1.12: Table of cytokine correlations: Fast TST convertors at month 6 in response to *M.tb* PPD

Cytokine	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MCP-1	MIP-1α	MIP-1β
IL-1α	0.67*	0.58*								
IL-12p40		0.58*		0.84***						
IL-17		0.61*								
MCP-1		0.64*			0.53*					
MIP-1α	0.52*		0.71*							
MIP-1β	0.58*		0.60*			0.65*			0.89***	
TNF-α		0.74**			0.61*	0.53*		0.56*	0.51*	
IFN-γ	0.54*	0.79**		0.61*	0.62*	0.51*		0.58*		
IL-2		0.53					0.52*			
IL-5		0.82***				0.72*				
IL-10		0.76**				0.70*				
IL-13		0.84***		0.53*	0.52*	0.68*				0.52*
IL-1β	0.86***			0.69*						
IL-6	0.73*			0.76**	0.65*					
IL-8		0.81***				0.55*	0.61*	0.54*		
IL-12p70	0.55*									

Cytokine	TNF-α	IFN-γ	IL-5	IL-10	IL-13	IL-1β
IL-2	0.68*					
IL-4		0.52*				
IL-10	0.73*	0.72*				
IL-13	0.57* 0.	51* 0.	71*			
IL-1β	0.82***	0.72*	0.94***	0.72*		
IL-6		0.57*				
IL-8		0.63*	0.59*		0.60*	0.82***

Appendix 1.12: Spearman correlation values with * denoting $p<0.05$, ** $p <0.001$ and *** $p\leq 0.0001$ (printed "p< or = 0.0001").

Appendix 1.13: Table of cytokine correlations: Fast TST convertors at month 6 in response to ESAT6/CFP10 fusion protein

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MIP-1α	MIP-1β	TGF-α
IL-1ra	0.74**										
IL-1α		0.78**									
IL-17			0.58*								
IP-10			0.63* 0.				65*				
MCP-1							0.59*				
MIP-1α		0.62* 0.			71*						
MIP-1β		0.85***			0.81***				0.80***		
TGF-α			0.64* 0.		56* 0.				69* 0.	62*	
TNF-α		0.77**			0.82***	0.54*			0.67*	0.86***	
VEGF	0.67*										
IFN-γ			0.60*		0.50*			0.52*	0.55*		0.64*
IL-2			0.83*** 0.				55* 0.				56*
IL-5	-0.63*			-0.62*			0.65*				
IL-10		0.70* 0.			89*** 0.				65* 0.	77** 0.	65*
IL-13	-0.50*	-0.64*	0.53*								
IL-1β		0.89*** 0.			91*** 0.				55* 0.	77**	
IL-6		0.88***		0.52*	0.87***				0.55*	0.81***	
IL-8						0.50 0					.51*

Cytokine	TNF-α	IFN-γ	IL-2	IL-5	IL-10	IL-13	IL-1β	IL-6
IL-2		0.50*						
IL-10	0.87***	0.64*						
IL-13			0.60*	0.55*				
IL-1β	0.79**				0.82***	-0.71*		
IL-6	0.87***				0.83**	-0.69*	0.95***	
IL-8	0.53				0.61*			0.53*

Appendix 1.13: Spearman correlation values with * denoting p<0.05, ** p <0.001 and ***p< or = 0.0001.

Appendix 1.14: Table of cytokine correlations: Fast TST convertors at month 6 in response to Rv1733c

Cytokine	G-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MIP-1α	MIP-1β	TNF-α	IL-1β	IL-6
IL-1α	0.79**										
IP-10					0.52*						
MIP-1β							0.59*				
TGF-α		0.73* 0.						54*			
TNF-α					0.53*						
VEGF	0.55* 0.	58*									
IFN-γ						0.55*					
IL-10	0.63* 0.		67* -0	.56* 0.			61* 0.	69*			
IL-1β	0.85***		0.87***								
IL-6	0.64* 0.		67* 0.					70* 0.		75**	
IL-12p70						0.57*			0.62*		0.56*

