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Biomarkers for TB treatment response and cure; Immunological profiles of individuals infected by *Mycobacterium tuberculosis* complex in Ghana

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Dedication: To my precious sons, Kwesi Akai and Kweku Nyamekye Nakai

ABSTRACT

Background: Given the shortcomings associated with the use of classical microbiological methods such as "sputum culture status'' as a biomarker for TB treatment response and cure, measurement of Mtb- specific antigen induced responses in blood has been proposed as a better option. Characterization of the cellular response to Mtb antigens during treatment is therefore required.

Methods: Peripheral blood mononuclear cells (PBMC) of sputum smear positive TB patients and Quantiferon TB test positive (QFT+) and negative (QFT-) household contacts of TB cases were stimulated with Early Secretory Antigenic Target-6 and Culture Filtrate Protein-10 kDa (ESAT-6/CFP-10) fusion protein and latency associated antigens (Rv1733, Rv2029 and Rv2628). Secreted cytokines (IFN-γ, IL- 17, IL-10, TNF-α, sIL2-Rα and Granzyme B) levels in the six-day culture supernatant was measured at baseline and at the 2nd week of treatment. Frequency of IFN-γ+ CD4 and CD8 T cells was also assessed by multi-colour flow cytometry at four time points during anti TB therapy.

Results: High quantities (pg/ml) of IFN-γ, followed by Granzyme B, TNF-α and IL-17 and lower quantities of IL-10 and sIL2R-α characterized secretion by the antigens in TB patients (n=20) at baseline with increased levels of IFN-γ, Granzyme B, IL-17, and sIL2R-α responses at week two. Additionally the T cell response to ESAT-6/CFP-10 was characterized by a lower frequency of IFN-γ +CD4 + T cells than IFN-γ+ CD8 T cells at baseline, and a decline in the frequency of IFN-γ+ CD8 T cells (P=0.0024) and increased frequency of IFN-γ +CD4 T cells (P=0.0008) at week two. In patients (n=21), followed up till treatment completion, frequency of IFN-γ +CD4 T cells increased steadily till treatment completion, while that of IFN-γ +CD8 T cells declined in response to ESAT-6/CFP-10. However, there were no significant changes in T cell response to Rv1733 although there was a trend of increased frequency of IFN-γ+ CD4 and decline in the frequency of IFN-γ+ CD8 from baseline to week 2. After successful TB treatment, the frequency of ESAT-6/CFP-10 -specific IFN-γ +CD4 T cells were significantly increased in comparison to pretreatment levels (P<0.01) as well as levels in QFT+ (n=19) (P<0.01) and QFT- (n=23) (P<0.001) household contacts.

Conclusion: Anti-TB therapy is associated with increased frequency of IFN-γ+ CD4 and decreased IFN-γ+ CD8 T cell response to ESAT-6/CFP-10 and improved protective cytokine responses which can be exploited in biomarker discovery studies.

Key words:

TB, biomarkers, ESAT--6/CFP-10, DosR, Quantiferon test, immune response, multiplex assay

LIST OF ABBREVIATIONS

HIV/AIDS	Human Immune Virus/Acquired Immune deficiency Syndrome
Mtb	Mycobacterium tuberculosis
TB	Tuberculosis
DOTS	Directly Observed Treatment-Short Course
MDR	Multi-drug resistant
XDR	Extensively/Extremely Drug-Resistant
TDR	Totally Drug Resistant
IFN-γ	Interferon Gamma
IGRA	Interferon Gamma Release Assays
ESAT-6	Early Secreted Antigenic Target 6
CFP-10	Culture Filtrate Protein 10
FDA	Food and Drugs Administration
LITB	Latently Infected TB
BCG	Bacille Calmette Guerin
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
MVA85A	Modified Vaccinia Ankara virus carrying Antigen 85A
TNFα	Tumour Necrosis Factor alpha
sIL2Rα	soluble interleukin 2 R alpha
IL-	Interleukin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8

CRP	C-reactive protein
sICAM1	soluble Intercellular adhesion molecule 1
DosR	Dormancy survival regulon
RPF	Resuscitation promoting factor
RD	Region of difference
NO	Nitric Oxide
CO	Carbon Mono-oxide
MHC	Major Histocompatibility Complex
PBMC	Peripheral blood mononuclear cells
Th-	T Helper cell
FoxP3	Fork head Box P3
AFB	Acid Fast Bacilli
MAF1	*Mycobacterium africanum* 1
MAF2	*Mycobacterium africanum* 2
MTBC	Mycobacterium *tuberculosis* complex
Mtb	*Mycobacterium tuberculosis*
Maf	*Mycobacterium africanum*
rRNA	ribosomal ribonucleic acid
WHO	World Health Organization
ZN	Ziehl -Neelsen
PPD	Purified Protein Derivative
FBS	Foetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
RPMI	Roswell Park Memorial Institute

RT	Room temperature
QFT	Quantiferon Gold In Tube Test
LJ	Lowenstein Jensen
FITC	Fluorescien isothiocynate
PE	Phycoerythyrin
Percp	Peridinin chlorophyll protein complex
APC	Allophycocyanin

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CHAPTER ONE

Introduction

1.1 The epidemiology of Tuberculosis

Despite the availability since the 1980's of an inexpensive, effective, and reasonably well-tolerated therapy, that can cure 90% of cases, tuberculosis (TB) continues to be a major global health problem ranking as the second leading cause of death from an infectious disease worldwide after HIV, the human immunodeficiency virus (WHO, 2012). With an estimated 9 million new cases of active TB and 1.4 million deaths annually; 990,000 among HIV-negative and 430,000 among HIV-positive TB patients, TB is now the leading killer of people living with HIV (WHO, 2012).

The average incidence of TB in African countries is estimated to have more than doubled between 1990 and 2005, from 149 to 343 per 100,000 population (WHO, 2007). Much of this problem is due to the human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) plague as illustrated by the enormous HIV prevalence (22%) among TB patients in the WHO Africa region (Kaufman and Parida, 2008). Thus, in Sub Saharan Africa, where the TB/HIV prevalence is high, TB is the number one killer of HIV-infected individuals with one third of the 640,000 deaths due to TB occurring in HIV co-infected individuals.

When the World Health Organization (WHO) declared TB a global emergency in 1993, efforts to improve TB care and control intensified at national and international levels leading to the adoption and implementation of the DOTS (Directly observed treatment-short course) strategy by all countries a decade later. This culminated in increased case detection (WHO, 2012). however, these efforts have achieved limited success by only slowing the rate of increase but failing to make substantial progress towards the ultimate goal of eliminating TB (Wallis et al., 2009). The spread of HIV/AIDS in TB endemic regions and the global emergence of multi-drug resistant (MDR), extensively drug resistant (XDR) and now totally drug resistant (TDR) TB have largely frustrated these efforts (WHO, 2008).

1.2 Human immune response to tuberculosis infection

The major route of entry of the tubercle bacillus into the body is via the respiratory tract through the inhalation of infectious droplet nuclei. Only small sized droplet nuclei (1 to 2 μm or less) are able to gain entry into the lower respiratory tract while larger ones are excluded by physical barriers of the nasopharynx and upper respiratory tract (Riley et al., 1995). The alveoli is the first point of entry for the bacilli and there, they interact with professional phagocytic cells such as macrophages and dendritic cells through different receptors (Ernst, 2012). Host-pathogen interaction finally results in the initiation of an adaptive immune response and migration of antigen-specific lymphocytes to the lungs to fight the infection.

Earlier publications described four possible outcomes: Complete solution in which the host immune system is able to completely get rid of all invading bacilli such that there is no probability of disease establishment; Containment of the bacilli in granuloma leading to latent TB infection in the host characterised by no symptoms and a positive tuberculin skin test; Primary active infection where the bacilli are able to grow and multiply to cause clinical disease or reactivation where the latent bacilli exit the dormancy mode through resuscitation to establish active infection (Schluger and Rom, 1998). However, recent advances in studying the immune response to TB, suggest a paradigm shift from this old model of well defined outcomes towards a view of the outcome of infection with *M. tuberculosis (Mtb),* as a continuous spectrum generated by a range of lesions providing multiple microenvironments that support bacterial replication, persistence or killing (Barry et al., 2009). This continuous spectrum extends from sterilizing immunity, to subclinical active disease, to fulminate active disease, with conventional designations of latent infection and active disease corresponding to partially overlapping regions of biological heterogeneity (Young et al., 2009).

It is not completely understood what determines which outcome will manifest in any individual exposed to *Mtb* but nutrition, hygiene, sex, age, host genetic factors, infecting strain and more recently HIV infection have all been implicated. What is clear is that most people are resistant to *Mtb* infection as only 5-10% of infected people (if HIV negative) ever develop a primary disease at some point in the lifetime.

1.2.1 Innate immune response to tuberculosis

In mice, the early innate immune response to *Mtb* is characterized by the progressive accumulation of neutrophils, inflammatory monocytes, interstitial macrophages and Dendritic cells (DCs) in the lungs (Ernst, 2012). In humans also, upon entry into the body, the *Mtb* components are recognised by multiple pattern recognition receptors (PRR) of the host including toll like receptors (TLR), specific members of the C-type lectin receptor (CLR) family, including DC-SIGN, dectin 1, the mannose receptor and Mincle-monocyte-inducible C-type lectin (Ernst, 2012). The stimulation of these receptors either individually or collectively induces the expression of pro-inflammatory cytokines, selected chemokines and cell adhesion receptors that contribute to local and systemic immune cell mobilization and activation.

Phagocytic cells engulf the invading microbe in a membrane-bound tight vacuole created when the pseudopods surround the bacterium and fuse distally (Schlesinger, 1996). Engulfment inside the phagosome leads to killing of pathogenic bacteria via several pathways; fusion of the phagosome with the lysosome to form the phagolysosome resulting in release of cytotoxic granules, generation of reactive oxygen intermediaries (ROI's) and reactive nitrogen intermediaries (RNI,s) (Schluger and Rom, 1998).

Mtb however, accomplishes intracellular survival through several evasion strategies including neutralization of the phagosomal pH and interference with autophagy, which serves as a cell autonomous defence mechanism (Gutierrez et al., 2004; Deretic, 2006; Russell, 2007), invasion of the cytosolic compartment (van der Wel et al., 2007) and finally inhibition of apoptosis by production of prostaglandins.

In contrast to other infectious diseases, where the recruitment of phagocytic cells restricts and even eliminates invading pathogens, the recruitment of phagocytes to sites of mycobacterial infection actually benefits the pathogen during the early stages of infection, by providing additional cellular niches for bacterial population expansion (Davis and Ramakrishnan, 2009). The early response to mycobacterial infection leads to the establishment of the early granuloma formed from the progressive accumulation of neutrophils, inflammatory monocytes,

interstitial macrophages and dendritic cells which become infected by the expanding population of mycobacteria in the lung.

1.2.2 Adaptive immune response to tuberculosis

The adaptive response is critical to effective control of tuberculosis infection as its onset typically results in the arrest of the progressive growth of the bacterial population and may result in transient disease symptoms, including fever and an unusual skin rash termed erythema nodosum (Poulson, 1950). Subsequently, most humans become asymptomatic, do not shed bacteria and are considered to have latent TB infection (Ernst, 2012), defined by a detectable memory *Mtb*-specific T cell response signifying the important role of lymphocytes as co-effectors in mycobacterial host defense.

Most of our understanding of the T cells involved in protective anti–TB immunity is based on cause and effect evidence from studies of TB in mice in which the expression of immunity can be measured in terms of the control of infection in major organs in the absence of selected T cell subpopulations (Cooper et al., 1997). On the other hand, our knowledge of the T cells involved in immunity to TB in humans, is based on correlative evidence that comes from experiments designed to identify T cells that respond to appropriately presented *Mtb* antigens in vitro (Mogues et al., 2001). Many studies are in agreement that many types of T-lymphocytes (including α/β CD4+ and CD8+ cells, cytotoxic T-lymphocytes, and γ/δ T-lymphocytes) play a role in host defense against *Mtb*, in both humans and mice (Boom et al., 1996; Murray et al., 1999; Stenger and Modlin 1999). However, although undoubtedly the major effector cell in cell-mediated immunity in TB is the CD4+ T-lymphocyte with CD8+ T cells thought to play a supporting role (Boom, 1996), others believe that CD8+ T cells are more important (Orme and Collins. 1984), equally important (Caruso et al., 1999; Flynn et al., 1992) or plays no protective role at all (Leverton et al., 1989). Other murine studies have suggested (D'Souza et al., 1997) that T cells, like B cells (Johnson et al., 1997), contribute little to protective immunity in mice in spite of numerous publications showing that *Mtb*-specific T cells are generated in response to *Mtb* infections (Boom, 1999), and one publication showing that these T cells contribute significantly to protective immunity in mice (Ladel et al., 1995). A classic single study that

compared CD4+, CD8+ and other T cells using mice of a single strain infected via the natural route with small numbers of a given virulent strain of *Mtb* proved that CD8 in contrast to CD4 T cells are not essential for control of infection in mice (Mogues et al., 2001). Undoubtedly mouse studies have contributed immensely to our current understanding of human TB infection, however the role of CD8+ T cells in human TB infection is still unravelling.

1.2.3 Role of CD4+ and CD8+ T cells in adaptive immune response

Whilst inside the phagosome, *Mtb* secretes proteins which after appropriate degradation are presented as small peptide fragments in the context of major histocompatibility complex (MHC) class II molecules. CD4+ T cells expressing α/β T-cell receptor recognizes these MHC II complexed peptides on the surface of antigen presenting cells such as monocytes, macrophages and dendritic cells resulting in CD4+ T-cell activation.

Stimulation of CD8+ T cells, which requires peptide presentation by MHC I products, generally takes place in the cytosol and as *Mtb* does not readily access this environment, two possible pathways have been reported for this mechanism; direct loading and cross priming. In the former, *Mtb* can enter the cytosol of infected dendritic cells leading to direct loading of MHC 1 molecules (van der Wel et al., 2007) and in the latter, infected macrophages undergo apoptosis and resulting vesicles carrying mycobacterial antigens are taken up by local DCs, which can present antigenic peptides with high efficacy both in the context of MHC II and MHC I to CD4+ and CD8+ T cells, respectively (Winau et al., 2005). The CD8+ T cell response to *Mtb* has normally been of a lower magnitude than the CD4+ T cell response; however, CD8+ T cells may modulate phagocyte activity or produce molecules such as granulysin that may be directly cytotoxic to the mycobacteria (Bruns et al., 2009; Stenger et al., 1998). *Mtb* has evolved mechanisms to subvert the antigen presentation process by inhibiting MHC class II processing and thus impairing CD4+ T cell stimulation (Harding and Boom, 2010) or blocking cross priming of CD8+ T cells through modulating the lipoxygenase pathway (Divangahi et al., 2010). Under the influence of specific T lymphocytes, the loose aggregates of mononuclear phagocytes and polymorphonuclear granulocytes transform into solid granulomas composed of macrophages of

different activation and maturation stages and different T cell populations in a structured arrangement (Ulrichs and Kaufmann, 2006).

The classic experiment by Engen et al., 2008 using *intravital imaging*, has gifted us with live images of tuberculous granulomas of the mouse (first live images of mycobacterial infection in a mammalian host), demonstrating the influx and incessant wandering of T lymphocytes. These surveys reveal that, relative to their potential, effector T cells migrating within mycobacterial granulomas produce an extremely muted response as a consequence of the limited local antigen presentation and/or identification. *Mtb* is contained within these solid granuloma, but not eradicated thus when the immune response fails, necrotic areas develop and may become caseous later. Finally Mtb grows and thrives to cause lung damage and spreads to other organs climaxing in TB disease.

To deal effectively with TB infection, there is a need to consider a double pronged approach as only up to 10% of exposed individuals actually develop active disease upon exposure to the pathogen and can be completely cured by the existing drug regimen if infected with a drug sensitive strain. The vast majority of exposed individuals (90%) develop only a latent TB infection (LTBI) with about 5-10% of this latently infected population developing disease sometime during their lifetime as a result of bacteria reactivation (Israel et al., 1941). The major stumbling block to eradicating TB remains this huge population of latently infected individuals who will need therapy and or vaccination that can kill dormant bacteria or prevent reactivation respectively.

It has become apparent that strategies aimed solely at expanding the pool of antigen-specific effector T cells in individuals infected with some persistent pathogens, such as Mtb, may meet with limited success, because there may be insufficient antigen present at sites of infection to support additional effector responses (Engen et al., 2008). The best chance at successfully reversing the course of the disease could lie with utilizing immunotherapeutic approaches designed to both increase levels of local antigen presentation and maintain a high frequency of effector T cells within infected tissues (Engen et al., 2008).

1.3 New tools for an ancient disease

In 2002, WHO projected that by 2020, 1 billion people will be newly infected with TB, 200 million people will become sick with 35 million deaths, recommending that in order to avert this situation there was the need for novel diagnostics, new drugs, new vaccines as well as diagnostic/detection and treatment biomarkers (WHO, 2002). There was a general acknowledgement within the TB community that indeed these new approaches would be required to improve diagnosis, shorten treatment, improve outcomes, especially in MDR and XDR TB and enhance protection offered by vaccination if the goal of tuberculosis elimination was to be realized (Wallis et al., 2009).

A decade after these projections were made, increased donor, governmental and corporate investment for the diagnosis, treatment, prevention and control of TB have led to a substantial advancement in the development of novel diagnostics, new drugs and vaccines (WHO, 1994; WHO, 2012).

1.3.1 Novel diagnostics

The Interferon-gamma (IFN-γ) release assays (IGRAs) for detecting infection with Mtb, are recent additions to TB diagnostics based on the ability of the *Mtb* antigens; Early Secretory Antigen Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) to stimulate host production of IFN-γ. These assays commercialized as QuantiFERON-TB Gold In-Tube (FDA approved since 2007) and T-SPOT. TB (UK-based) quantifies the total amount of IFN-γ when whole blood is exposed to the antigens ESAT-6, CFP-10 and TB 7.7 and counts the number of activated T lymphocytes that secrete IFN-γ respectively (Ferrara et al., 2006). These antigens are not present in non-tuberculous mycobacteria (NTM) or in any Bacille Calmette Guerin (BCG) vaccine variant hence these tests can distinguish between actual TB infection and NTM or vaccine induced responses. Systemic reviews of IGRAs have concluded the tests have excellent specificity to distinguish latent TB from prior vaccination (Dinnes et al., 2007; Menzies et al., 2007). In a recently published meta analysis, with data from both developed and developing countries, QuantiFERON-TB Gold In Tube had a pooled sensitivity for TB infection (active or latent) of 81% and specificity of 99.2%, whereas T-SPOT. TB had a pooled sensitivity of 87.5%

and specificity of 86.3%. Although both IGRA'S and TST cannot distinguish between active and latent disease, nor past or previous exposure, in head-to-head comparisons, the sensitivity of IGRAs surpassed TST confirming that IGRAs are "superior" to the TST for detecting TB infection (Diel et al., 2010).

The Xpert MTB/RIF is a polymerase chain reaction-based diagnostic test for TB, which detects DNA sequences specific for *Mtb* and rifampicin resistance in about 100 minutes within the paltform of a cartridge-based, automated system. (Van Rie et al., 2010; Helb et al., 2010). The Xpert® MTB/RIF purifies and concentrates *Mtb* bacilli from sputum samples, isolates the genomic material from the captured bacteria by sonication and subsequently amplifies the genomic DNA by PCR and identifies all the clinically relevant Rifampicin resistance inducing mutations in the RNA polymerase beta (rpoB) gene in the *Mtb* genome in a real time format using fluorescent probes called molecular beacons. Results are obtained from unprocessed sputum samples in 90 minutes, with minimal Biohazard and very short technical training needed to operate (Boehme et al., 2010). In 2010, WHO endorsed the Xpert MTB/RIF for use in TB endemic countries after 18 months of rigorous assessment of its field effectiveness in the diagnosis of TB, MDR-TB and TB with HIV co-infection (Small et al., 2010). Since the rollout of Xpert MTB/RIF 1.1 million tests had been purchased by 67 low-and middle-income nations.

1.3.2 New anti-TB drugs

The development of new drugs and new vaccines is also progressing with new or re-purposed TB and novel TB regimens to treat drug sensitive or drug resistant TB advancing in clinical and regulatory review. Bedaquiline, a diarylquinoline anti-tuberculosis drug became the first new medicine to fight TB in more than forty years after rifampicin, after being certified by the WHO for treatment of MDR (WHO, 2013). Bedaquiline had only been through two Phase IIb trials for safety and efficacy (Singh et al., 2013) but there is a huge interest in its potential to treat MDR hence was granted accelerated approval by the United States FDA in 2012.

1.3.3 New Vaccines

Currently there are 12 candidate vaccines being tested in clinical trials (Brennan and Thole, 2012) and for the first time since BCG was last assessed in infants as part of the Chingleput-Madras trial in 1968 (Baily, 1980), a TB vaccine MVA85A which is a recombinant strain of modified Vaccinia Ankara virus expressing the immunodominant *Mtb* protein, antigen 85A went into phase 2b trials. Developed as a heterologous boost for BCG (McShane et al., 2010) it failed to confer significant protection against tuberculosis or *Mtb* however immense lessons can be learned from the data to aid in the design of the next vaccine (MacShane et al., 2013).

While these successes are commendable the discovery of TB biomarkers has lagged behind.

1.3.3 Biomarkers for TB

Biomarkers are objective characteristic that indicates a normal or pathogenic biological process or a pharmacological response to a therapeutic intervention or vaccination (Biomarkers working group, 2001). Thus, they can provide information about disease status, risk of progression, likelihood of response to treatment or of drug toxicity and protective immunity after vaccination. In clinical trials they are especially useful as surrogate endpoints, replacing typical clinical endpoints that describe how a patient feels, functions or survives (Wallis et al., 2013). The requirement for biomarkers in TB stems from two critical features of human *Mtb* infection: its long and varied natural history, and the essential role played by minority bacillary sub-populations. Non-replicating persisters are thought to be the main impediment to shortening therapy, because they are relatively unaffected by most TB drugs (Rao et al., 2008).

Over the past decade, both human and *Mtb* biomarker studies have focused on three specific areas of research: biomarkers predicting treatment efficacy and cure of active TB, the reactivation of latent tuberculosis infection and the induction of protective immune responses by vaccination (Wallis et al., 2013). This study was set up to provide new information that will be useful for studies aimed at looking for immunological biomarkers predicting early treatment response and cure.

1.4 Biomarkers predicting treatment response and cure

Biomarkers for TB treatment response and cure are urgently required for proper management of the patient as well as for clinical trials for novel drugs and vaccines. For the TB patient, 6 months of anti-TB therapy makes adherence very difficult and this long duration also puts pressure on health care systems in developing countries (Walzl et al., 2008). It has been shown (Balasubramanian et al., 1990; Hong Kong Chest Service/British Medical Research Council, 1991 that it will be possible to reduce the duration of therapy for patients who show signs of early response to treatment. Tools such as surrogate biomarkers that provide an indication of treatment efficacy early on during chemotherapy or markers that stratify patients into risk groups requiring different durations of treatment even prior to the start of therapy would improve therapeutic strategies and possibly reduce drug resistance due to non-adherence. It will also make it easier to focus more attention on patients who have a high risk of poor treatment outcomes and ease pressure on healthcare systems, especially in developing countries (Walzl et al., 2008). Such tools would also be crucial in validation of novel anti-TB drug candidates, thereby accelerating new drug development through shortening of clinical trials.

For many years the ultimate success of chemotherapy has been assessed by the rate of relapse within the first two years after completion of treatment. The long duration of clinical trials that rely on this outcome renders clinical TB, research, despite its importance, unattractive to the pharmaceutical industry and biomarkers offer the possibility of a surrogate endpoint that can substitute for clinical endpoints (WHO/TDR, 2006). Early evaluation of the response to anti-tuberculosis (TB) treatment may improve routine clinical management and assessment of novel anti-TB drug candidates during clinical trials (Gandhi et al., 2006)

1.5 Problem statement

Currently there are no biomarkers for TB treatment efficacy and cure; response to TB treatment has traditionally been based on the decrease of acid fast bacilli in sputum during the course of anti-TB chemotherapy. TB patients on treatment are thus required by health facilities under the

DOTS strategy to produce sputum for AFB examination at month 2, month 5 and month 6 of therapy. Microbiological indicators such as "sputum smear positivity", "early bactericidal activity" (EBA) and "time to detection" (TTD) of mycobacteria in sputum culture have all not been sufficiently validated as biomarkers. Month 2 sputum culture status, however, is used as a surrogate marker of treatment response and cure (Mitchison, 1993) and is the only marker that has been accepted by the International union against tuberculosis and lung disease (IUATLD) for sterilizing activity. Month two sputum culture conversion has also recently been reported as candidate markers for TB relapse (Wallis et al., 2010) as well as a possible biomarker predictor of the required duration of treatment based on modelling studies (Wallis et al., unpublished). The increasing reliance on this biomarker is at variance with the numerous limitations to its applicability such as; the sample involved (Sputum), the technique (Culture) and the time frame (month two).

Most TB patients cannot produce sputum after two months on anti-TB therapy primarily because coughing ceases or reduces dramatically making expectorating difficult. Children as well as patients co-infected with HIV also have the same difficulty albeit for different reasons. In TB/HIV co-infected individuals the immunosuppression leads to disseminated disease as granuloma formation is impaired. Hence TB manifests mainly as extra pulmonary TB characterised by paucibacillary sputum (Sharma and Mohan, 2006). Therefore, the sputum culture status will be more difficult to monitor in patients with HIV co-infection (Sharma et al, 2005) and is not applicable in the context of extra-pulmonary disease (Sharma and Mohan, 2006) as obtaining sputum samples from these categories of people for biomarker analysis (month 2 sputum culture status) is usually not possible.

Sputum culture is the Gold standard for TB diagnosis, however, its sensitivity is limited as it requires about 100 bacilli per ml of sputum to yield a positive culture (van deun, 2004). A negative result can be obtained merely as a result of low bacterial numbers resulting from paucibacillary sputum or decrease in bacilli load resulting from the decontamination process. Culture is also very expensive and thus is not easily available in most developing countries where the burden of the disease is and where these biomarkers are most needed. Lastly,

sputum culture takes between 3 to 6 weeks to yield positive results and this delay makes it unattractive as a biomarker.

''Month 2 sputum culture status'' can only be determined after the patient has been on treatment for 2 months. Waiting for 2 months to make a decision on the treatment outcome could be detrimental to a patient as it unduly delays the need for drug sensitivity testing or changes in treatment regimens. Additionally, during this 2-month period, primary multi-drug resistant organisms will remain untreated and drug-resistant mycobacteria may have time to develop resistance to additional drugs (Sharma and Mohan, 2006). Most importantly "month 2 sputum culture status'' has not been validated to predict treatment duration, a very important factor to consider in validating a marker for treatment outcome and cure.

1.6 Immunological profiling of individuals infected by *Mycobacterium tuberculosis* complex

Given the shortcomings associated with the use of classical microbiological methods such as "sputum culture status'' as a biomarker for TB treatment response and cure, blood has been proposed as a better sample for identification of biomarkers. A blood-based biomarker would be ideal as blood is easier to obtain and assessment of immunological parameters can be done within days and could be easily adapted for field use. Also, if validated as a surrogate marker, it will be useful in clinical trials. Identification of immunological parameters in blood that correlates with culture sterilization may also provide important information about host factors most relevant to anti-TB therapy.

Understanding the interplay between the host immune system and *Mtb* may provide a platform for the identification of suitable biomarkers, through both unbiased and targeted hypotheses-driven approaches (Walzl et al., 2011). In this study both approaches were used to generate immunological profiles of TB patients during the early stages of treatment as well as over the entire course of treatment. Such information can be exploited in studies aimed at

defining surrogate biomarkers for TB treatment outcome and cure respectively. The immunological profiles were generated by;

(a) Measuring in peripheral blood mononuclear cells (PBMC) culture supernatant TNF-α, IL-10, IL-17, sIL-2Rα and Granzyme B in addition to IFN-γ, (6-plex assay) secretion after 6 days of stimulation with *Mtb*-specific antigens (ESAT-6/CFP-10, Rv1733, Rv2029, Rv2628) before and at two weeks of treatment (when most of the actively replicating bacteria are thought to be eliminated rendering TB patients no longer infectious) to determine their utility as predictive markers of early treatment response.

(b) Determining by intracellular cytokine staining, frequency of IFN-γ+ CD4 and CD8 T cells in PBMC of TB patients stimulated with *Mtb*-specific antigens (ESAT-6/CFP-10, Rv1733,) at four time points (before treatment /baseline, 2 weeks on treatment, 2 months on treatment and 6 months/treatment completion).

(c) Comparing the immune profile of TB patients after treatment with their latently infected TB contacts and non-infected controls (baseline measurement).

1.7 Rationale/Study justification

1.7.1 Measurement of antigen induced responses

Several studies (reviewed in Walzl et al., 2008) that have investigated the role of immune products that can be measured directly in blood or serum as biomarkers for TB treatment response without further in vitro re-stimulation with antigen have been unsuccessful. This is because many of these products are non-specific markers of immune activation and can be detected in other infections. However, a few promising ones have been found to be associated with the extent of disease or treatment response based on increased levels in blood of active TB patients that declines with therapy as well as a correlation between high baseline levels and negative treatment outcomes. Additionally, persistently high levels even after therapy has been associated with risk of reactivation or relapse. Such promising markers

include Neopterin - a nonspecific marker of macrophage activation (Immanuel et al., 2001; Djoba Siawaya et al., 2008), soluble intercellular adhesion molecule type 1 (sICAM-1) expressed by endothelial cells (Demir et al., 2002; Mukae et al., 2003) and C-reactive protein (CRP) - an acute phase protein produced by the liver. These studies have been inconclusive and it has been suggested that multivariate analyses may be helpful in future studies of these markers to determine the extent to which they are associated with other recognized baseline predictors of relapse, such as the bacterial burden and the presence of cavitary disease (Wallis et al., 2009). Antigen induced responses on the other hand, offers the opportunity to measure recall responses that are specific to TB infection, thus obviating the need for further complicated analysis and interpretation.

Most treatment response biomarker discovery studies based on the T cell response to Mtb antigens have focused on the dynamics of the effector T cell (Tem) response hence have been limited to short term cultures. There is a paucity of information on the kinetics of the central memory response to *Mtb* antigens during anti TB therapy. This study employed long term stimulation (6 days) to determine the effector response of central memory T cells (Tcm) to antigenic stimuli and its utility as a platform for TB treatment response biomarker.

1.7.2 Selection of Mtb-specific antigens

It is now known that the *Mtb* genome (4.42Mb) contains over 4000 protein-encoding genes of which 52% can be assigned a function and only 376 putative proteins are considered unique to *Mtb* because they share no homology with known proteins (Casmus et al., 2002). With this wide array of antigens to choose from, we based our selection on antigens that are thought to be secreted by *Mtb* during certain stages of infection. Some of these promising *Mtb*-specific antigens have been identified as immune-dominant and are currently being tested as potential TB vaccine candidates (Sander and McShane, 2007). These so called "stage specific'' antigens are secreted during latency, reactivation and active disease and are known respectively as Dormancy survival regulon (DosR), resuscitation promoting factors (RPF) and region of difference 1 (RD1) proteins. DosR is a set of about 48 co-regulated genes induced by conditions that inhibit respiration (hypoxia, NO and CO) and are thought to be unregulated by

mycobacteria (Voskuil et al., 2009) to cope with these conditions in the granuloma. So far, only one study has examined the immune response of latently infected African populations to the entire set of antigens of the dormancy survival regulon (Black et al., 2009). We selected ESAT-6/ CFP-10 fusion protein as well as 4 of the DosR antigens for our panel based on their known immunogenecity in African populations. The RD1 proteins are the most studied of all *Mtb* proteins, hence it is well known that of the 9 proteins, ESAT-6 and CFP-10 are the most immunogenic and immunodominant.

1.7.3 Measurement of multiple cytokines

In search of biomarkers for TB treatment outcome and cure, many cell-mediated immune response analytes of the host have been studied and CD4 and CD8 T lymphocytes, which secrete IFN-γ, have been shown to be important for protection of mice and humans. They don't only secrete cytokines such as IFN-γ that regulate immune responses to mycobacteria, but they also serve as cytotoxic effectors in an antigen-specific major histocompatibility complex (MHC) restricted manner (Boom and Wallis, 1991).

A variety of studies have attempted to characterize the T-lymphocyte responses associated with TB infection; Surcel et al. (1994) studied proliferative responses and cytokine production in PBMCs and found that patients with active TB had an increased proliferation of cells secreting IL-4 but not IFN-*γ* in response to stimulation with mycobacterial antigens *in vitro*. Sanchez et al. (1994) reported similar results in patients with pulmonary TB and tuberculin skin-test-positive controls and concluded that patients with active TB had a Th2-type response in their peripheral blood, whereas tuberculin positive patients had a Th1-type response. IL-12 production has also been suggested as an important regulator of T-cell phenotypes in TB (Zhang et al., 1994). Further elucidation of the role of IL-12 as a regulator of the T-cell phenotype response has been found in other studies (McDyer et al., 1997; Taha et al., 1997). The trigger for IL-12 release appears to be phagocytosis of *Mtb* by macrophages, as has been shown by several investigators, with the release of IL-12 appearing to be an early and perhaps somewhat nonspecific response to phagocytosis (Fulton et al., 1996). Ladel et al. (1997), showed that IL-12 was released by macrophages *in vitro* after infection with MTB or

phagocytosis of latex beads, but TNF-α and IL-12 were released together only after infection with the mycobacteria.

Undoubtedly, some studies utilizing single parameters to distinguish between active and latent TB infection have produced encouraging results. The diagnostic potential of T-cell response quality was established when utilizing the expression of CD27 on peripheral blood tuberculin specific CD4+ T-cells, Streitz et al, 2007, proved that a single parameter could be used to diagnose active TB infection even in a BCG vaccinated population. In a related study, Schuetz et al., 2011 showed in subjects from a *Mtb* and HIV endemic region; that down-regulation of CD27 on *Mtb*-specific CD4 T cell could be used as a biomarker of active TB, potentially preceding clinical TB disease. The recent report of single-positive TNF-α *Mtb*-specific CD4 T cells in subjects with active disease being the strongest predictor of diagnosis of active disease versus latent infection (Harari et al., 2011) is welcome as it is one of only a few such studies using a single parameter that has been validated in a cohort study with a sensitivity and specificity of 67% and 92% respectively.

Interesting as some of these results have been, no single parameter has been able to predict early treatment response leading to the view that in this respect, multiple cytokines may hold the key. In the search for biomarkers, it has been suggested that multiple cytokines would increase the predictive value (Walzl et al., 2008, Mustapha, 2002; Bertholet et al., 2008). Of the multiple cytokines involved in the pathogenesis of TB, IFN-γ, TNF-α, IL-10, sIL-2R-α, Granzyme B and IL-17 were targeted in this study not only because they are involved in the control of TB infection but also because of their critical role during the early stages of infection.

1.3.7.1 Interferon gamma (IFN-γ)

Interferon gamma (IFN-γ) is the first identified human immunologic factor essential for resistance against mycobacterial infection (Ottenhoff et al., 1988) due to its critical role of inducing macrophage synthesis of the enzyme inducible nitric oxide synthase (NOS2). Upon secretion by activated CD4 T cells, IFN-γ activates macrophages to generate nitric oxide and

other reactive nitrogen intermediates (RNIs), the best characterized anti-tuberculous effector molecules in mice (Chan et al., 1999) and humans (Nicholson et al., 1996).

Since the strength of the host immune response against *Mtb* infection is directly proportional to the level of cellular (CD4+) production of IFN-γ, (Feng et al., 1999), IFN-γ level has been widely used for diagnosis of TB infection following stimulation with *Mtb* specific antigens (Goldsack and Kirman., 2007; Flynn et al., 1993; Newport et al., 1996).

Due to its pivotal role in TB pathology, several studies have looked into the role of IFN-γ levels to monitor reaction to anti- TB therapy. These studies have, however, reported varied results, mainly because although it is known that IFN-γ plays an important role against *Mtb* infection, a complex network of other cytokines are involved (Lalvani and Millington, 2008) and studies involving multiple cytokines are needed.

1.7.3.2 Tumour necrosis factor alpha (TNF-α)

Tumor necrosis factor alpha (TNF-α) α is one of the most important pro-inflammatory cytokines and critical to the control of tuberculosis infection prior to initiation of the adaptive immune response. It is produced mainly by macrophages in response to stimuli activating toll-like receptors, but can as well be expressed by activating T cells, B cells, and NK cells (Old, 1988). In concert with IFN-γ, it increases the phagocytic ability of macrophages and enhances the killing of mycobacteria and may also induce apoptosis of permissive macrophages (Bekker et al, 2001). Baseline levels of TNF-α are thus thought to be low in peripheral blood and high at the sites of infection during the early phase of active tuberculosis infection. The importance of TNF-α especially in the early levels of TB infection had long been shown in mouse experiments proving that they play a vital part in the establishment of the early granuloma (Ehlers et al., 1999; Bean et al., 1999; Benini et al., 1999) however, the observation that there was an increased incidence of TB in persons given anti-TNF-α treatment for autoimmune diseases (Stenger, 2005) reinforced the protective function of TNF-α in TB in humans as easily. For example, progression from LTBI to active disease can occur following TNF- α blocking treatments for chronic inflammatory diseases (Keane et al., 2001).

TNF-α has been investigated in many studies exploring the immune response during TB infection and it has been found that during the early stages of the disease serum TNF-α levels are high and decrease as treatment progresses. However, in severe TB, TNF-α levels have been found to increase transiently, due primarily to the fact that the initiation of therapy in individuals with severe TB often begins with clinical deterioration (even death) before improvement occurs (Bekker et al., 1998).

1.7.3.3 Interleukin 10 (IL-10)

Interleukin-10 is a potent immunomodulatory cytokine that has been shown in vitro to directly or indirectly affect multiple cell types, including macrophages, monocytes, dendritic cells, CD4 T cells, and CD8 T cells (Moore et al., 2001). Its main biological function seems to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells, such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes (Asadullah et al., 2003). Produced by macrophages and T lymphocytes during infection with *Mtb,* IL-10 reduces the secretion of interferon-gamma (IFN-γ) by T-cells through the negative regulation of IL-12 production and co-stimulatory molecule expression (Lago et al., 2012). It also has a TNF-*α* opposite effect protecting against tissue damage by regulating inflammation and apoptosis (Rojas et al., 1999). Aside IFN-γ and TNF-α, IL-10 down regulates the production of other protective cytokines such as IL-1, and IL-12 and it has been demonstrated that it promotes mycobacterial persistence by acting on macrophages (Murray et al., 1997). On the other hand, the absence of IL-10 accelerates mycobacterial clearance (Van creval et al., 2002). Among the Th1 and Th2 cytokines, IFN-γ and IL-10 are considered the main cytokines responsible for protection against and pathogenesis of TB, respectively. IL-10 has multiple effects that interfere with the functions of protective cells and cytokines (Van creval et al, 2002), thereby helping mycobacteria to survive intracellular, despite the abundant production of IFN-γ (Murray et al., 1997). The interplay between IFN-γ and IL-10 is so critical that the IFNγ/IL10 ratio provides a useful objective marker of disease activity in TB and can be important in disease management (Jamil et al., 2007; Salina and Morozova, 2004). High IFN-γ/IL-10 ratios strongly correlate with

protection and TB cure, whereas low ratios correlate with disease severity. Although IL-10 is reported to be present in advanced TB, different responses of IL-10 according to infection status have been observed (Kim et al., 2012). Lower IL-10 levels have been reported in patients with TB (Frahm et al., 2011) in agreement with our study while others have reported that IL-10 is highest in patients with chronic TB (Handzel et al., 2007). IL-10 has been identified as an important clinical biomarker of TB disease progression (Jamil et al., 2007) as high levels at the end of treatment may function as a risk factor for TB recurrence.

1.7.3.4 Interleukin 17 (IL-17)

IL-17 (IL-17A) is produced by a newly described CD4+ Th cell population identified and referred to as Th 17 with signature cytokines including also IL-17F, IL-21 and IL-22. Due to its novelty, there is limited insight into its role in immunoregulation; however, recent data suggests a broader and more complex role for these cells and cytokines in different infections (Khader et al., 2009). During primary TB, (IL-17) is reported to be induced together with IFN-γ and both being potent inflammatory cytokines, are capable of inducing the expression of chemokines that promote cell recruitment and granuloma organization (Torrado and Cooper, 2010). . IL-17 produced by Th17 cells has been reported to be associated with protection against TB (Khader et al., 2007; Scriba et al., 2008) as reduced IL-17 production could limit the recruitment of CD4+ T cells into the lungs (Khader et al., 2007). It has been suggested that excessive production of IL-17 lead to an extensive neutrophil recruitment and tissue damage hence to control bacterial growth and limit immunopathology during the chronic phase of TB, there needs to be a balance between Th1 and Th17 responses. It has also been suggested that with MDR-TB, the severe tissue damage caused by IL-17 producing T cells may be associated with the low effectiveness of the second-line drugs employed in the treatment (Basile et al., 2011).

The role of IL-17 in TB infection and pathogenecity has been the subject of many recent studies on immunoregulation of TB. A recent meta analysis on the subject concluded that IL-17 acts as an effector molecule similar to IFN-γ after BCG vaccination and *Mtb* infection and contributes to protection against TB dependent or independent on IFN-γ, however, it found no evidence of

IL-17 as an inducer of tissue damage (Li et al., 2012). The Effect of TB treatment on IL-17 secretion needs to be investigated.