Appendix 1.14: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***p< or = 0.0001

Appendix 1.15: Table of cytokine correlations: Fast TST convertors at month 6 in response to Rv1737c

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-17	IP-10	MCP-1	MIP-1α	MIP-1β
IL-1α		0.53*								
IL-17			0.57* -0.51		*					
MCP-1			0.51*			0.51*				
MIP-1α		0.62* 0.			60* 0.		50*			
MIP-1β		0.72*							0.51*	
TGF-α										0.67*
TNF-α							0.90***		0.56*	
VEGF	0.55* 0.			73**						
IFN-γ			0.70*			0.56*		0.60*		
IL-10		0.85*** 0.			61* 0.				54* 0.	62*
IL-1β		0.82***		0.50*	0.63*				0.52*	0.52*
IL-6									0.50* 0.	75**
IL-12p70							0.67*	0.64*		

Cytokine	TGF-α	TNF-α	VEGF	IFN-γ	IL-10
IL-10			0.57^{*}		
IL-1β			0.59^{*} 0.		83^{***}
IL-6	0.83^{***}			0.50^{*}	
IL-12p70		0.66^{*} 0.		67^{*}	

Appendix 1.15: Spearman correlation Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***$p\leq 0.0001$ (printed "p< or = 0.0001").

Appendix 1.16: Table of cytokine correlations: Fast TST convertors at month 6 in response to Rv2029c

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MIP-1α	MIP-1β
IL-1α		0.77*								
IL-17			0.76**							
IP-10							0.59*			
MCP-1		-0.57*								
MIP-1α		0.61*			0.69*					
MIP-1β		0.74* 0.			67* 0.				80***	
TGF-α				0.62*						
TNF-α						0.76*** 0.		57* 0.		52*
VEGF			-0.56*	0.54*						
IFN-γ			0.53* 0.				68* 0.	58*		
IL-2		-0.61*			-0.66*		0.59*		-0.58*	-0.61*
IL-10	0.52* 0.			53* 0.	59* 0.					69*
IL-1β		0.75**			0.87***					0.59*
IL-6		0.77** 0.			71* 0.				61* 0.	78**
IL-8								-0.51*		
IL-12p70						0.64 0.		52*		

Cytokine	TGF-α	TNF-α	VEGF	IL-2	IL-10	IL-1β	IL-6	IL-8
IL-10	0.70*							
IL-1β			0.61*	-0.65*	0.69*		0.77**	0.59*
IL-6		0.54*		-0.71*	0.53*			
IL-12p70		0.87***					0.59*	

Appendix 1.16: Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and ***p< or = 0.0001.

Appendix 1.17: Table of cytokine correlations: Slow TST convertors at month 6 in response to *M.tb* PPD

Cytokine	GM-CSF	IL-1ra	IL-17	IP-10	MIP-1α	TNF-α	IFN-γ	IL-2
IL-17		0.88						
IP-10			0.79					
MIP-1α	0.96							
MIP-1β					0.69			
TNF-α	0.96				0.93			
IFN-γ	0.88		0.56 0	.89 0	.77	0.85		
IL-2	0.93				0.85	0.86	0.93	
IL-10								0.52
IL-8						0.54	0.59	0.79

Appendix 1.17: Spearman correlation values, all $p<0.05$.

Appendix 1.18: Table of cytokine correlations: Slow TST convertors at month 6 in response to ESAT6/CFP10 fusion protein