1.7.3.5 Soluble interleukin 2 receptor alpha (sIL-2Rα)

Interleukin-2 receptor alpha chain is a protein that in humans is encoded by the *IL2RA* gene (Leonard et al., 1985) and together with the interleukin 2 (IL-2) receptor alpha (IL-2Rα) and beta (IL-2Rβ) chains and the common gamma chain (IL2Rγ), constitute the high-affinity IL2 receptor. Interleukin-2 receptor (IL-2R) molecules are expressed on the surface (Cantrell and Smith, 1983) of activated T-lymphocytes upon the interaction of mycobacteria and alveolar macrophages and soluble IL-2R (sIL-2R) molecules are released into the circulation (Rubin et al., 1985). It is possible that sIL-2R could play a regulatory role in the immune response as this soluble receptor retains some of the biological activities of the cell-associated IL-2R molecule, including its capacity to bind IL-2 efficiently (Rubin et al., 1986). The exact immunological role of sIL-2R is not well-established, but it has been suggested that it may serve as a marker of disease activity in patients with systemic lupus erythematosus, rheumatoid arthritis (Semenzato et al., 1988), hematological malignancies (Chilosi et al., 1989) and pulmonary disorders, such as asthma (Lai et al., 1993), lung cancer [(Chan et al., 1993) and TB (Chan et al., 1991).

Serum sIL-2Rα levels are known to be directly proportional to the number of producing cells as well as the number of molecules per cell, making sIL-2Rα blood values an index of the number and the functional state of producing cells, both normal and neoplastic. While sIL-2Rα could just be a byproduct without biological significance, it levels have been reported to correlate with disease progression and/or response to therapy making their measurement a useful index of activity and extent of disease. Active pulmonary TB is associated with markedly elevated sIL- 2R levels (Chan et al., 1991), however, the effect of anti-TB chemotherapy on the cellular immune response is unclear. It has been suggested by some studies that anti-TB drugs may have an immunosuppressive effect (Ruben et al., 1974) which may be reflected in the sIL-2R levels. Other studies have reported elevated levels of sIL-2Rα in active tuberculosis patients, which declined with therapy (Tsao et al, 2002).

1.7.3.6 Granzyme B

Granzyme B is a serine protease that in humans is encoded by the *GZMB* gene (Dahl et al., 1990) and expressed by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells both of which share the remarkable ability to recognize specific infected target cells. They are thought to protect their host by inducing apoptosis of cells that bear on their surface 'nonself' antigens, usually peptides or proteins resulting from infection by intracellular pathogens. Thus the protein encoded by this gene is crucial for the rapid induction of target cell apoptosis by CTL in cell-mediated immune response (Bots and Medema, 2006). Originally thought to induce apoptosis by entry through pores created within the cell membrane by perforin, it has now been established that Granzyme B is rather part of a multimeric complex (Granzyme B, perforin and granulysin). This complex enters the cell through endocytosis using the mannose 6 phosphate receptor and remains arrested in endocytic vesicles until it gains access into the cell when perforin bores holes in the vesicle and allows it to pass through (Buzza and Bird, 2006). Within the cytosol, Granzyme B targets caspase-3 directly or indirectly through the mitochondria, initiating the caspase cascade to DNA fragmentation and apoptosis (Lord et al., 2003). Granzyme B and other molecules involved in lymphocyte cytotoxicity have been implicated in disease pathogenesis. In the case of TB infection, it has been suggested that the apoptotic environment may be deleterious to mycobacteria, however, studies have shown that mice-deficient in perforin or Granzyme B do not exhibit a dramatically increased susceptibility to *Mtb* infection (Lewinsohn et al., 2003).

Granzyme B levels could therefore be a marker of disease activity, therefore would be expected to be high in active TB patients and decline with treatment and could be useful as part of a biomarker panel.

1.7.4 Selection of time points

During anti-TB therapy under the DOTs strategy, sputum of TB patients is assessed again for acid fast bacilli (AFB) at month 2, month 5 and upon completion of anti-TB therapy at month 6.

For this study, follow- up started in week two in order to detect early treatment responses (if any) which could lead to the identification of a biomarker that can be used earlier than month 2 which may also be useful in identifying early responders. It has been established that clinical, bacteriological and radiological improvements in TB patients are achieved early (within 2 months) upon effective chemotherapy with multidrug regimens, however, due to persister phenotypes, it requires 6 months of treatment to achieve complete cure and prevent frequent relapses (Stratton et al., 1986). Earlier studies assessed cellular responses in TB patients only at single time points, e.g. 4 months (Dieli et al., 1999) or 6 months (Garcia et al., 2002), and thus no data are available to show if there are any differences in the cellular responses in the early stages versus the end of anti-TB chemotherapy and most especially during the first two weeks of treatment when most of the actively replicating bacteria are eliminated.

1.7.5 Immune responses in *M. tuberculosis* versus *M. africanum* infected TB patients

Human TB is caused by a group of closely related *Mycobacterium* species known collectively as the *Mycobacterium tuberculosis* complex (MTBC). These species; *M. tuberculosis, M. africanum, M. bovis, M. caprae, M. microti* and *M. pinnepidii* are characterized by a 99.9% similarity at the nucleotide level and a 16S rRNA sequence (Boddinghaus et al., 1990; Sreevatsan et al., 1997). They don't only differ, in their host tropisms but also in their phenotypes and pathogenicity (Brosch et al., 2002). Of the six, *M. tuberculosis* (MTB) and *M. africanum* (MAF) are the most frequent cause of human pulmonary TB, but while the former occurs globally the latter is restricted to the West African region.

First described in 1968 in Dakar, Senegal (Castets et al., 1968), *M. africanum* causes up to 50% of all pulmonary tuberculosis cases in West Africa (de Jong et al, 2010). *M. africanum* strains were previously classified into two major subgroups per geographic origin and biochemical properties: *M. africanum* subtype I (Cluster G) from West Africa, which exhibits *M. bovis*-like properties, and *M. africanum* subtype II from East Africa (Cluster F), which exhibits *M. tuberculosis*- like properties (Källenius et al.,1999; Mostowy et al., 2004; David et al., 1978; Sola

et al., 2003). *M. africanum* is estimated to have lost about 68 kilobases compared with the *M. tuberculosis* genome (Motoswy et al., 2004) and recent studies using regions of difference (RD) to discriminate members of the *M. tuberculosis* complex isolates characterized as *M. africanum* have since refined the old classification. Using molecular techniques *M. africanum* has been divided into two sub-species; *M. africanum* West African 1 (MAF1), common around the Gulf of Guinea, and *M. africanum* West African 2 (MAF2), mainly found in Western West Africa (Brosch et al., 2002; Gagneux et al., 2006). These two subtypes are indistinguishable phenotypically and also share some genetic markers like deletion of region of difference (RD) 9 and presence of RD12 and a specific *gry*B polymorphism (Niemann et al., 1997; de Jong et al., 2010). However, MAF 2 has additional deletions of RD7, RD8 and RD10 and giving the mounting evidence that the strain differences affect the host pathogen interaction (Malik et al., 2005), in studying the immune profiles of TB patients in Ghana, it is incumbent that infecting species are considered.

The geographic restriction of *M. africanum* to human populations in West Africa is not well understood and basic research on these clinically important mycobacteria was neglected until recently; however, an improved understanding of the biology of this mycobacterial lineage will also give clues about gene functions in the closely related *M. tuberculosis* (Gehre et al., 2013).

Measuring levels of changes in multiple cytokines before and after two weeks of chemotherapy might help in the identification of cytokine profiles associated with early treatment response while determining longitudinal changes in T cell subsets frequency during the course of TB treatment may provide an insight into which subset would be more useful in monitoring treatment response.

1.8 Objectives

1.8.1 Main objective

To define **putative** biomarkers of TB treatment response and cure based on the immunological profiles of patients infected by *M. tuberculosis* and *M. africanum* in response to **ESAT-6/CFP-10** fusion protein, **Rv1733, Rv2029, Rv2628** antigens of *Mtb.*

1.8.2 Specific objectives

- Genotype Mycobacteria species isolated from sputum cultures of smear positive TB patients to identify *M. africanum* (*Maf*) and *M. tuberculosis* (*Mtb*) infected patients.
- Determine cytokine/immunological factor (IFN-γ, TNF-α, IL-10, IL-17, sIL-2Rα and Granzyme B) expression profiles in the PBMC culture supernatant of *Mtb* and *Maf*-infected patients before and after 2 weeks of anti-TB therapy in response to *Mtb*-specific antigens (ESAT-6/CFP-10 fusion protein, Rv1733, Rv2029, Rv2628**)** using a Luminex bead assay (6-plex assay).
- Determine T-cell subset (CD4/CD8) specific cytokine expression profile of *Mtb* & *Maf*-infected patients during anti-TB therapy through intracellular cytokine staining for IFN-γ expressing CD4+ and CD8+ T cells from *in vitro* re-stimulation of PBMCs with *Mtb* specific antigens.
- Compare Immune profile of TB patients after treatment with LTBI contacts (Baseline measurement) and non-infected controls.

CHAPTER TWO

Genotyping of Mycobacteria species isolated from sputum samples to identify *M. tuberculosis* and *M. africanum*-infected patients

2.1 Background

Mtb strains with distinct genotypes have been shown to evoke different immunopathological events in mouse models (Dormans et al., 2004) and variable clinical manifestations in human population based studies (Dole et al., 2005). In studies done in the Gambia where there is a reported prevalence of 38% MAF2 and no MAF1, *M. africanum* has been shown to be less virulent and more opportunistic than *M. tuberculosis* thus frequently associated with HIV disease, malnutrition and old age. It has also been reported to be less likely to reactivate in latently infected individuals, in whom ELISPOT responses to a known virulence protein ESAT-6 is thought to be lower compared to latently infected *M. tuberculosis* individuals (de Jong et al., 2006, 2008, 2010).

In a study done in Ghana however, the rate of *M. africanum* infections were similar in HIV-positive and HIV-negative patients and no significant differences were found clinically and radiographically, except that *M. africanum* caused lower-lobe disease less frequently than *M. tuberculosis* (Meyers et al., 2008). In that same study, when MAF 1 and MAF2 were compared, there was no difference in virulence, as assessed by the severity of radiological presentation. In Ghana, 70-80% of tuberculosis infections are caused by *M. tuberculosis* with *M. africanum* accounting for 20-30% (Addo et al., 2006; Addo et al., unpublished). MAF 1 is more common (21%) than MAF 2 (9%).

Given the genotypic difference between the two lineages of *M. africanum*, it is possible that differences also exist in terms of the human immune response to these two lineages which may require different biomarker signatures. The human immune response to *Mtb*-specific antigens have not been investigated in a Ghanaian cohort of TB patients. Ghana offers an ideal setting to study the immunological profiles of individuals infected by *M. africanum* (MAF1 and MAF2)

and *M. tuberculosis* as both strains coexist in the country. Identifying the nature of the immune response to *Mtb* antigens in a cohort of TB patients infected by *M. tuberculosis* or *M. africanum* is important as some of these *Mtb* antigens are also potential vaccine candidates and as such have to be shown to be immunogenic and immunodominant in individuals infected with *M. africanum* as well.

2.2 Setting

Ghana is a West African country with a TB incidence of 79 per 100,000 population and 21.6% of active TB patients are HIV positive (NTP, 2009). Ghana ranks 13th in Africa for the highest estimated number of new TB cases per year (WHO, 2006). The country adopted the DOTS strategy for controlling TB in 1994 and having achieved 100% coverage by 2005, is now implementing WHO's Stop TB Strategy. TB diagnosis is by sputum smear microscopy and patients (smear positive as well as smear negative patients confirmed by chest radiography) are monitored daily during the intensive phase with sputum samples being examined again at month 2, month 5 and month 6 upon completion of treatment.

Accra, the capital city where the study was conducted has a population of 2.4 million and a high BCG vaccination coverage (Ghana DHS, 2008). With 20 government-run health facilities, Accra has the highest concentration of health centers.

2.3 Study design

2.3.1 Study period

The study was a prospective longitudinal study where patients were recruited over a period of one year (June 2011 to June 2012) and were followed up for up to six months until they completed TB treatment (December 2011-December 2012).

2.3.2 Study sites

The study recruited participants from three public health facilities in Accra namely Achimota, Maamobi and University of Ghana Hospitals. Selection of health facilities was based on proximity (not more than 3 hours drive) to the Noguchi Memorial Institute for Medical Research (NMIMR) so that samples could be picked up early enough to allow PBMC separation to be done within 5 hours of blood draw as per study protocol and best practices.

2.3.3 Sample size

Given a 20-30% prevalence of *M. africanum* in Ghana, (de Jong., 2010) it was calculated that for 50 TB cases recruited, about 10-15 could be infected with *M. africanum.* To increase the probability of getting a representative number of *M. africanum* infected patients in comparison with *M. tuberculosis* infected patients, a sample size of 100 participants was targeted for recruitment from which 10-15 *M. africanum* and 20 to 30 *M. tuberculosis* cases could be selected for the immunological profiling experiments using their peripheral blood mononuclear cells (PBMC).

2.3.4 Inclusion/exclusion criteria

TB patients 16 years of age or older and newly diagnosed with sputum smear positive and /or culture positive pulmonary TB, including those co-infected with HIV (results were analyzed separately) were included, whereas patients with extra pulmonary TB were excluded.

2.3.5 Administration of informed consent

Details of the study were discussed with the potential study participants by the nurses stationed at the TB treatment centers (DOTS center) of the study facilities who had been thoroughly briefed about the study. Potential participants were then given the informed consent to read or the contents were translated into their local dialect for them. This consent form was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (Certified protocol No.030/10-11). Those who were satisfied with the explanation of the purpose of the research study, and agreed to enroll in the study were asked

to sign or thumb-print the consent form. Details of sex, age, contact details, HIV status, and previous history of tuberculosis were taken using a structured questionnaire.

2.3.6 Sputum Sample collection

The participants were counseled about sputum production at the DOTS center and given wide mouthed sputum containers to produce sputum for microbiological analysis. Study participants who could not produce sputum on the spot were asked to bring an early morning sample when coming for their medication the following day. For each patient, two sputum specimen was collected; the positive specimen from the laboratory and an on-spot one from the DOTS center.

2.3.7 HIV testing

All study participants were offered voluntary counselling for HIV testing by trained health personnel in accordance with the National TB control program guidelines at the health facilities. Appropriate post- test counselling and referral for further treatment advice was offered to those with positive results. Two rapid HIV diagnostics kits were used; First response anti-HIV1/2 (Premier Medical Corporation, India) and Determine HIV 1/2 (Abbot Diagnostics, USA).

2.3.8 Final study cohort

In all, 104 sputum smear positive TB patients signed on for the study, 55 from Achimota, 30 from Maamobi and 19 from the University of Ghana Hospitals. PBMC from these participants were selected for in-vitro assays in accordance with set objectives.

2.2 Laboratory analysis

2.2.1 Isolation of Mycobacterium species from sputum

Sample Processing: The oxalic acid method was used for decontamination based on its superior performance compared to the Nalc-NaoH method in our laboratory setting. This method, although specially recommended for decontamination of clinical specimens that may be contaminated by *Pseudomonas aeruginosa* e.g., pulmonary specimens from cystic fibrosis patients and urine specimen (Della Latta., 2004). Briefly, each sputum sample was transferred

into a 50 ml sterile centrifuge tube and an equal volume of 5% oxalic acid was added. The mixture was homogenized using a vortex mixer and then allowed to stay at room temperature for 30 minutes. The tubes were then filled with sterile distilled water up to the 50ml mark and centrifuged at 3000g for 30 minutes. The supernatant was poured off and the 0.1µl of resulting sediment was used as inoculum for the culture of Mycobacterium species and also for smear preparation for AFB staining using the Ziehl-Neelsen (ZN) method.

Inoculation and incubation: Four tubes of self-made egg-based media Lowenstein-Jensen (LJ); 2 containing glycerol and 2 with 0.4% Sodium pyruvate (to enhance isolation of *M. africanum*) were used per sample for primary isolation. For cultivation, 0.1 ml of the sediment from each sample was spread on the surface of each tube of media using a sterile Pasteur pipette and incubated at 37°C for 12 weeks, with weekly observation for the appearance of Mycobacterium colonies. Initial identification was based on growth rate, colonial morphology and colonial pigmentation. Positive cultures were sub-cultured onto another set of media (2 slopes of each medium per culture) and incubated for another 3 to 4 weeks for further identification after a Ziehl-Neelsen staining to confirm whether they are acid fast bacilli.

Ziehl-Neelsen staining*:* This is a differential staining procedure used to identify acid- fast bacteria. A tiny bit of the suspected bacterial culture was aseptically transferred onto a drop of distilled water on a slide and emulsified. It was then left to air dry for some time. The smears were then heat fixed on the slides and arranged on a staining rack with enough spaces between them to prevent cross contamination. The slides were then flooded with carbol fuchsin to cover the entire surface of the slides. The underside of the slides was then heated with a flame until steam came out and then they were left for about 5 minutes to cool down. The slides were then washed with a gentle stream of water to remove all excess carbol fuchsin. The slides were then covered with 20% Sulphuric acid for about 5 minutes (decolourisation) and drained. They were then washed with water and counter-stained with 0.3 % Methylene blue for about 1 minute. The slides were then rinsed for a final time and drained. They were then observed under oil immersion for the presence of acid fast bacilli which appear as red under a blue background.

2.2.2 Confirmation of MTBC using Capilia TB-Neo test®

All AFB-positive cultures were further screened with the Capilia TB- Neo test to determine whether or not they belonged to the *Mycobacterium tuberculosis* complex species. The test was based on a slight modification of the protocol developed by TAUNS Laboratories, Inc.` Numazu, Japan (Abe et al., 1999). Briefly, 200 µl of the extraction buffer (using an in-house prepared buffer) were dispensed into 1.5 ml sterile Eppendoff tubes. One loop (0.1µl) of bacteria obtained from mycobacterial colony was suspended in the extraction buffer and mixed by a vortex. The resulting suspension was used as specimen for Capilia TB-Neo Test®. Using a pipette, approximately 80-100 µl of the specimen was dropped in the specimen placing area of the Capilia TB-Neo test® plate. The reading was then made between 15 and 60 minutes. A positive reading is indicated by the presence of a purple- red colour line in the reading areas of both the control band (C) and the test band (T). Likewise, a negative result is indicated by the presence of the purple-red colour line at only the control band (C) and not the test band (T).

2.2.3 Hain Genotyping to differentiate between *Mtb* and *Maf*

The Genotype MTBC® kit (Hain Life Sciences, Germany) is based on the DNA STRIP technology and permits amongst other things on the basis of gyrase B gene polymorphisms the genetic differentiation of the species/strains belonging to the *Mycobacterium tuberculosis* complex. The whole procedure is divided into three steps: DNA extraction from cultured material (culture plates/liquid medium); a multiplex amplification with biotinylated primers, and a reverse hybridization. The hybridization includes the following steps: chemical denaturation of the amplicons to the membrane-bound probes, stringent washing, the addition of a streptavidine/alkaline phosphatase (AP) conjugate and an AP mediated staining reaction. A template ensures an easy and fast interpretation of the banding pattern obtained.

***DNA extraction*:** For isolation of DNA, 2 loops of bacteria from 4 week-old subcultures on LJ media was suspended in distilled water (1ml) and heated at 90^{o}C for one hour using a heating block. This suspension was cooled and stored at -20 until ready to be used as DNA in the amplification assay. For use in a reaction the suspension was thawed, given a quick spin and the supernatant containing mycobacterium DNA was harvested into a separated vial.

***Multiplex amplification*:** The GenoType MTBC assay was performed as recommended by the manufacturer. Briefly, for an amplification, 35 μl of a primer nucleotide mixture (provided with the kit), 5 μl of amplification buffer containing 1.5 mM $MgCl_2$ and 1 U of Platinum Taq polymerase (Invitrogen, USA) (sold separately), and 5 μl of DNA in a final volume of 50ul were used. The amplification mix (45 μl) was prepared in a DNA-free hood. The DNA sample was added in a separate hood.

Per tube mix:

35 μl PNM-provided

5 μl Polymerase incubation buffer (10x)

2 μl MgCl2 solution (25mM)

0.2 μl HotStarTaq (1U)

3 μl Water (molecular biology grade) to obtain a volume of 45 μl

5 μl DNA solution (20-100ng DNA) leading to a final volume of 50 μl

A master mix was prepared containing all reagents except the DNA solution and mixed well. An aliquot of 45 μl was then put in each of the prepared PCR tubes. As a negative control one of the tubes contained water instead of DNA. In a separate room, 5 μl of each DNA was added per tube and then the tubes were placed in a thermocycler for amplification.

***Cycling conditions*:** The amplification protocol consisted of a cycle of 15 seconds of denaturation at 95°C, followed by 10 cycles comprising 30 seconds at 95°C and 120 seconds at 58°C, an additional 20 cycles comprising 25 seconds at 95°C, 40 seconds at 53°C, and 40 seconds at 70°C, and a final extension at 70°C for 480 seconds.

Amplification Profile: **GenoType MTBC**		
15 min	95°C	1 Cycle
30 sec	95°C	10 Cycles
2 min	58°C	
25 sec	95°C	20 Cycles
40 sec	53°C	
40sec	70°C	
8 min	70°C	1 Cycle

***Hybridization*:** The shaking water bath/ TwinCubator was pre-warmed to 45^0C, (the maximum tolerated deviation from the target temperature is +/-10^0C) while the solutions HYB and STR were pre-warmed to 37-45^0C before use to dissolve all precipitates. All remaining reagents were warmed to room temperature except the CON-C and SUB-C. Using a 15ml falcon tube, the conjugate concentrates (CON-C, orange) and substrate concentrate (SUB-C, yellow) were mixed at a ratio of 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. These were mixed well and brought to room temperature. For each strip, 10ul concentrate was added to 1ml of the respective buffer. CON-C was diluted before each use, while diluted SUB-C (stable for 4 weeks if stored at room temperature and protected from light) was prepared for multiple usage.

The denaturation solution (20 μl) was dispensed in a corner of each of the wells used. To this was added, 20 μl of amplified sample. Using a pipette the mixture was mixed well and incubated at room temperature for 5 minutes. While incubating, the strips were taken out of the tube using tweezers and marked with a pencil underneath the coloured line (gloves were always worn when handling the strips). To each well, 1ml of pre-warmed hybridization buffer (HYB, green) was carefully added and the tray was shaken until the solution had a homogenous colour. A strip was then placed in each well with the coated side (identified by a coloured line near the lower end facing upward. Tweezers were used to turn over strips which might have turned when immersed in the solution. The tray was then placed in the shaking water bath/ TwinCubator and incubated for 30 minutes at 45^0C.

After 30 minutes the hybridization buffer was completely aspirated by pouring it out into a discard jar and turning the tray upside down and gently striking on absorbent paper. Stringent solution (1ml) was added to each strip and incubated for 15 minutes at 45^0C in the TwinCubator. The Stringent Wash solution was also removed in the same manner after which each strip was washed once with 1ml of Rinse solution for I minute on the TwinCubator at room temperature.

After pouring out the Rinse solution, 1ml of conjugate was added to each strip and incubated for 30 minutes in the TwinCubator. The solution was removed after incubation and each strip was rinsed twice with for 1minute with 1ml of Rinse solution and once with 1ml of distilled water on the TwinCubator. All water was removed after the last wash and then 1ml of diluted substrate was added to each strip and incubated (protected from light without shaking) for 10 minutes. The reaction was stopped by rinsing with distilled water. Using tweezers the strips were removed from the tray and dried between layers of absorbent paper.

The strips were protected from light and pasted on an evaluation sheet provide with the kit in the designated fields by aligning the bands CC (conjugate control) and UC (universal control) with their respective lines on the sheet. The species' was then determined with the help of the interpretation chart and the names of the species identified was entered in the last column.

2.2.4 Spoligotyping

To differentiate between MAF1 and MAF2, all the isolates that were confirmed as *M. africanum* by Hain genotyping were further typed by spoligotyping (Kamerbeek et al., 1997). The method described in Yeboah Manu et al., 2011 was used as follows; the direct repeat region of each genome was amplified using primers DRa (59-CCG AGA GGG GAC GGA AAC-39) and biotinylated Drb (59-GGT TTT GGG TCT GAC GAC-39). The amplified DNA was tested for the presence of specific spacers by hybridization with a set of 43 oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 (the GenBank accession no. for the sequence of *M. tuberculosis* H37Rv is Z48304, and that for *M. bovis* BCG P3 is X57835). Bound fragments were revealed by chemiluminescence after incubation with horseradish peroxidase-

labeled streptavidin (Boehringer Mannheim). In order to prevent cross contamination, PCR amplifications and pre-PCR procedures were conducted in physically separated rooms. Negative water controls were PCR amplified and included on each blot to identify any possible amplicon contamination. In addition, Positive controls (H37Rv and *M. bovis* BCG DNA) was amplified and included on each blot.

2.3 Data analysis

Data was entered into Microsoft® Excel 2007 (Microsoft Corp., USA) and age profile (mean, range), sex ratios and HIV status determined. For comparison of the mean ages between groups, students T tests were used and P values < 0.05 were considered significant. Graphs were generated using the same software.

Spoligotypes were analyzed as character types. The obtained spoligotyping patterns were compared with those available in the international spoligotype database (SpolDB4) (Brudey et al., 2006) containing 35,925 spoligotypes comprising 39,295 isolates from 122 countries.

2.4 Results

2.4.1 Participant characteristics

Study participants comprised 74 males and 30 females giving a male/female ratio of 2.5 to 1. A total of 15 participants tested positive for HIV; 8 males and 7 females, giving a TB/HIV co-infection of 14.42% of the study population (Table 2.1). Of the 15 HIV positive participants, only one tested positive for both HIV1 and 2, all others were infected with HIV-1 only. There was no statistical difference between the number of male and female infected with HIV (P=0.126) in our cohort using Fishers exact test (2 sided), however, there is still a 2 times increased HIV rate in the female TB cases compared to males.

The mean age was 35.13 (SD±12. 83) ranging from 16 to 78 with the majority (85.72%) within the 20 to 50 year group (Fig 2.1). Males (Mean ± SEM =29. 53 ± 2.016) were significantly

younger (P=0. 0045) compared to females (Mean ± SEM =37. 27 ± 1.444) with a 95% CI (-13.03 to -2.449). However, there was no statistical difference (P=0. 297) between the mean age for the HIV-positive (Mean ± SEM =35. 47 ± 1.409) compared to the HIV-negative (Mean ± SEM =31.73 ± 2.381) participants, 95% CI (-3.336 to 10.80). Within the HIV-positive participant group, the mean age of the females (Mean ± SEM =29. 00 ± 2.610) and males-(Mean ± SEM =34.13 ± 3.796) was not statistically different P=0.299, 95% CI (-15.37 to 5.125).

Table 2.1 Sex distribution and HIV prevalence among sputum smear positive study participants

		HIV(-) n(%)	HIV(+) n(%)	Total
Sex				
	Male	66 (89.19)	8 (10.81)	74 (71)
	Female	23 (76.67)	7 (23.33)	30 (29)
Total		89 (85.58)	15 (14.42)	104 (100)

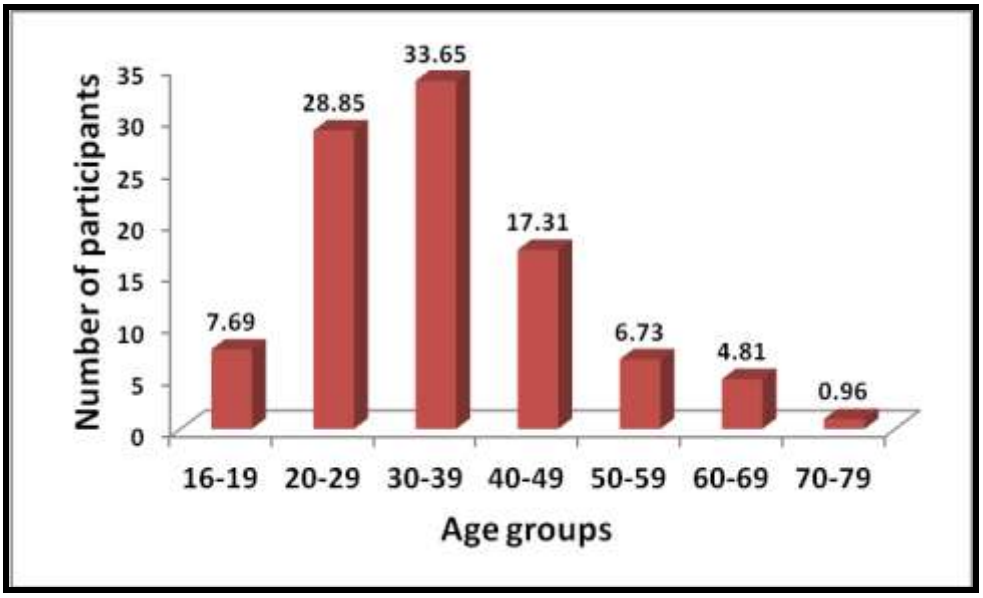

Figure 2.1 Age profile of sputum smear-positive participants

2.4.2 Differentiation of Mycobacterium isolates

Identification of the infecting species followed a stepwise process of sputum culture to spoligotyping (Fig 2.2). Sputum samples could not be obtained from 2 of the participants leaving 102 samples available for culture. After about 12 weeks of incubation, 7.8% (8/102) of the cultures yielded no growth and were discarded together with 8.8%, (9/102) of the samples that got contaminated before isolates could be harvested. For the cultures that yielded growth, the Capilia neo TB test® confirmed 84 out of the 85 as belonging to the *Mycobacterium tuberculosis* complex (Fig 2.3). Subsequently Hain genotyping® identified 74 as *M. tuberculosis* and 10 as *M. africanum* (Fig 2.4) giving an *M. africanum* prevalence of 11.9%. The spoligotype patterns of the africanum species identified 1 as MAF2 and the 9 as MAF1 (Fig 2.5a, b).

The mean age of the *M. africanum* infected patients was 34.1 ±6. 83, range 26-50 and they comprised of 7 males and 3 females. Of the 10, only one tested positive for HIV. None of the *M. africanum* isolates was multi-drug resistant (MDR) Table 2.2.

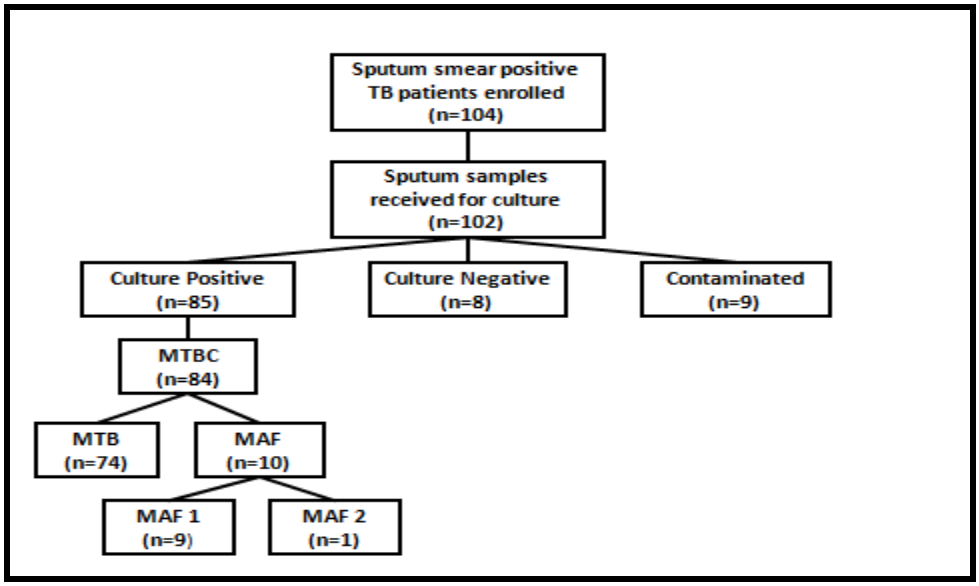

Figure 2.2: Differentiation of Mycobacterium isolates

Figure. 2.3 Capilia Neo tb test: 2 bands indicating a valid positive test for MTBC

Figure. 2.4 Hain GenoType test: sheet showing samples (strips) identified as *M. tuberculosis* and *M. africanum* (MI011)

[a]RD9	RD701	RD720	BSpoligoprofile	Sublineage	[c]Spoldb4
Del[d]	Del	Undel	11111100011101111100001010000111111111101111	West African 1	
Del	Del	Undel	10111110000011111111000001111111111110001111	West African 1	
Del	Del	Undel	11111110000011111111000011111111111110001111	West African 1	331
Del	Del	Undel	11111110000011111110000001011000001100000111	West African 1	
Del	Del	Undel	11100110000011111111111111111111111110001111	West African 1	331
Del	Undel[e]	Del	11111100011111111111000111111111111101111	West African 2	326
Del	Del	Undel	11100000000010011001111111111111111111101111	West African 1	
Del	Del	Undel	10111100000010011111000011111111110100011101	West African 1	319
Del	Del	Undel	10111110000011111111000001111111111110001111	West African 1	
Del	Del	Undel	10111110000011111111000001111111111110001111	West African 1	

[a]RD: Regions of difference
[b]Spoligoprofile: presence of the spacer (1); absence of the spacer (0).

[c]Spoldb4: coded patterns in the international spoligotype database.

[d]Undel: Undeleted, [e]Del: Deleted.

Figure 2.5: Spoligotyping profile for isolates identified through Hain genotyping® as *M. africanum* as defined by RD's

2.5 Discussion

The male/female ratio of 2:1 in this population is consistent with reports stating that the tuberculosis notification in most countries is twice as high in males as in females. Although worldwide the male/female ratio of tuberculosis is 1.9±0.6 (WHO, 2009), there are wide variations between countries. Previously attributed solely to socioeconomic and cultural factors denying women access to health leading to under-reporting of TB in women, biological factors are now being considered as possible factors for this observation (Neyrolles et al., 2009). This is based on the numerous studies linking female sex as a protective factor. Specifically related to TB, there is some evidence that protective Th1 responses associated with IFN-γ production is stronger in females partly because of estrogens (Roberts et al., 2001). More evidence based studies will be needed to fully explain this gender difference.

It is also well known that TB affects the most productive age group, hence the majority of participants in this study being within the 20-50 year group is consistent with the global trend.

The Global TB/HIV prevalence stands at 13% (WHO, 2012) and in Ghana, a nationwide survey to determine the prevalence of HIV in TB patients that concluded in 2008, recorded a prevalence of 14.1% in 503 newly diagnosed TB patients from 66 health facilities across the country (Addo et al, unpublished). That study also recorded an HIV 1 prevalence of 96% and an HIV 1+ 2 prevalence of 4% with no HIV 2 alone being detected in any patient. Those results are consistent with the 14.4% HIV prevalence recorded among this study cohort as well as the preponderance of HIV 1, although recent reports attributed to the National tuberculosis Control program in Ghana indicate that the TB/HIV prevalence has gone up to 21.6 % (NTP, 2012).

The actual prevalence of *M. africanum* in Ghana is difficult to ascertain, as genotyping is not a routine practice, but a recent study put the prevalence at 20% out of 232 samples collected (Yeboah-Manu et al., 2011), making the prevalence of 11.2% recorded in this study on the lower side. This could probably be due to the small sample size in our study or to regional variation as the former study recruited participants from the Central and Western Regions of Ghana whilst our study recruited from Greater-Accra Region only. Regional variation in prevalence of *M. africanum* has been previously reported in Senegal (Diop et al., 1976). The proportion of MAF1/MAF2 (9 out of 10) in this study is comparable to the study in the Central/Western Region of Ghana which reported MAF1 prevalence greater than 80%, however, another study in a different region (Ashanti) of Ghana reported an almost 50-50 proportion of MAF1-MAF2 (Goyal et al., 1999) emphasizing that within the same country, there is also wide variation in the distribution of the 2 genotypes in different regions. Only large scale population-based studies will be able to conclusively confirm these observations.

Also in contrast to reports of an association between *M. africanum* and HIV infection (de Jong et al., 2010) no such association was seen in this study. Given the mean age of 34.4 years in the *M. africanum* infected population in this study, *M. africanum* could not be associated with old age as has been previously reported (de Jong et al, 2008). These observations are, however,

consistent with other studies conducted in Ghana with a higher prevalence of MAF1 which also reported no association between MAF and HIV or older aged individuals (Meyer et al., 2008). Thus, it can be inferred that this association with HIV and old age is peculiar to MAF2 and not to the entire *M. africanum* genus or the sample size was just too small to see any such associations.

CHAPTER THREE

Mtb-specific multiple cytokine and CD4/ CD8 T cell responses before and after two weeks of treatment

3.1 Background

Cytokines are soluble proteins that are secreted by cells of the immune system and can alter the behavior and properties of different cell types (de Jager et al., 2003). Different cytokines possess biological overlapping functions, and they have the ability to regulate the production of other cytokines. Therefore, analysis of the function of the complete set of cytokines expressed within micro-environments (e.g., a site of inflammation) are often of more value than the analysis of a single isolated cytokine (O'Garra and Murphy, 1994). The human immune response to infection is mediated by cytokines and in *Mtb* infection in humans, the best described mediators of immunity are tumour necrosis factor (TNF-α) and IFN-γ (Harris and Keane, 2010; Jouanguy et al., 2000), owing to the known effect of the usage of TNF-blocking therapeutic agents and the characterization of mutations in the IFN-γ receptor gene.

The IFN-γ knockout (KO) mouse experiments also provide compelling evidence of the protective function of IFN-γ in tuberculosis infection (Mogues et al., 2001). Again, using both in-vivo neutralization and a KO mouse (with a disruption in the gene for the 55 kDa TNF receptor), Flynn et al., 1995 established that TNF-α and the 55 kDa TNF receptor are essential for protection against tuberculosis in mice, and for reactive nitrogen production by macrophages early in infection.

However, several other mediators have been characterized for their specific roles in the human immune response to *M. tuberculosis.* Interleukin 10 (IL-10), an anti- inflammatory cytokine with immune inhibitory functions, produced by macrophages and T cells is known to down-regulate interleukin 12 (IL-12) production, leading to decreased IFN-γ production and exacerbating infection (Raja, 2004). Other studies suggest that Granzyme B, a serine protease secreted by CTL and NK cells by exocytosis to induce apoptosis (Stenger and Modlin, 1998) as well as the soluble form of IL-2Rα (sIL-2Rα) released by mononuclear cells following activation

are markers of disease severity (El-Mesallamy et al., 2013; Seidler et al., 2012) while other molecules such like IL-17, contribute to the immune control of *Mtb* in mice, but has not yet been shown to be significant in humans (Reviewed by Torrado and Cooper, 2010).

Despite extensive investigation, a clear, reproducible correlate of human immunity to *M. tuberculosis* infection has not yet been identified (Ernst, 2012). Probably the full repertoire of T cell subsets and molecular mediators of protective immunity are still unravelling or there is a lack of appreciation of the fact that no single parameter alone will mediate or correlate with protective immunity in tuberculosis. Although in mouse model of tuberculosis infection, single cytokines are essential, it is clear that protection is mediated by a complex immune response that involves many different cell subsets and cytokine pathways. Assessing cytokine levels after antigenic stimulation of PBMC as well as specific T cell subset dynamics therefore could be useful in monitoring treatment response during tuberculosis infection.

So far only a few studies have integrated combinations of markers to predict treatment outcome. Most studies aimed at identifying host immune responses to *Mtb* antigens have mostly studied IFN-γ production resulting in poor specificity. It has been suggested that sets of markers rather than a single marker may increase the predictive ability (Walzl et al., 2008).

There has been a long held belief that patients with drug-susceptible TB are non-infectious after two weeks of therapy (Rouillon et al., 1976; NICHE, 2006) although recent microbiological and epidemiological evidence has challenged this dogma (Rouillon et al., 1976; Escombe et al.,2007; Menzies et al., 1997; Riley et al.,1962;Fitzwater et al., 2010). However, the nature of the *Mtb*-specific immunological response during this period of TB treatment has not been adequately investigated. Levels of immune markers (IFN-γ, TNF-α, IL-17, IL-10, sIL-2R and a soluble mediator, Granzyme B) released in response to *Mtb* were assessed for their utility for monitoring early response to TB treatment. In addition, to determine the functional T cell phenotypes contributing to the cytokine secretion during the first two weeks of treatment, IFN-γ+ CD4+ and CD8+ T cells were assessed at baseline and at two weeks of treatment in the same patients.

3.2 Experimental Design

3.2.1 Preparation of cells for culture

3.2.1.1 Blood collection

From each participant, up to 30 ml of venous blood was drawn using butterfly needles (BD) into 10 ml vacutainers (BD) containing sodium heparin to prevent clotting. Blood samples were taken at four time points; before treatment (baseline), and at 2 weeks, 2 months and 6 months of TB treatment. All samples were sent immediately to the laboratories of the Noguchi Memorial Institute for Medical Research for the appropriate analysis

3.2.1.2 PBMC separation

Using sterile 10 ml disposable pipettes (Sarstedt) blood from the three 10 ml vacutainers per participant was transferred into a sterile 50 ml centrifuge tube labeled with the participant unique identification number. An equal volume of pre-warmed (37^0C) RPMI 1640 (GIBCO) was added to the blood in the falcon tube to achieve a 1:1 dilution and mixed gently. The diluted blood was layered gently onto 15 ml of Histopaque (Sigma: Cat. No. H8889) without breaching the Histopaque-blood barrier. To attain a ratio of 2:1 for blood and Histopaque, 25-30 ml of blood was layered on 15 ml of Histopaque. Both the blood and Histopaque were used at room temperature. The tubes were centrifuged at 800 g for 30 min at room temperature with the brake off. The milky-white PBMC band at the interface between Histopaque (transparent) and plasma (yellow) was then aspirated with a sterile pastette into sterile 50ml tubes, topped up to the 50ml mark with pre-warmed Hank's Balanced Salt Solution or HBSS (Sigma: Cat. No. H9394) and centrifuged at 400 g for 10 minutes. The supernatant was discarded and the pellet (cells) re-suspended once more in HBSS and centrifuged at 400 g for 5mins. After this wash the supernatant was again discarded and the pellet suspended in 1 ml of the filtered growth medium [RPMI, 10% FCS (Fetal Calf Serum) (Sigma: Cat. No. F9665), 1% Pen-Strep (Penicillin/Streptomycin) (Sigma:Cat No. 15070063)] for counting.