Cytokine	G-CSF	GM-CSF	IL-1α	IL-17	IP-10	MCP-1	MIP-1α	MIP-1β	TNF-α	IFN-γ	IL-2	IL-5	IL-10	IL-1β	IL-6
IL-1α		0.78*													
IP-10		0.90* 0.		90*											
MCP-1				0.76*											
MIP-1α		0.76* 0.	72*												
MIP-1β	0.71*						0.83*								
TNF-α	0.71* 0.	78* 0.			71* 0.		90* 0.	88*							
IFN-γ		0.88*		0.86*	0.97***										
IL-2		0.78* 0.		88* 0.	91* 0.					94*					
IL-5		0.81*	0.79*	0.73*	0.87*					0.84*	0.80*				
IL-10	0.86* 0.	71* 0.	52* 0.				83* 0.	81* 0.	79*						
IL-1β		0.73*	0.81*				0.93**	0.81*	0.90*			0.74*	0.76*		
IL-6	0.81* 0.	88* 0.	74* 0.		76* 0.		79* 0.		83* 0.	77* 0.		74* 0.	90* 0.	79*	
IL-8		0.85*		0.76*	0.90*				0.90*	0.83*	0.72*	0.74*		0.74*	0.81*
IL-12p70	0.74* 0.							82* 0.					88*		

Appendix 1.18: Table of cytokine correlations: Slow TST convertors at month 6 in response to ESAT6/CFP10 fusion protein. Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and *** $p\leq 0.0001$ (printed as "p< or = 0.0001").

Appendix 1.19: Table of cytokine correlations: Slow TST convertors at month 6 in response to Rv1733c

Cytokine	EGF	G-CSF	GM-CSF	IL-1α	IL-12p40	IP-10	MCP-1	TGF-α
Eotaxin	-0.84							
GM-CSF		0.83						
MIP-1α		0.79	0.93					
TNF-α		0.76	0.79					
VEGF							0.88	
IFN-γ						0.74		
IL-10								0.81
IL-1				0.90				
IL-6				0.81				
IL-8					0.74			

Appendix 1.19: Table of cytokine correlations: Slow TST convertors at month 6 in response to Rv1733c. Spearman correlation values, all $p<0.05$.

Appendix 1.20: Table of cytokine correlations: Slow TST convertors at month 6 in response to Rv1737c

Cytokine	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IL-17	IP-10	MCP-1	MIP-1α	MIP-1β	TGF-α	TNF-α	IFN-γ	IL-1β
GM-CSF	0.88*													
IL-12p40			0.76*											
IL-17		-0.73*												
MCP-1	-0.81*	-0.81*												
MIP-1α	0.74*	0.88*		0.88*		-0.78*		-0.79*						
MIP-1β		0.81*												
TGF-α					0.75*									
TNF-α	0.83*	0.81*		0.83*				-0.88*	0.90*					
VEGF											0.77*			
IFN-γ	0.74*	0.79*					0.86*			0.83*		0.76*		
IL-10		0.74*					0.79*			0.79*			0.90*	
IL-1β		0.81*		0.93*					0.98**			0.88*		
IL-6				0.98**					0.90*			0.86*		0.95*
IL-12p70								-0.79*				0.81*	0.83*	

Appendix 1.20: Table of cytokine correlations: Slow TST convertors at month 6 in response to Rv1737c. Spearman correlation values with * denoting $p<0.05$, ** $p<0.001$ and *** $p \leq 0.0001$.

Appendix 1.21: Table of cytokine correlations: Slow TST convertors at month 6 in response to Rv2029c

Cytokine	EGF	G-CSF	GM-CSF	IL-1ra	IL-1α	IL-12p40	IP-10	MIP-1α	MIP-1β	TNF-α	IFN-γ	IL-10	IL-1β	IL-6
GM-CSF	0.90													
IL-1α	0.88		0.88											
IL-12p40							0.79							
MCP-1									-0.81					
MIP-1α		0.76			0.67									
MIP-1β								0.74						
TNF-α		0.79				0.81	0.76	0.90						
VEGF														
IFN-γ		0.76				0.71	0.74	0.95		0.98				
IL-10		0.79				0.74		0.79		0.95	0.90			
IL-1β				0.74	0.74	0.79		0.86		0.86	0.81	0.76		
IL-6		0.79		0.81		0.76		0.79		0.88	0.81	0.88	0.93	
IL-8							0.81	0.83		0.79	0.83			
IL-12p70		0.74				0.76				0.90	0.83	0.98	0.79	0.93

Appendix 1.21: Table of cytokine correlations: Slow TST convertors at month 6 in response to Rv2029c. Spearman correlation values, all $p<0.05$.