3.2.1.3 Cell counts

The Cell suspension was diluted 1 in 2 with 0.2% Trypan blue (GIBCO: Cat. No. 15250-061) by adding 20 μl of Trypan blue solution to 20 μl of cell suspension in an Eppendorf tube. Using a coulter counting chamber, 10 μl of the cell suspension was dispensed into the counting chamber and cover slip was firmly attached. Live lymphocytes (translucent white cells) and dead cells (blue cells) were counted using the x10 magnification, within 5 of the 25 *triple* ruled squares of the hemocytometer. The cell count was then calculated as ff;

Viable Cell concentration =

Average number of viable cells counted x multiplier x dilution x 10^4/ ml of suspension

I.e.: [No. of cells/5] x25 x10 x 10^4/ ml

3.2.1.4 Cryopreservation of PBMC

Cells that were to be used later, as well as any remaining cells from any assay were cryopreserved. For cryopreservation, the "Mr Frosty" (Nalgene: Cat. No. 5100-0001), filled with 250 ml 2-propanol was placed in a fridge (4^0C) at least one hour before use and in case it had been used for the fifth time, the 2-propanol was refreshed. While counting, the rest of the cells were placed on ice for at least 30 minutes and at the same time X ml freezing medium [RPMI, 20% FCS, 10% DMSO/Dimethyl Sulphoxide (Sigma: Cat. No. D2650)] per sample was also placed on ice in the same period (for the preparation of the freezing medium the DMSO was added at the last minute. The freezing medium was added drop-wise to the cells and quickly the cell suspension was transferred to X cryotubes (Cat. No. 377267). The tubes were put in the Mr Frosty and stored overnight in a -80^0C freezer and after at maximum 1 week the cells were transferred into liquid nitrogen.

3.2.2 Preparation of antigens/recombinant proteins

3.2.2.1 Antigens/recombinant proteins used

ESAT-6/CFP-10 fusion, Rv1733c, Rv2029c, Rv2628 and Rv1115 (**Table 3.1**) were obtained from the Leiden University Medical Centre, the Netherlands in a dehydrated form and reconstituted using growth medium.

3.2.2.2 Reconstitution of antigens/recombinant proteins

Using sterile techniques an appropriate volume of freshly prepared growth medium was added to each vial of protein to reconstitute it into a stock concentration. The stock was then distributed into separate cryotubes, labelled with the antigen name, volume, date of reconstitution and concentration and stored as aliquots at -20^{0}C. Antigens in the working concentration of 5µg/ml were prepared from these stock aliquots by dilution with growth medium, labelled similarly and stored at -20^{0}C until ready for use. The working concentration of 5 µg/ml was chosen based on a pilot study using 2.5, 5 and 10 µg/ml. While the 2.5 µg induced minimal secretion of IFN-γ, there was no significant difference between the secretion at 5 µg and 10 µg.

Table 1Table 3.1: Antigens used in the study showing their protein size and function

NAME	PROTEIN SIZE (a .a)	MTB GENE FUNCTION/PROTIEN FUNCTION
ESAT-6/CFP-10	-	Fusion product, Classical antigen
Rv1733c	210	Possible trans membrane protein
Rv2029c	339	*pfkB* (Phospho fructo kinase B)
Rv2628	120	HP (Hypothetical protein)
Rv1115	232	Possible exported Protein

3.2.3 Cell culture with antigens/recombinant proteins

Frozen antigen aliquots (25 μl at 10 μg/ml) were brought to room temperature and each aliquot added in duplicate wells of the culture plates (Nunc; Cat. No. 163320). In the negative control well, growth medium was added instead of antigen. After cell counting, the thawed cells were re-suspended in pre-warmed sterile filtered growth medium at 500,000 cells per 25 μl and aliquots of 250 μl (500,000 cells) added per well of antigen and growth medium (negative control). Blank spaces were filled with HBSS to prevent evaporation. The plate was covered and sealed with Micropore tape (Cat. No.1530-125) and incubated for 6 days at 37°C in a 5% CO_2 incubator. The culture form was then filled indicating the subjects IDs start and end date. The positive control (Staphylococcus enterotoxin B (SEB) (Sigma: Cat. No. S4881) was added on the 4^{th} day of culture.

3.2.4 Harvesting culture supernatant and inhibition of cytokine secretion

To ensure sterile working condition for this procedure, the hood was thoroughly cleaned with 70% ethanol**.** For the last 12-16 hours of the 6 day incubation, the culture plates containing previously cultured PBMCs were removed from the incubator and placed in the hood. Using a sterile pipette, aliquots of 255 μl of culture supernatant was put into pre-labelled screw-capped Eppendorf tubes and frozen at -20^{o}C for multiplex assay. Without disturbing the cells, 5 μl of Brefeldin A (BFA) (Sigma: Cat. No. B7651) at 250 μg/ml) was carefully added to the remaining cell suspension to achieve a final concentration of 5 μg/ml. The culture plate was covered, sealed with Micropore tape and returned into CO_2 incubator at 37^{0}C overnight.

3.2.5 FACS Analysis

3.2.5.1 Surface staining

After the 6-day culture, cells were harvested into appropriately labeled FACs tubes. Each well was washed out gently with sterile FACS buffer (1 X PBS, 1% HI-FCS, 0.1% NaN_3) to completely collect all cells. All tubes were placed on ice ensuring cells are kept cold during staining. Two (2

ml) of FACS buffer was added per tube and centrifuged for 5 minutes at 1350 rpm. The supernatant was decanted and the pellet re-suspended in 100 µl FACS buffer and incubated with the appropriate amount of monoclonal antibody (Mab) for surface staining (CD4/CD8) at 4^0C in the dark for 30 minutes wrapped in aluminum foil. After incubation, 2 ml of FACs buffer was added per tube and centrifuged as before and the supernatant decanted as before. The cell pellet was then re-suspended in 0.5 ml of 2% PFA (paraformaldehyde) at room temperature in the dark for 15 minutes to fix the cells for intracellular staining.

3.2.5.2 Intracellular staining:

After fixing, 2 ml of sterile filtered Perm wash (FCS, 10% NaN3, 10% Saponin) pH, 7.5 was added and centrifuged as before. The supernatant was decanted, the pellet re-suspended in 1 ml of perm wash for 25 minutes before centrifugation. After centrifugation, 100 µl of perm wash and appropriate volumes of intracellular antibody (IFN-γ) were added to the tubes and incubated for 30 minutes at 4^0C in the dark. After incubation, 2 ml perm wash was added and centrifuged as before and the supernatant decanted. The pellet was then re-suspended in 0.3 ml of 2% PFA and the cells were acquired immediately on a FACS Calibur (BD). Using FLOWJO software Version 7.6.2, we defined an R1 gate for lymphocytes in a dot plot of Forward Scatter Channel (FSC) versus Side Scatter Channel (SSC). To identify CD4+ and CD8+ T cells, events from R1 were analyzed in a plot of either CD3-FITC versus CD4-PerCP or CD8-PerCP respectively or CD4-Percp and CD8-APC (R2). Finally, gated CD4+ and CD8+ T cells were analyzed for IFNγ-PE or IFNγ-FITC. Data were reported as percentages of CD4+ and CD8+ T cells. Compensation settings were defined using anti-mouse kappa Comp Beads (BD Biosciences) stained with each fluorochrome–conjugated antibody. A threshold of 0.2% IFN-γ+ CD4+/CD8+ T cells defined positive T-cell responses against antigens (Shuck et al., 2009).

Table 2Table 3.2: Flourochrome -conjugated monoclonal antibodies used in the study

Antigen	Clone	Isotype	Material No.	Company
CD3 FITC	UCH-T1	Mouse IgG1, k	555332	BD Biosciences
CD4 PerCp	SK-3	Mouse IgG1, k	345770	BD Biosciences
CD4 APC	RPA-T4	Mouse IgG1, k	555349	BD Pharmingen
CD8 PerCp	SK-1	Mouse IgG1	345774	BD Biosciences
CD8 APC	RPA-T8	Mouse IgG1	561421	BD Biosciences
IFN-γ PE	4S.B3	Mouse IgG1,k	559326	BD Pharmingen
IFN-γ FITC	4S.B3	Mouse IgG1,k	554551	BD Pharmingen

3.2.6 Thawing of previously stored culture supernatant

The six-day culture supernatant, previously harvested and stored at -80^{o}C were brought to room temperature to thaw slowly until all ice had completely melted. Whilst still ice cold, the samples were centrifuged to remove cell debris after which it was dispensed into new tubes and transported on ice for analysis.

3.2.7 Human 6-plex (IFN-γ, TNF-α, IL-17, IL-10, sIL-2Rα and Granzyme B) assay

Luminex xMAP technology for multiplexed quantification of cytokines, chemokines, and growth factors in human was performed using the Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta-Canada). The six markers were measured in the cell culture supernatant using an Affymetrix Human Cytokine/Chemokine Custom plex kit (Affymetrix, Inc, Santa Clara, CA, USA) according to the manufacturer's protocol. The 6-plex consisted of IFN-γ, IL-10, IL-17, IL-2Rα, Granzyme B, and TNFα,. The assay sensitivities of the 6-plex markers ranged from 0.1 – 0.4 pg/ml, and 5 pg/ml for IL-2Rα and Granzyme B.

3.2.8 Data analysis

Data was entered into Microsoft Excel 2007® or transported to GraphPad Prism®4 for analysis and graphs. To compare the cytokine expression profile for each antigen, the median concentration of each cytokine in pg/ml was determined at baseline and after 2 weeks on TB treatment. The minimum detectable concentrations of the cytokines or soluble mediator in

this assay were 5.7, 0.2, 0.26, 3.35, 1.39, 2.13 pg/ml for Granzyme B, IFN-γ, IL-10, IL-17, sIL-2R-α and TNF-α respectively.

To determine the number of positive responders to each antigen, values of S/U (*where S/U is defined as follows: cytokine concentration in antigen- stimulated cultures divided by the cytokine concentration in un-stimulated (negative control)* as described in Al-Attiya et al, 2008) that were greater than or equal to 2 were considered positive responses. In experiments where the concentrations of cytokines in control cultures lacking antigens were not detectable, the S/U values were determined by dividing the concentration of a given cytokine in antigen-stimulated cultures with the minimum detectable concentration of the same cytokine.

To determine changes in cytokine expression profile after 2 weeks on TB treatment, the Mann-Whitney test was used to compare the median cytokine concentrations for each antigen before and after two weeks of treatment, while the Wilcoxon signed rank, text was also used to compare changes in cytokine expression profile in the same individuals at baseline and 2 weeks of treatment (matched observations). The Mann-Whitney test was used to compare cytokine responses at baseline and after 2 weeks of treatment in each participant (single). For flow cytometric analysis, percentage of IFN-γ+ cells were calculated by subtracting the percentage of IFN-γ+ cells in un-stimulated cultures from stimulated ones. A threshold of 0.2% IFN-γ+ CD4/ CD8 T cells defined positive T-cell responses against antigens. Samples with a negative SEB response were excluded from the analysis. Differences in the percentage of IFN-γ+ CD4/CD8 T cells to antigenic stimulation were analyzed using the nonparametric Mann-Whitney U-test.

In all cases, P values of 0.05 were considered significant.

3.3 Results

3.3.1 Early response to TB treatment: Cohort Characteristics

The early response to TB treatment cohort consisted of the first 20 sputum smear positive patients recruited for the study with Mean age 34.05 years (range: 21 to 55) and 80% male. Participants were categorized based on AFB smear grading as 3+ (one or both smears were 3+), 2+ (one or both smears were 2+ or lower), 1+ (one or both smears were 1+ or lower) and SC (one or both smears were scanty "less than 10 AFB per field" or negative). Accordingly, nine (9) were classified as 3+, Four (4) as 2+, three (3) as 1+ and four (4) as SC (scanty). Two of the participants were infected with MAF and one was HIV+. All but one had converted to smear negative at month 2 **(Table 3.3).**

Table 3Table 3.3: Detection of early responses to TB treatment; Participant's Characteristics

PARTICIPANT	AGE (YEARS)	GENDER	SPUTUM SMEAR MICROSCOPY RESULTS DIAGNOSIS^		CATEGORY	MONTH 2
01	44	M	3+	3+	3+	Neg
02	32	M	3+	3+	3+	Neg
03	52	M	2+	2+	2+	Neg
04	21	F	1+	Neg	1+	Neg
05	31	M	2+	2+	2+	Neg
06	29	M	3+	SC	3+	SC
07*	34	M	1+	1+	1+	Neg
08	55	M	3+	3+	3+	Neg
09	23	M	2+	2+	2+	Neg
10#	23	F	2+	2+	2+	Neg
11	37	M	3+	3+	3+	Neg
12	24	M	1+	1+	1+	Neg
13	37	F	3+	3+	3+	Neg
14	45	M	3+	3+	3+	Neg
15	32	M	3+	3+	3+	Neg
16	38	M	SC	SC	SC	Neg
17	33	M	SC	SC	SC	Neg
18	33	M	3+	3+	3+	Neg
19*	30	M	SC	Neg	SC	Neg
20	28	M	SC	Neg	SC	Neg
Average	**34.05**	**M (80%)**			**+ (100%)**	**(+) 5%**
Range	**[21-55]**					

***** *M. africanum* (MAF) **#** HIV + participant

SC (Scanty) < 10 AFB per 100 fields ^ Showing two smear results required for TB diagnosis

3.3.2 Antigen-induced secretion of cytokines by PBMC in response to "stage specific" mycobacterial antigens

To determine the immunogenicity of each antigen, we compared the levels of cytokines (pg/ml) induced by each antigen after 6 days of PBMC culture to that of the un-stimulated controls. In all cases, the levels of cytokines were significantly higher ($P<0.001$) in stimulated than un-stimulated controls, indicating the antigens were immunogenic in our cohort of TB patients. This observation was true at both baseline **(data shown in Fig 3. 1),** and after two weeks of TB treatment (data not shown).

3.3.3 Positive responders and median cytokine concentration

Determining the number of positive responders to *Mtb* antigens is critical to prioritizing the antigens as there continues to be a search for the TB antigen with universal immunogenicity. Based on our calculation of positive cytokine responses, SEB (positive control) induced the most positive responses, with about 84-100% responders for all 6 cytokines **(Table 3. 2).** Compared to ESAT-6/CFP-10 (65-84%), and the latency associated proteins Rv1733 (67-83%), Rv2029 (55-73%) and Rv2628 (50-80%). The important role of IFN-γ in protection during TB disease was evident as regardless of the antigen in question, the cytokine with the highest median concentration (pg/ml) was IFN-γ (Range 372.6 - 1931). This was followed by Granzyme B (Range 585 - 1144), TNF-α (Range 216.5 - 491.5) or IL-17 (Range 142.6 - 334.2) while sIL-2R-α (Range 155.1 - 187.1) and IL-10 (Range 108.2 - 172) were secreted in lower quantities **(Figure 3. 2**). After two weeks of treatment, this trend did not change in terms of cytokine secretion profile.

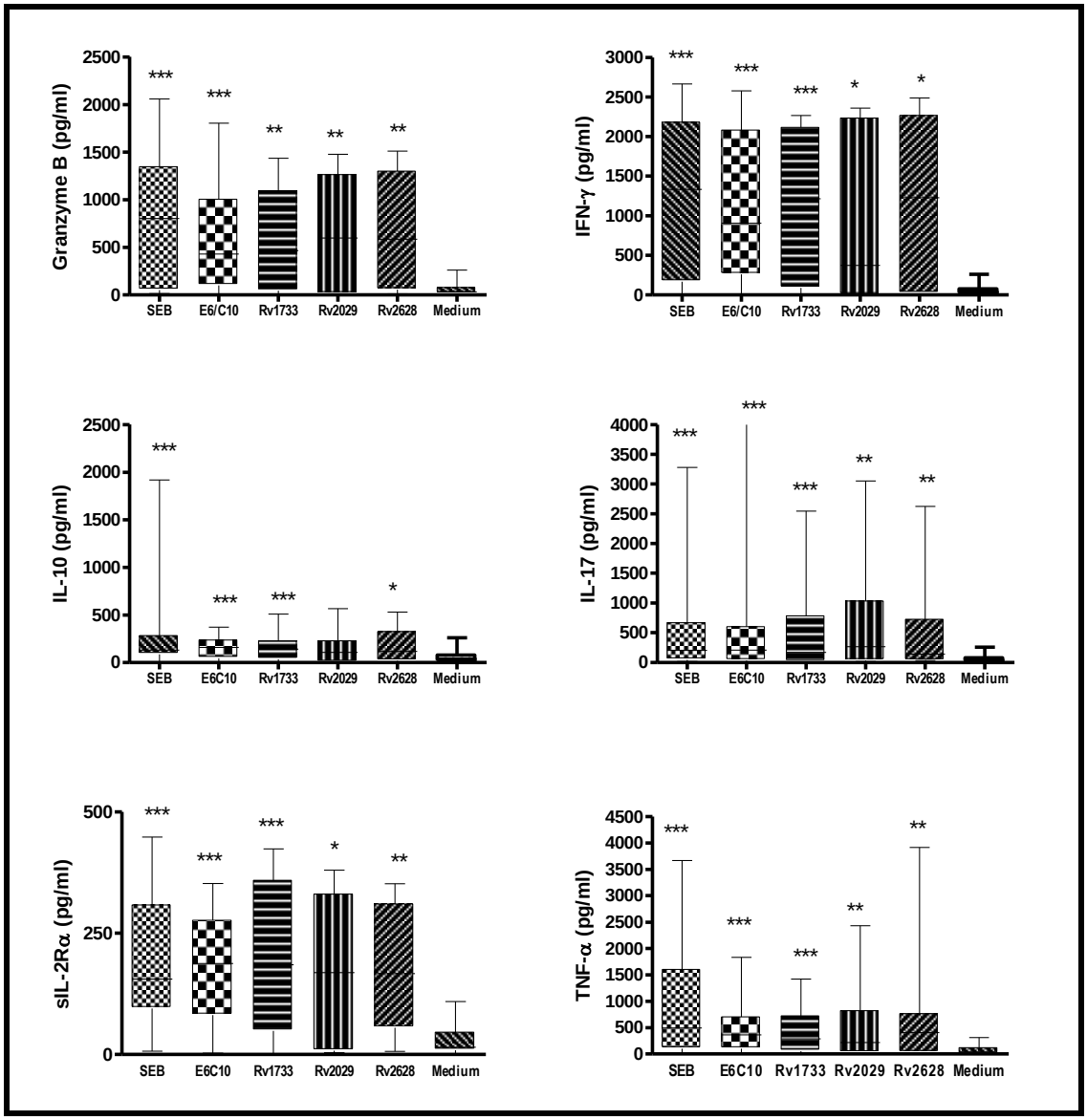

Figure 3. 1: Median cytokine concentration in response to different antigenic stimulation of PBMC of TB patients at baseline (before start of TB therapy).

Secretion of Granzyme B, IFN-γ, IL-10, IL-17, sIL2Rα and TNF-α (pg/ml) by PBMCs obtained from 20 sputum smear positive TB patients at baseline (before treatment). PBMC was stimulated *in vitro* with the stage specific *M. tuberculosis* antigens; ESAT-6/CFP-10 (n=19), Rv1733 (n=18), Rv2029 (n=11), Rv2628 (n=10), a negative control (Growth medium) and *Staphylococcus aureus* B (SEB) as positive control. A six-plex Luminex assay was done on 6-day culture supernatants. The box plots show the 25th, 50th, and 75th percentiles, and the whiskers represent the minimum and maximum levels of cytokine (pg/ml) induced by each stimulus. To determine immunogenicity of antigens in the study population, each antigen-induced response was compared to the un-stimulated or negative control culture (medium) using a Mann-Whitney U test. *P<0.0001 (***), P<0.001 (**), P<0.01 (*).*

Table 3.4: Positive cytokine responses per antigen before and after 2 weeks of TB treatment

	No. of positive responders per antigen [n^1/N^2 (%)]				
	SEB	**E6/C10**	**Rv1733**	**Rv2029**	**Rv2628**
Granzyme B					
Before	19/19 (100)	13/19 (68.4)	13/18 (72.2)	7/11 (63.6)	7/10 (70)
After 2wks	14/15 (93.3)	12/15 (80.0)	9/10 (90.0)	7/7 (100.0)	5/5 (100)
P value	0.4412	0.6974	0.3746	0.1193	0.5055
IFN-γ					
Before	18/19 (94.7)	16/19 (84.2)	15/18 (83.3)	8/11 (72.7)	8/10 (80.0)
After 2wks	12/15 (80.0)	13/15 (86.6)	10/10 (100)	7/7 (100)	5/5 (100)
P value	0.2994	1.0	0.5330	0.2451	0.5238
IL-10					
Before	18/19 (94.7)	14/19 (73.7)	12/18 (66.7)	7/11 (63.6)	5/10 (50.0)
After 2wks	12/15 (80)	9/15 (60.0)	7/10 (70.0)	4/7 (57.1)	5/5 (100)
P value	0.2994	0.4748	1.0	1.0	0.1009
IL-17					
Before	19/19 (100)	13/19 (68.4)	14/18 (77.8)	6/11 (54.5)	8/10 (80.0)
After 2wks	13/15 (86.6)	12/15 (80.0)	9/10 (90.0)	6/7 (85.7)	5/5 (100)
P value	0.1872	0.6974	0.6264	0.3156	0.5238
sIL-2R-α					
Before	18/19 (94.7)	14/20 (70.0)	12/18 (66.7)	7/11 (63.6)	8/10 (80.0)
After 2wks	11/15 (73.3)	12/15 (80.0)	9/10 (90.0)	6/7 (85.6)	5/5 (100)
P value	0.1458	0.7003	0.3642	0.5956	0.5238
TNF-α					
Before	16/19 (84.2)	13/19 (68.4)	12/18 (66.7)	7/11 (63.6)	7/10 (70.0)
After 2wks	11/15 (73.3)	9/10 (90.0)	9/10 (90.0)	5/7 (71.4)	5/5 (100)
P value	0.6722	0.3667	0.3642	1.0	0.5055

Freshly isolated PBMC from Sputum smear positive TB patients were stimulated for 6 days **before initiation of therapy** (baseline) with SEB (n=19), ESAT-6/CFP-10 (n=19), Rv1733 (n=18), Rv2029 (n=11) and Rv2628 (n=10) and ***after 2 weeks on anti-TB therapy*** with SEB (n=15), ESAT-6/CFP-10 (n=15), Rv1733 (n=10), Rv2029 (n=7) and Rv2628 (n=5). Culture supernatant was assessed by multiplex cytokine analysis. Percentage of positive cytokine (Granzyme B, IFN-γ, IL-10, IL-17, sIL2R-α and TNF-α) responses per antigenic stimulation were calculated. Positive responses were determined as follows; *Cytokine concentrations were divided by the negative control sample (medium only, un-stimulated) and values greater than or equal to 2 were considered positive*.

[1]Number of positive responses [2]Number of samples analyzed

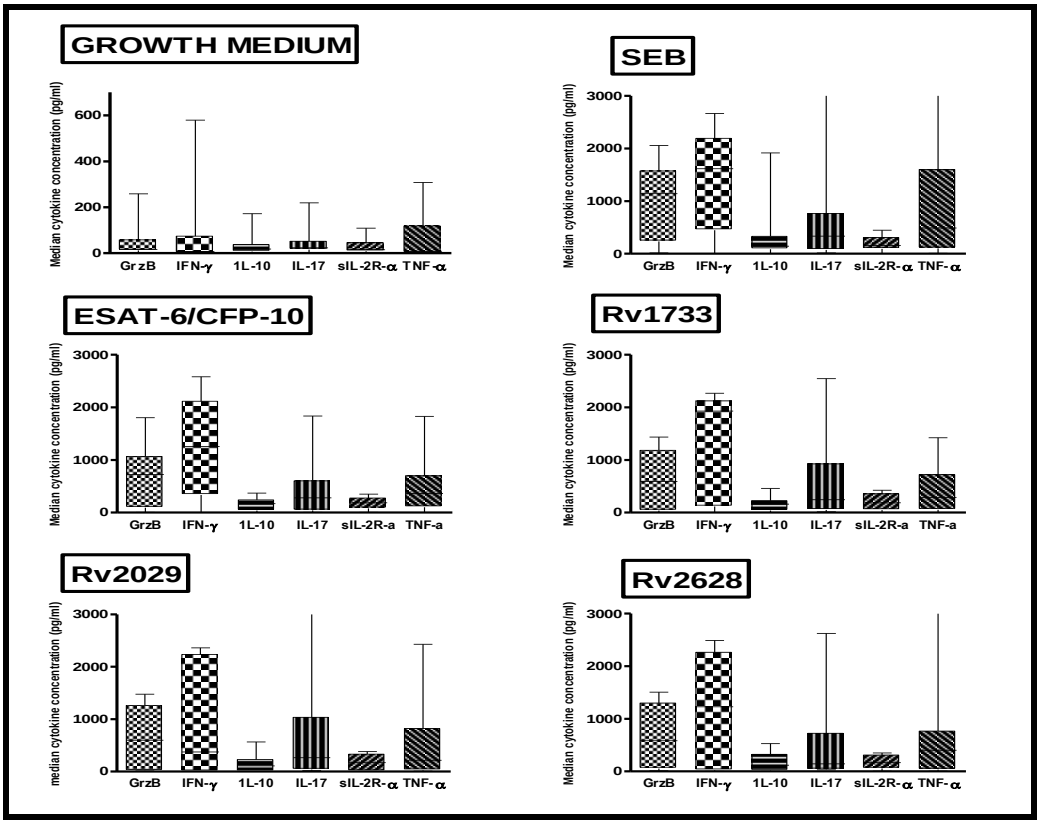

Figure 3.2: Cytokine profile in response to Mtb-specific antigens. High levels of IFN-γ and Granzyme B are secreted in response to all antigens.

Freshly isolated PBMC obtained from sputum smear positive TB patients (n=19) at baseline (before treatment) were stimulated in vitro with the stage specific *M. tuberculosis* antigens; ESAT-6/CFP-10, Rv1733, Rv2029, Rv2628 (latency associated), positive control *Staphylococcus* enterotoxin B (SEB) and negative control (Growth medium) for 6 days. The harvested supernatant were used in a six-plex Luminex assay for of Granzyme B (GrzB), IFN-γ, IL-10, IL-17, sIL2Rα and TNF-α. The box plots show the 25th, 50th, and 75th percentiles, and the whiskers represent the minimum and maximum levels of cytokine (pg/ml) induced by each stimulus. In response to all antigens, high levels of IFN-γ followed by Granzyme B and TNF-α and low levels of IL-17, sIL2Rα and IL-10 were observed.

3.3.4 Effect of two weeks of anti-TB treatment on cytokine response to *Mtb*-specific antigens

To determine whether there are significant changes in the antigen-induced cytokine secretion by PBMC of individuals undergoing anti-TB therapy after the first two weeks of initiation of treatment, two approaches were used. First, to identify differences in cytokine responses to antigens associated with active infection (ESAT-6/CFP-10) and latency (Rv1733), the Wilcoxon matched pairs test was used to compare the levels of each of the six cytokines in response to ESAT-6/CFP-10 and Rv1733 at baseline and 2 weeks of treatment using patients (n=9) with data available for all cytokines at both time points. Secondly, each cytokine response to the same antigen was compared at baseline and week 2. There was no difference ($P>0.05$) in levels of the 6 cytokines induced by ESAT-6/CFP-10 and the latency associated Rv1733 at baseline (T0) and 2 weeks of treatment (T1). The median concentration (pg/ml) of all cytokines (except TNF-α) in response to ESAT-6/CFP-10 were higher at TI (week two) compared to T0 (baseline) and the same trend was observed with responses to Rv1733, while IL-10 levels remained almost unchanged during this period. However, only the median increase of Granzyme B secretion in response to Rv1733 fusion protein (474.2 to 1264.9 pg/ml, P=0. 01), was statistically significant (**Figure 3.3).**

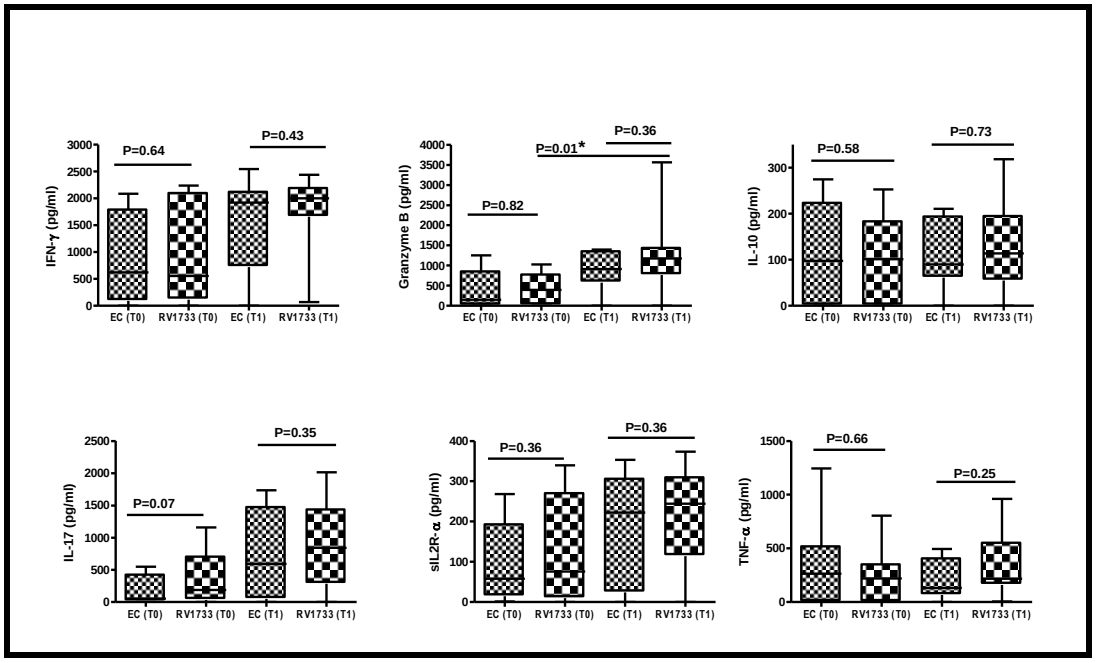

Figure 3.3: ESAT-6/CFP-10 fusion protein and latency associated Rv1733 induce comparable levels of the 6 cytokines in TB patients.

Cytokine levels induced by ESAT-6/CFP-10 (EC) fusion protein and Latency associated Rv1733 at baseline (T0) and 2 weeks (T2) on treatment were compared using the Wilcoxon matched pairs test in Patients who had data for both time points available (n=9). Cytokine levels were obtained after subtracting values in un-stimulated wells from stimulated wells and negative values were converted to zero. There is no difference (P>0.05) in levels of the 6 cytokines induced by ESAT-6/CFP-10 and the latency associated Rv1733 at baseline (T0) and 2 weeks of treatment (T1). In the same patients (n=9), the median concentration of all cytokines except TNF-α in response to ESAT-6/CFP-10 were higher at TI compared to T0 and the same trend was observed with responses to Rv1733. However, only the median increase of Granzyme B secretion in response to Rv1733 fusion protein was statistically significant (P=0. 01).

3.3.5 Cytokine response to antigenic stimulation in individual patients before and after two weeks of anti-TB treatment

To determine if the trend of improved cytokine responses (Granzyme B, IFN-γ, IL-17) was a reflection of individual cytokine responses and not a group effect, analysis of cytokine responses to ESAT-6/CFP-10 at baseline and 2 weeks of treatment was done for individual patients (n=12) who had both data available. A wide inter-individual variation was observed in cytokine response profile. As such, cytokine responses to ESAT-6/CFP-10 fusion protein were categorized based on the three distinct response patterns observed from baseline to week 2 as; (a) Increased median cytokine concentration, (b) decreased median cytokine concentration and (c) Fluctuations in median concentration for all 6 cytokines **(Figure 3.4).** To understand this variation in cytokine response pattern, a Uni-variate analysis was done for "age'' and "sputum smear result at diagnosis" with cytokine response to antigenic stimulation (increased/decreased/fluctuating) as outcome variables. However, no association could be established.

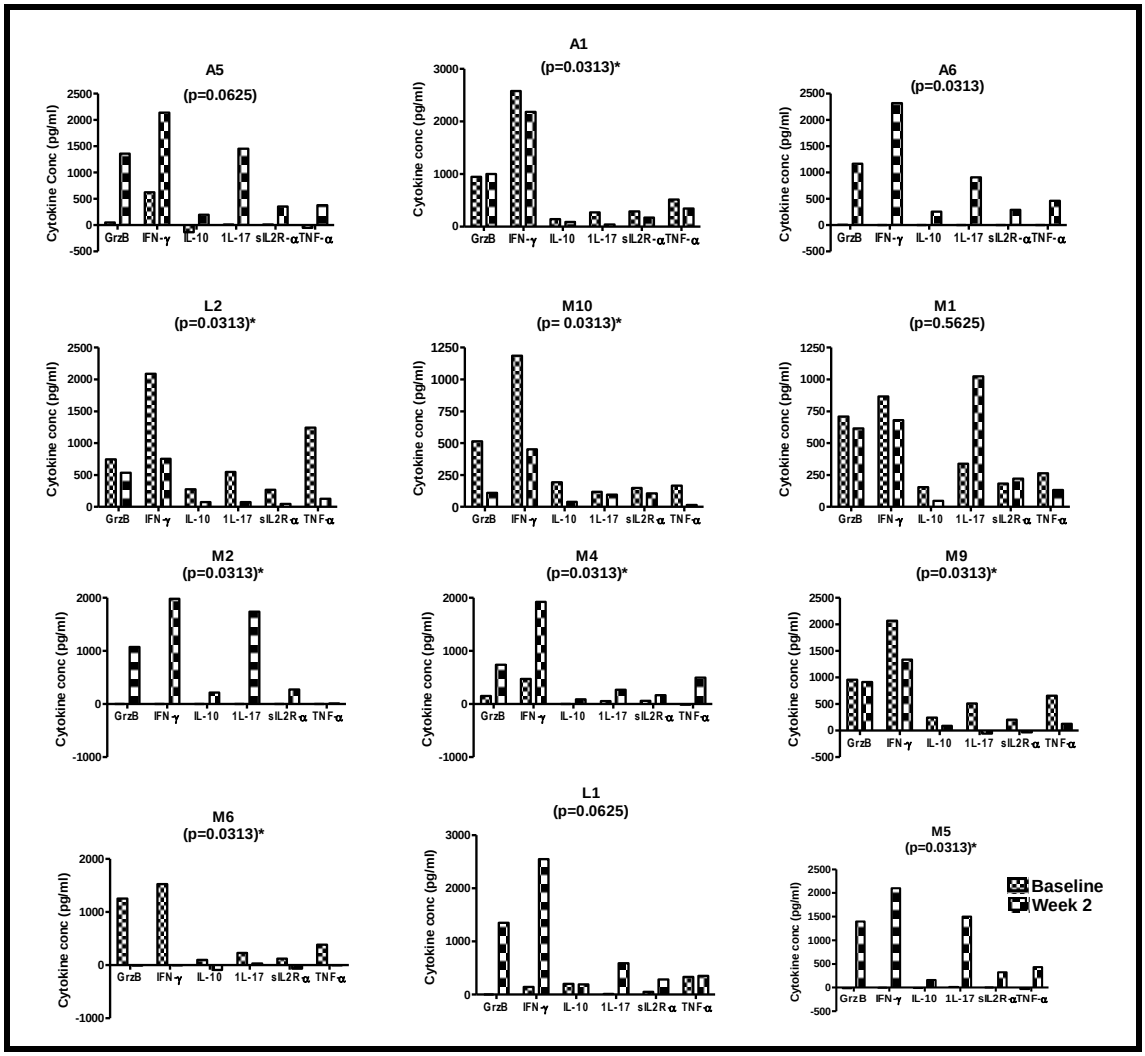

Figure 3.4: Changes in cytokine levels after 2 weeks of TB treatment follows three patterns.

Shown are the cytokine profiles of (n=13) patients with both time points available for response to ESAT-6/CFP-10 fusion protein. In patients A1, L2, M10, M9 there is a significant *decrease* in all cytokine levels at week two, while in patients A6, L4, M4, M2, M6, M5 there is a significant *increase* in all cytokine levels. The increase and decrease in cytokine levels in A5 and L1 respectively, are not significant while M1 shows fluctuating levels.

3.3.6 Dynamics of *Mtb*-specific CD4 and CD8 T cell responses before and after two weeks of treatment

Effective treatment of active TB patients with anti-TB drugs has been shown to improve cellular responses (antigen-induced proliferation and IFN-γ secretion) of PBMC to various complex mycobacterial antigens and some immunodominant single antigens, e.g. ESAT-6, 16-kDa and 38-kDa proteins (Wilkenson et al., 1998; Ulrichs et al., 2000). However, it is not known if the improvement in cellular responses is limited to these proteins or is a generalized improvement of cellular responses to a large number of antigenic proteins of *Mtb*. In view of this, the IFN-γ T cell responses to 3 latency associated antigens were also evaluated at baseline and week 2 in this subset of patients. To determine the CD4 and CD8 T cell phenotypes contributing to the cytokine secretion during the first two weeks of treatment, we assessed the IFN-γ+ CD4+ and CD8+ T cells at baseline and at two weeks of treatment. **Figure 3.5** illustrates the gating strategy.

Rv1733 was the strongest inducer of IFN-γ in both CD4 and CD8 T cells compared to Rv2029 and Rv2628. After two weeks of treatment, the median frequency of antigen- specific IFN-γ+ CD4 T cell responses increased compared to baseline values; however, only the increase in the ESAT-6/CFP-10-specific response was significant (Median: 1.660 vs 3.495 pg/ml, P=0. 0008). In contrast, the median frequency of antigen- specific IFN-γ+ CD8 T cell responses declined during week two, with the decline in ESAT-6/CFP-10- specific (from 3.295 to 1.665 pg/ml, P=0.0024) and Rv2029- specific CD8 T cell response (from 2.225 to 0.480 pg/ml, P=0.009) being significant (**Figure 3.6 A and B**). The Wilcoxon matched pairs test analysis was done on subjects for whom T cell responses at both time points in response to ESAT-6/CFP-10 and Rv1733 (the strongest inducer of T cell responses among the 3 DosR proteins) were available. Again, the median frequency of IFN-γ+ CD4 T cells in response to ESAT-6/CFP-10 was significantly increased (P=0. 0078) at week two, while the frequency of IFN-γ+ CD8 T cells was significantly decreased (P=0. 0039, **Figure 3.6 C).** There were no significant changes in response to Rv1733 (**Figure 3.6 D**).

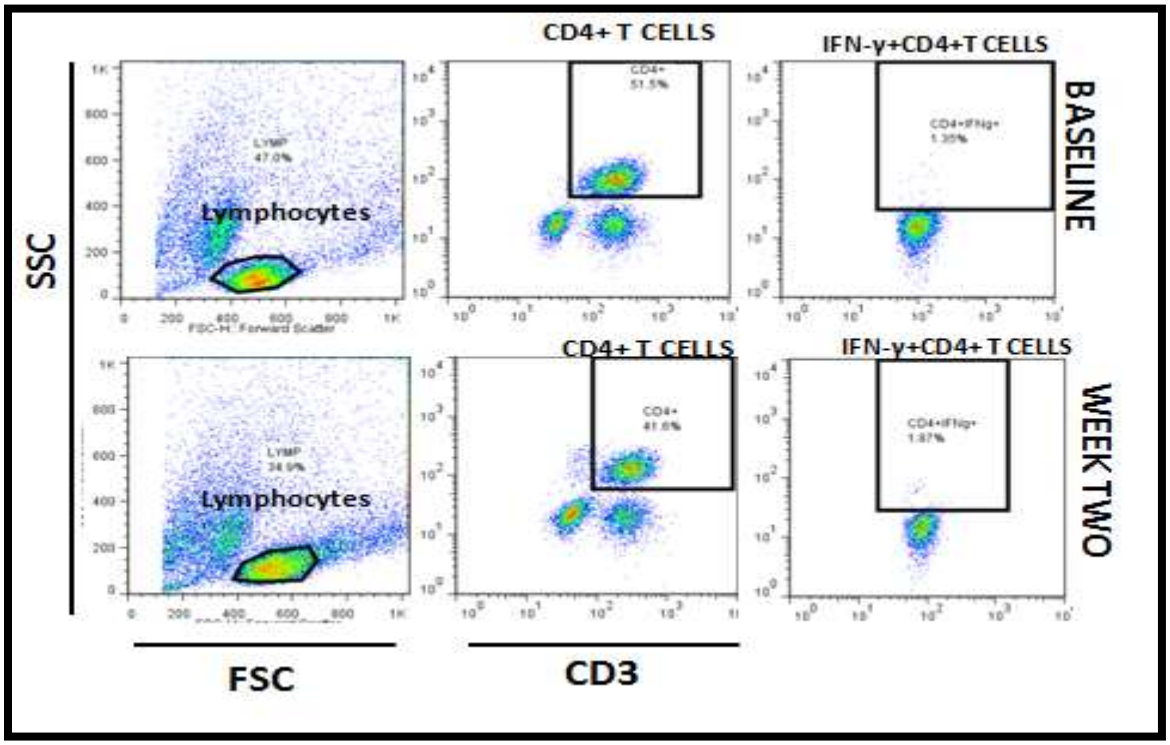

Figure 3.5 Gating strategy for identification of IFN-γ+ CD4 T cells after ESAT-6/CFP-10 re-stimulation at baseline and week 2.

Freshly isolated PBMC from smear positive TB patients (n=20) were stimulated for 6 days with *Mtb* antigens. Supernatant was harvested on the 5th day for multiplex assay and BFA was added to the remaining cells overnight. Cells were harvested and fixed in paraformaldehyde, stained for CD3 and CD4/CD8, permeabilized, and stained for IFN-γ. Cells were gated on lymphocytes by forward and side scatter and analyzed by three-color FACS®. CD3+ CD4+ or CD3+ CD8+ T Cells were analyzed for each stimulant, and dot plots from a representative sample are shown. Percentages of gated cells are indicated.

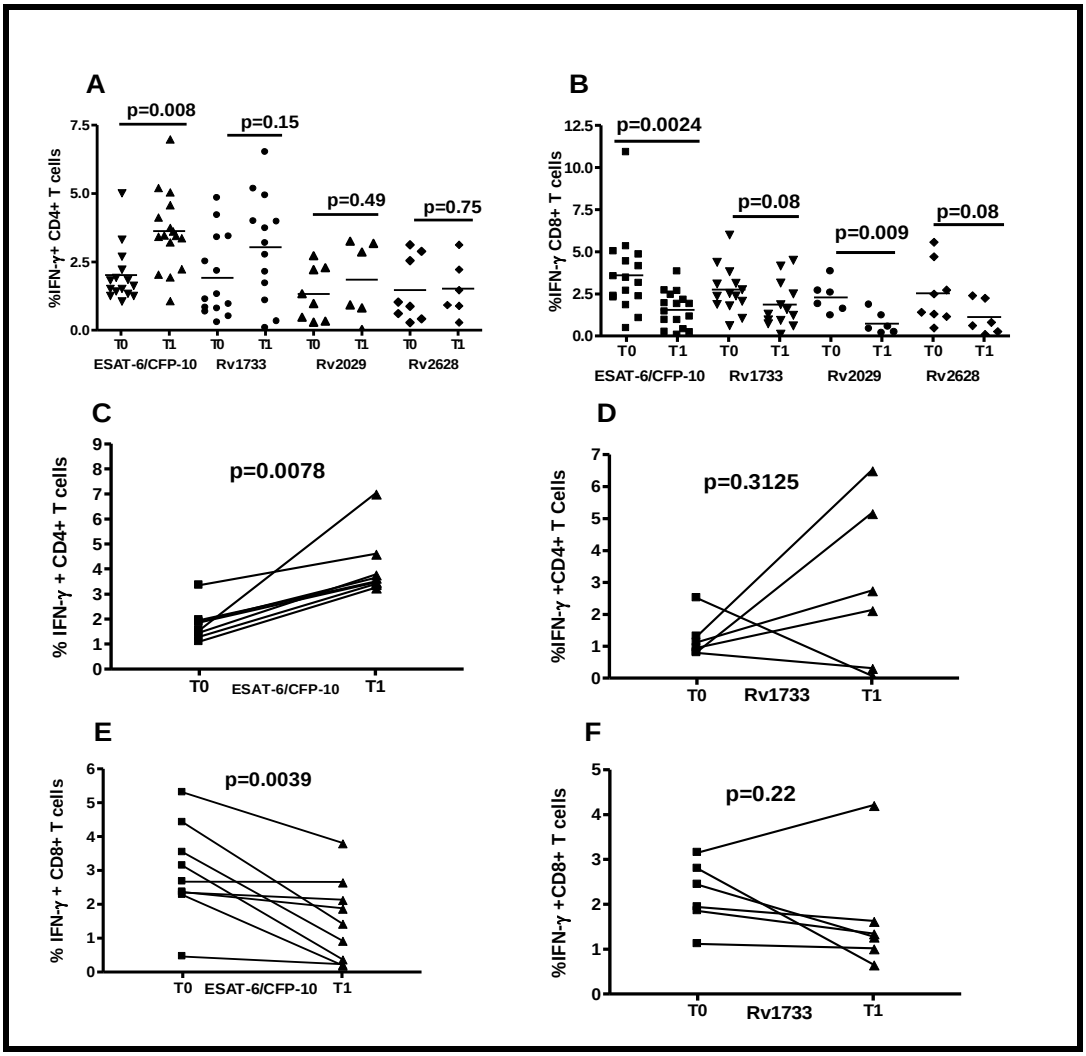

Figure 3.6: CD4 and CD8 T cell responses to *Mtb* specific antigens before and after two weeks of treatment

Freshly isolated PBMC from newly diagnosed sputum smear positive and HIV-negative TB patients (n=19) were stimulated for 6 days with ESAT-6/CFP-10, Rv1733, Rv2029 and Rv2628, SEB and an un-stimulated control (Medium). IFN-γ+ CD4+ and IFN-γ+ CD8+ T cells were assessed by flow cytometry by subtracting the percentage in un-stimulated cultures from stimulated ones. A threshold of 0.2% IFN-γ+ CD4/ CD8 T cells defined positive T-cell responses against antigens. The Mann-Whitney U test ($P<0.05$) was used to compare frequency of IFN-γ+ CD4 (A) and CD8 (B) T cells at baseline (T0) and week two (T1) in response to ESAT-6/CFP-10 (n=11), Rv1733 (n=10), Rv2029 (n=6), Rv2628 (n=6). Also shown (E &F) is the frequencies IFN-γ + CD4 and CD8 T cell at baseline (T0) and at 2 weeks (T1) into treatment for individual participants with data available in response to ESAT-6/CFP-10 (n=8) and Rv1733 (n=6). P values computed with the Wilcoxon signed rank test ($P<0.05$).

3.4 Discussion

Cytokine expression in response to mycobacterial antigens has been studied extensively in identifying immunological differences between active TB and latent infection or in TB patients before and after treatment. Determining the antigen-induced cytokine expression profile would also expand the knowledge of the performance of potential immunogenic *Mtb* antigens just as is required in designing new diagnostics or vaccines (Bartholet et al., 2000; Sable et al., 2007). All the four antigens used, were immunogenic in this cohort as evidenced by the highly significant differences ($P<0.01$ to $P<0.0001$) in cytokine secretion seen between stimulated and un-stimulated cultures.

The cytokine profile in response to all antigenic stimulation consisted of high levels of IFN-γ followed by granzymeB (grzB) and TNF-α or IL-17 and lower levels of sIL2Rα and IL-10. This finding was contrary to expectations that ESAT-6/CFP-10 being virulent factors would induce both pro- and anti-inflammatory cytokines whiles Rv1733, Rv2029 and Rv2628 being latency associated antigens would only induce pro-inflammatory responses and relatively minor anti-inflammatory response. The cytokine profile observed in this study suggests that the immune responses in the active stage of the disease (TB patients) are characterised by both pro- and anti-inflammatory cytokines irrespective of the nature of the antigen. This is supported by studies that have shown that the functional signature in response to *Mtb* antigens depends on the infection state in the host (active/latent infection) rather than the nature of the antigen (RD1, DosR, PPD) (Petruccioli et al., 2013; Lalvani et al., 1998). This suggests that a discriminatory cytokine profile could be observed using different cohort groups (active/latent TB).

The level of inflammatory versus anti-inflammatory cytokines determine the clinical outcome of *Mtb* infection (Kassa et al., 2012). Although antigens that evoke strong IFN-γ responses are candidates for TB vaccine development (Feng et al., 1999), it has been argued that care should be taken when antigens that induce high levels of IL-10 for instance, are considered for vaccine formulations for TB, as IL-10 down regulates the production of protective cytokines, including IFN-γ, TNF-α IL-1, and IL-12 (Mustapha et al., 2011; Van Crevel et al., 2002). Hence, high

concentrations of IFN-γ as observed in this study, on its own is not indicative of protection without being accompanied by low concentrations of anti-inflammatory cytokines such as IL-10.

Except for sIL2R-α, median IL-10 levels were lowest in response to all antigens, resulting in high IFN-γ/IL-10 ratios (not shown). Low IL-10 levels early during treatment is a good indicator of early response and this could probably be the reason why 95% (19/20) participants were "smear-negative" by month 2 signifying the successful clearance of mycobacteria. It has previously been confirmed that, IL-10 levels are significantly higher in slow responders very early during treatment, indicating that an early increased anti-inflammatory response during treatment may lead to the delay of sputum culture conversion in patients (Djoba Siawaya et al., 2009).

Levels of TNF-α in this study were higher than that of IL-17, IL-10 and sIL2R in response to all antigens, an indication of its importance in TB pathogenesis. The median cytokine concentration of IL-17 in response to all antigens was higher than only that of sIL2R and IL-10. The detection of IL-17 positive responses in 65% of supernatant of ESAT-6/CFP-10 induced PBMCs may indicate nonspecific inflammation during active TB (Nemeth et al., 2011) while addition IL-17 positive responses to the latency antigens may indicate that these play a role in inflammation and pathogenesis of TB.

Antigen induced secretion of sIL2R was low compared to the other cytokines, except IL-10. Elevated sIL-2R during TB infection could indicate an inappropriate activation of T-lymphocytes and macrophages/monocytes, which may have harmful consequences (Chan et al., 1991).

There was high secretion of grzB to all antigens with median cytokine concentrations only less than that of IFN-γ. Similar findings were reported by Toosii et al., 2004 where IFN- γ and grzB, were the only *Mtb* effector molecules that were induced in PBMC from *Mtb*-sensitized subjects. In the latter study, however, *Mtb*-induced expression of IFN-γ, but not grzB, was significantly lower in TB patients as compared with healthy control subjects.

The role of grzB in TB pathogenesis has not been clearly established, but it is known to be a driving force of cytotoxicity. The resolution of infections with many intracellular pathogens requires the effector functions of both NK cells and CD8+ CTLs. In *Mtb* infections, the combined action of perforin and the antibacterial agent granulysin, both of which are expressed in the granules of CTLs and NK cells, influences the outcome of infection (Stenger et al., 1998). However, other molecules involved in cytotoxicity (FasL, perforin and granulysin) are not inducible by *Mtb* and/or a T-cell mitogen in primary cells (Toosii et al., 2004) making grzB the only mediator to assess for evidence of cytotoxicity in such situations. The high levels of grzB suggests that the early stages of disease are characterized by apoptosis of infected cells ostensibly to halt the spread of infection and this is supported by a higher frequency of CD8+ T cells than CD4+ at baseline observed in this study. This also re-enforces the role of CD8+ T cells in the control of human tuberculosis infection. Studies from mouse models indicate that the majority of *Mtb*-specific CD8 T cells are limited to either cytotoxicity or the secretion of gamma interferon (IFN-γ), with cytotoxicity being far more prevalent than IFN-γ secretion with memory response being less functional (Einarsdottir et al., 2009).

It has been demonstrated that Acid fast bacilli (AFB) counts fall by about 20-fold in the first 2 days and by a further 200-fold in the next 12 days to reduce the counts of an initially smear-positive patient to about 10^3 per ml at 2 weeks of short course chemotherapy (Jindani et al., 1980). These levels are below the estimates of $10^{3.5}$ to 10^4 per ml which are the limits indicating a change from smear-positive to smear-negative, culture-positive in untreated patients (Rouillon et al., 1976). To determine whether this change is characterized by an improvement in cellular response, the cytokine levels at baseline and two weeks of treatment were compared. There were marginal increases in median cytokine levels of IFN-γ, Granzyme B and IL-17 from baseline to week two signifying an improvement in cytokine response to therapy. However, only the increase in median Granzyme B response to Rv1733 was statistically significant (P=0. 013). This could be because the sample size was too small to show any differences or the levels of the six cytokines assessed, remain relatively unchanged or stable from baseline to week two of treatment. In the case of the former, the fact that there were

significant changes in IFN-γ positive T cell subsets in the same patient cohort might suggest that is not the case, while the latter cannot be determined as polyfunctional T cells producing two or more of the six cytokines were not assessed. In-spite of this general improvement in cellular responses, inter-individual variation was observed with 3 distinct patterns of *increased*, *decreased* or *fluctuating* levels of all cytokines. An association could not be established between "age'' and "sputum smear result at diagnosis" with cytokine response to antigenic stimulation. The variation could be best explained by host intrinsic factors beyond the scope of this study.

In contrast to other studies which reported higher frequencies of IFN-γ+ CD4+ Cells than CD8 T cells (Young et al., 2010) in response to ESAT-6/CFP-10 in active TB patients, we observed a lower CD4 T cell response at baseline which increased at week two. Such disparities could be related to the stage of TB disease and hence the bacterial load at the time of the experiment as in this present study, the frequency of IFNγ+ CD4 T cells increased and was higher than that of CD8 T cells by the second week of treatment when the bacterial load should have reduced significantly. Low levels of antigen- specific T cells have been reported in peripheral blood in active disease (Moresini et al., 2005) and it has been suggested that the low levels are due to migration of antigen specific T cells to the site of infection during acute stages of the disease. This sequestration of T cells at the site of infection has been reported for both CD4 and CD8 T cells (Caccamo et al., 2006; Dieli et al., 2000; Dilei et al., 1999) hence the relatively higher levels of IFN-γ+ CD8 T cells observed in this study pre-treatment could probably be attributed to a "compensatory'' increase arising out of a massive influx of antigen specific CD4 T cells which are the major producers of IFN-γ to the site of disease. While the proliferation of CD4 T cells producing IFN-γ could activate macrophages to fight against the early infection (Munk and Emoto, 1995), proliferation of CD8 T cells could promote bacterial schizolysis by secreting perforin, granulysin and extracellular enzymes. This cytotoxic function of CD8 T cells probably plays an important role during these early stages of disease as evidenced by the high quantities of Granzyme B, (a soluble mediator released by CD8 T cells) in addition to IFN-γ in the culture supernatant of active TB patients during the first 2 weeks of treatment.

The sub-study had some limitations, including the small sample size (n=20), and limited number of analytes tested for (6 cytokines). As applies in all biomarker discovery studies, regardless of the discovery platform used, the potential for reporting a significant finding which occurred by chance, given that 6 cytokines/soluble mediators were evaluated in 4 different *Mtb* infection phase-dependent antigen-stimulated supernatant is a risk. Sputum samples could not be taken at week 2 which would have allowed a direct comparison of the changes in the cytokine profile to smear status (bacterial load) at week 2 and perhaps would have helped to better explain the individual variation in response. We could also not compare the cytokine response profile of *Mtb* and *Maf*-infected individuals due to the rather low number (2/20) of *Maf*-infected patients in this subset. However, of the 2 *Maf* species included in the subset, IFN-γ secreted by M11 (MAF2) was very low (518pg/ml) in response to ESAT-6/CFP-10 compared to A11 (MAF1) (2260pg/ml). The mean IFN-γ response to ESAT-6/CFP-10 in this study was 1235.4 *(± 926.2)* at baseline (before TB treatment). This observation, although limited could suggest that MAF 2 infection does lead to reduced IFN-γ response to ESAT-6 as reported by de Jong et al, especially as all the five cytokines to ESAT-6/CFP-10 as well as cytokine responses to the other antigens were comparable between the two MAF 1 and MAF 2 cases. More cases of *Maf* would be needed to confirm this observation. Although in a similar study, a tendency towards increased IL-10 and TNF-α production was seen in TB cases infected with *Maf* (compared to *Mtb*) but this had no significant effect on the overall cytokine profile (Sutherland et al., 2010). It would have been interesting to compare the two as the latter study was done in the Gambia where there is a preponderance of MAF2 in contrast to MAF1 in Ghana.

These limitations notwithstanding, the results indicate that in addition to IFN-γ, multiple cytokines, including TNF-α, IL-17, sIL2Rα, IL-10 and soluble mediator grzB are expressed in PBMCs of TB patients in response to antigenic stimulation and that the cytokine profile reflects the immune status of the host and not the nature of the antigen. While the high levels of grzB was an interesting finding that lends credence to the view that in the early stages of TB disease,

cytotoxic activity is a critical part of TB control it warrants further investigation as quantity does not reflect function.

The results further show that effective chemotherapy improves cellular responses of TB patients to *Mtb*- stage specific antigens, as early as two weeks after therapy, however, most of the trends were not statistically significant. This could be due to the small sample size, however, changes in cytokine profile beyond week two, but before second month would need to be equally investigated for markers for early response. Cytokine levels vary considerably amongst individuals, so the range of values observed with the multiplexed assays was rather large. Using cytokine levels to monitor the efficacy of anti-TB treatment will be difficult due to this inter-individual variation. Therefore, studies in large populations of TB patients are required to identify the factors that determine variation in cytokine responses before cytokine based prediction scales are used for clinical management of TB. Future longitudinal studies with larger sample size to identify biomarkers should in addition to IFN-γ and TNF-α include grzB to investigate the functional significance of the high levels observed during the first two weeks.

CHAPTER FOUR

Longitudinal changes in IFN-γ expression in *Mtb* antigen-specific T cell subsets obtained from active pulmonary TB patients undergoing treatment

4.1 Background

Host defense against TB is T-cell-mediated, and among the T lymphocytes the CD4+ T-lymphocyte is undoubtedly the major effector cell (Boom, 1996). The role of CD4 T-cells in protection against *Mtb* is well documented, however, evidence from various studies using human and animal models suggests an involvement of CD8 T-cells (Flynn et al., 1992; Stenger et al., 1997). CD8 T cells are thought to contribute to the control of *Mtb* infection by mediating specific effector functions, including IFN-γ and TNF-α production upon recognition of mycobacterial antigens (Kaufman et al., 2005; Flynn et al., 2001), lysis of infected host cells, and direct killing of mycobacteria (Stenger et al., 1997, Ottenhoff et al., 2008). Consistent with the hypothesis that CD8 T lymphocytes are constantly being stimulated with antigen, CD8 T-cells specific for numerous mycobacterial antigens can be isolated at high frequency from human and mouse models (Lalvani et al., 1998; Ottenhoff et al., 2000).

Various studies have highlighted the immunologic and clinical relevance of measuring T cell response to TB infection. The development and introduction of the IGRA's (Interferon gamma release assays) into clinical practice for diagnosis (Pai et al., 2008) have also resulted in an explosion of studies aimed at using IFN-γ production for monitoring of tuberculosis infection. Conflicting results have been reported by these studies with some reporting decreasing or negative responses (Aiken et al., 2006; Dheda et al., 2007) and increased or persistently positive responses (Ferrand et al., 2005; Pai et al., 2007) after treatment. These differences have been attributed to assay characteristics, antigen load at different stage of the disease and the functional diversity of T-cell response (Sauzollo et al., 2009). Determination of the T cell cytokine profile at specific stages of infection, disease and recovery would aid in the

identification of specific markers that could serve as an end point in clinical trials for the development of new diagnostics and vaccines. Despite the conflicting results, the common denominator for all these IGRA based assays for monitoring TB treatment response, is the use of short-term incubation assays. Short-term stimulation (18-24hrs) essentially detects responses of activated effector/effector memory T cells that rapidly release IFN-γ when stimulated in vitro with antigen (Leyton et al., 2006). On the other hand, studies investigating antigenic responses after long -term stimulation are lacking. Longer period of in vitro stimulation by contrast detects the effector functions of long-lived central memory T cells, which may be less likely to release IFN-γ during the short period of exposure to antigens in the IGRA assay (Ketch et al., 2002). Thus the dynamics of central memory response during anti TB treatment have not been properly investigated and may be a better tool for monitoring of TB treatment response.

4.2 Experimental Design

4.2.1 Sample selection

Cryo-preserved PBMC of culture positive and HIV negative patients from the study cohort, which met the selection criteria were identified. PBMC samples of patients who were lost to follow up (**Figure 4.1**) were excluded and only samples of patients who honored all four study time points were eligible. In addition, selected samples included only those of patients whose infecting strain of Mycobacterium had been genotyped as *M. tuberculosis* or *M. africanum.* Based on this criteria, PBMC of 38 participants, known to be infected with *M. tuberculosis* were selected in addition to all PBMC of all 10 patients infected with *M. africanum*.

4.2.2 Thawing of cryopreserved PBMC

The vials of PBMC to be used were transferred from the nitrogen vessel and put in a transport vessel filled with liquid nitrogen or dry ice. For each vial to be thawed, a 15 ml tube containing 2 ml of RPMI 10% FCS was prepared. The vials were placed in a water bath of 37°C degrees and removed before the last clump of cells had thawed. The outside of the tube was thoroughly wiped clean with alcohol and 1ml of the thawing medium added drop-wise while gently shaking the tube. The contents of the tube were then transferred into the 15ml tubes and topped up

with R10 (RPMI, 10%FCS) to the 14 ml mark. The tubes were centrifuged for 7 minutes at 1400 RPM (439g), the supernatant was discarded and the pellet re-suspended in 1 ml of sterile filtered growth medium for cell counting. The cells were then made up to the concentration needed for the particular assay using growth medium and placed in the appropriate wells of an already labelled culture plate. All cryopreserved cells were rested for 2 hours at 37^0C, in the CO_2 incubator prior to the addition of antigens. All protocols from stimulation till intracellular flow cytometry followed procedures described in Chapter 3.

4.2.3 Assay characteristics

For the longitudinal study, CD4+ and CD8+ T cell expression of IFN-γ in response to ESAT-6/CFP-10 fusion protein and the most recognized of the three DosR proteins, Rv1733 was evaluated to determine T cell specific dynamics during treatment. To minimize experimental bias, for each selected sample cryo-preserved PBMC for all 4 time-points were thawed and cultured the same day under similar conditions.

For the evaluation of *M. africanum* response to ESAT-6/CFP-10 fusion protein, frequency of IFN-γ+ CD4 and CD8 T cell responses to ESAT-6/CFP-10 was compared at baseline between the *M. africanum* and Mtb samples (matched for age and sex).

4.2.4 Data analysis

Data was entered into Microsoft Excel 2007 (Microsoft Corp, USA) and analyzed using PRISM software version 4.0 (GraphPad prism software Inc., California, USA). Differences in the percentage of IFN-γ+ CD4/CD8+ T cells to antigenic stimulation were analyzed using the non-parametric Mann-Whitney U-test. Data in the longitudinal analysis during the treatment course of individual patients were evaluated with the non parametric Wilcoxon signed-rank test (two tailed). P–values of less than 0.05 were regarded as significant. Samples were excluded from analysis as a result of culture contamination or negative SEB result.

4.3 Results

4.3.1 The Study Profile

Of the 104 TB patients recruited, blood samples were collected from all at baseline, 2 weeks, 2 months after initiation of TB treatment and 6 months upon treatment completion for in-vitro assays. Due to losses to follow up, 95 participants were available for the week two, 93 for month two and 81 for month six. In all, 23 (22%) of participants recruited were lost to follow up for reasons described in **Figure 4.1**. Based on the selection criteria for the longitudinal study, 38 samples were selected. After eliminating samples due to culture contamination or negative SEB response, 21 out of 38 samples were included in the longitudinal study analysis. The 21 samples were from 13 males and 8 females with mean age of 32.7 years.

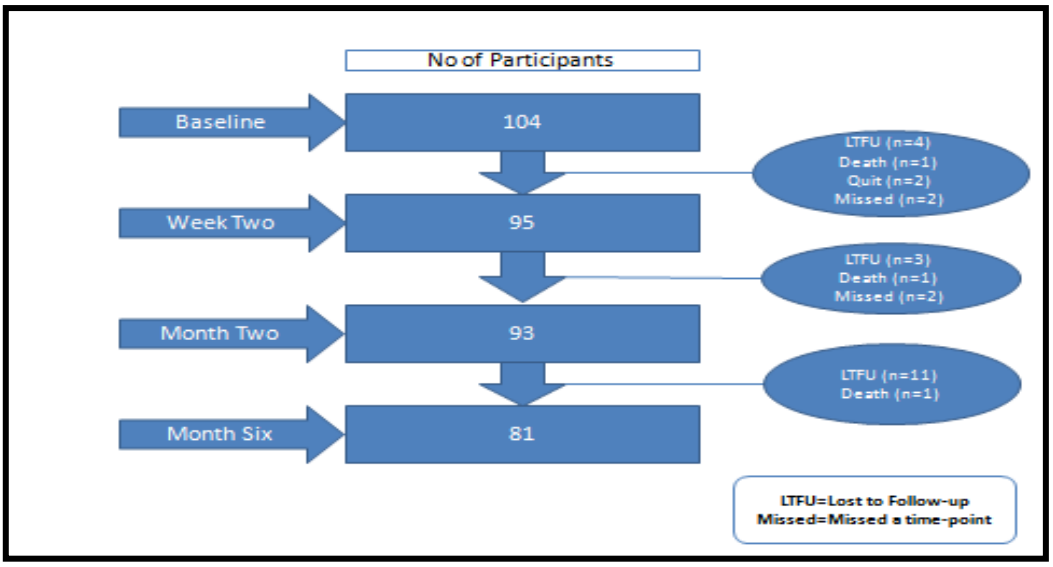

Figure 4.1: Study Profile shows losses to follow-up at different time points

Of the 104 TB patients recruited into the study, 81 were available for all the four time points for blood draw. A total of 20 were lost to follow up in the course of the study for various reasons, while 3 were as a result of death.

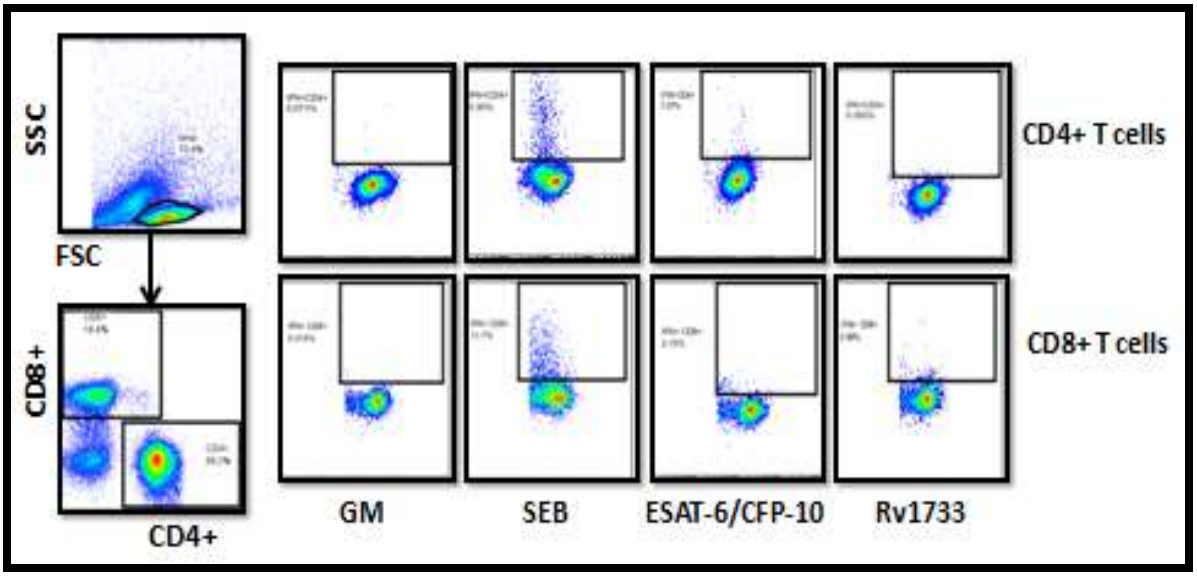

Figure 4.2: Representative sample showing IFN-γ secretion in un-stimulated (GM), SEB (positive control), ESAT-6/CFP-10 and Rv1733.

Intracellular IFN-γ staining of PBMC from TB patients during the course of anti-TB therapy. Cyropreserved PBMC were thawed, rested for 2 hours and then stimulated for 6 days with antigens (indicated), BFA was added on the 5th day overnight. Cells were harvested and fixed in paraformaldehyde, stained for CD4 and CD8, permeabilized, and stained for IFN-γ. Cells were gated on lymphocytes by forward and side scatter and analyzed by three-color FACS®. Cells were analyzed from each stimulant, and dot plots from a representative sample are shown. Percentages of gated cells are indicated.

4.3.2 Kinetics of IFN-γ+ T cell subset response to ESAT-6/CFP-10 fusion protein and Rv1733

At baseline, T cells from all 21 patients expressed IFN-γ in response to ESAT-6/CFP-10 but this reduced substantially in CD8+ T cells by completion of treatment **(Table 4.1)**. A similar reduction was observed in IFN-γ expression of both T cell subsets in response to Rv1733.

Table 4.1: Longitudinal assessment of Positive T cell responses to ESAT-6/CFP-10 and Rv1733 during anti TB therapy

	%IFN-γ+ CD4 T cells			%IFN-γ+ CD8 T cells		
[n/N]	**SEB [1] (%)**	**ESAT-6/CFP-10[1] (%)**	**Rv1733[1] (%)**	**SEB [1] (%)**	**ESAT-6/CFP-10[1] (%)**	**Rv1733[1] (%)**
T0	21/21 (100)	21/21 (100)	8/10 (80)	21/21 (100)	21/21 (100)	9/10 (90)
T1	21/21 (100)	21/21 (100)	9/15 (60)	21/21 (100)	21/21 (100)	11/14 (79)
T2	21/21 (100)	21/21 (100)	7/13 (54)	21/21 (100)	15/21 (71.4)	7/13 (54)
T3	21/21 (100)	21/21 (100)	6/11 (55)	21/21 (100)	11/21 (52.4)	2/11 (18)

Samples were assessed at four time points: T0 (baseline), T1 (two weeks of treatment), T2 (two months of treatment) and T3 (month 6).

[1]Number of participants (PBMC) with more than 0.2% IFN-γ CD4/CD8 T cells in response to 6 days of antigenic stimulation.

Also, as reported in the earlier experiment using freshly isolated PBMCs **(3.5.3),** the median frequency of IFN-γ + CD8+ T cells was higher (not significant) than that in CD4 T cells at baseline (T0) **(Figure 3.4)**

At week 2 (T1), there was a significant increase in the median frequency of IFN-γ+ CD4 T cells in response to ESAT-6/CFP-10 (from 1.3 to 2.58 %; $P<0.01$) and an insignificant decrease in the median frequency of IFN-γ+ CD8 T cells. There was no significant difference in the median frequency of IFN-γ+ CD4/CD8 T cells in response to Rv1733 from baseline (T0) to week Two (T1).

By month, two (T2), there was a highly significant increase (from 1.3 to 3.1%; P<0.001) in the median frequency of IFN-γ+ CD4 T cells and decrease in CD8 T cells (from 1.85 to 0.485 % P<0.05) in response to ESAT-6/CFP-10, compared to baseline. However, in response to Rv1733 there were no significant changes.

From Month 2 (T2) to end of treatment or month 6 (T3), there was an insignificant increase in the median frequency of IFN-γ+ CD4 T cells as well as an insignificant decrease in CD8 T cells. Compared to baseline values, the IFN-γ expression in CD4 T cells was significantly increased at the end of TB treatment (from 1.3 to 3.45 %; P<0.001) while that of CD8 T cells decreased (from 1.85 to 0.277 %; P<0.001).

Taken together, the data show that in response to ESAT-6/CFP-10 there is an increase in the median frequency of IFN-γ+ CD4 T cells by week two (T1) of treatment in comparison to the pre-treatment frequency with a further increase at month two (T2) which continues till the end of treatment (T3). On the contrary, there is a decline in the median frequency of IFN-γ+ CD8 T cells at week 2 compared to pre-treatment frequencies and this decline continued through to month 2. By the end of treatment there is a marked decrease in the median frequency of IFN-γ+ CD8 T cells compared to baseline values.

In response to latency associated Rv1733, there were no significant changes in median frequencies of IFN-γ+ CD4 or CD8 T cells at any time point, but there was a trend of a decline from pre-treatment frequencies to week 2, followed by an increased frequency by month two for both T cell subsets. At the end of treatment (month 6) there was a decline in the frequency of Rv1733-specific IFN-γ+ CD4 T cells in contrast to Rv1733-specific IFN-γ+ CD8 T cells which increased at the end of treatment. The longitudinal changes in frequencies of ESAT-6/CFP-10 and Rv1733 specific IFN-γ+ responses for each T cell subset is shown in **(Figure 4.3**) for patients with all four time points available.

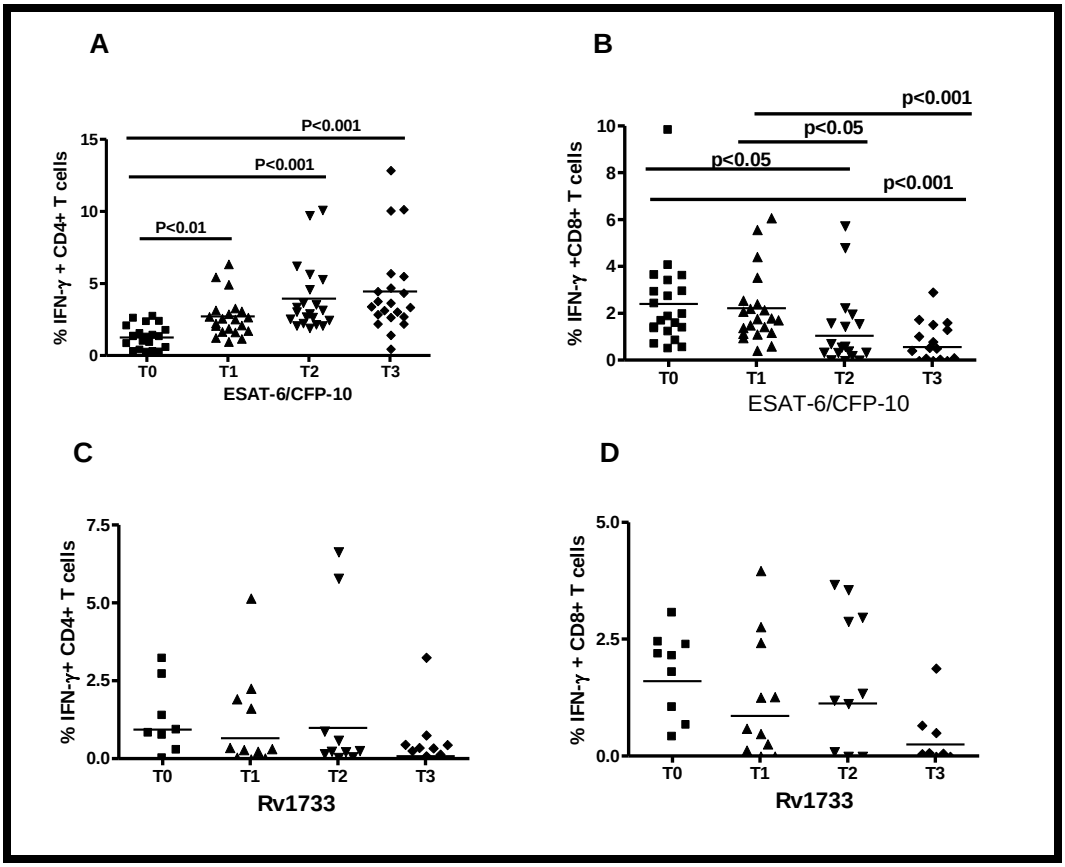

Figure 4.3: Longitudinal changes in frequencies of IFN-γ + CD4 and CD8 T cells in response to antigenic stimulation.

Cryo-preserved PBMC from culture-positive (genotyped as *M. tuberculosis*) TB patients were stimulated with ESAT-6/CFP-10 and Rv1733 for 6 days. Cells were analyzed by Flow cytometry for intracellular expression of IFN-γ in CD4+ and CD8+ T cells by subtracting the percentage in un-stimulated cultures from stimulated ones. A threshold of 0.2% IFN-γ+ CD4/CD8 T cells defined positive T-cell responses against antigens ESAT-6/CFP-10 (n=25) and Rv1733 (n=15). Samples with a negative SEB response were excluded, but values below this threshold were converted to zero for plotting. Bar indicates the mean at each time point: T0 (baseline,) T1 (two weeks into treatment), T2 (2 months into treatment) and T3 (month 6/treatment completion). Data were analyzed using a Kruskal-Wallis ANOVA followed by Dunn's post-test comparison and p-values indicated.

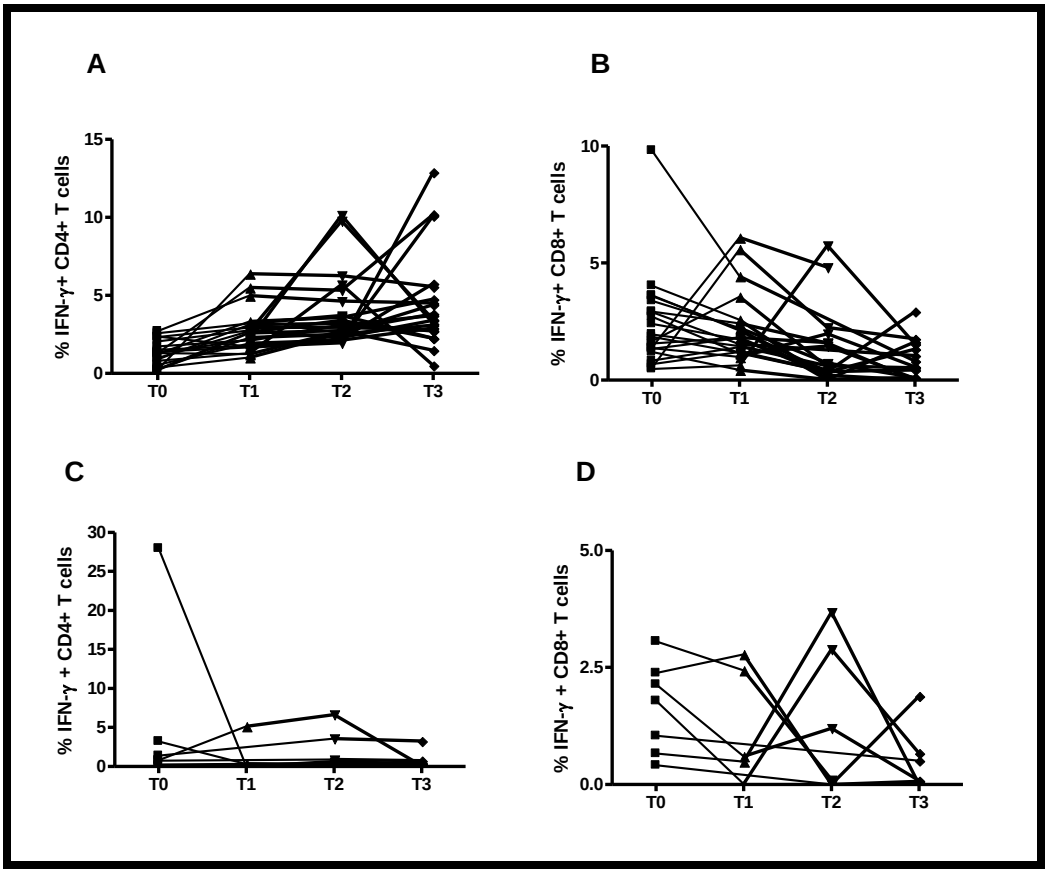

Figure 4.4: Kinetics of IFN-γ expression in CD4+ and CD8+ T cells for individual patients undergoing TB treatment

A longitudinal analysis of **IFN-γ** expression by CD4+ and CD8+ T cells at four time points T0 (pre-treatment), T1 (week 2 of treatment), T2 (month two of treatment) and T3 (end of treatment) was done including only patients with all four time points available for response to ESAT-6/CFP-10 (n=20) and Rv1733 (n=9).

4.3.3 Longitudinal changes in cytokine secretion profile in a subset of patients

The median cytokine secretion of the 6 cytokines at four time points (baseline, week two, month two and six months) was determined for the first five (5) patients to complete treatment with PBMC for all time points available. One way ANOVA was used to compare the median cytokine secretion upon stimulation with ESAT-6/CFP-10 and Rv1733 at each time point. A post test to identify statistically significant ($P<0.05$) means was done was the Kruskal-Wallis test. Cytokine secretion (pg/ml) was calculated by subtracting the values of the un-stimulated cultures from the stimulated cultures.

There was a general increase in all cytokine levels upon treatment completion compared to baseline values in response to both ESAT-6/CFP-10 fusion protein and Rv1733 **(Fig 4.6).** However, only the increase in IL-10 levels from baseline to month 2 and to treatment completion (month 6) in response to ESAT-6/CFP-10 were statistically significant ($P<0.01$).

IFN-γ secretion increased steadily in response to both ESAT-6/CFP-10 and Rv1733 from baseline to treatment completion (T0 to T3). In the former, there was a two-fold increase from baseline to week 2 and a four-fold increase by month 6 (treatment completion). Granzyme B, sIL-2Rα and IL-10 levels decreased at month 2 after an increase at week two before increasing again upon treatment completion.

TNF-α levels were dramatically low from baseline to month 2 before finally increasing upon treatment completion.

However, there was again wide individual variation in responses in response to both ESAT-6/CFP-10 (shown in **Fig 4.7**) and to Rv1733 (not shown). Due to the rather low number of samples these results would have to be confirmed in a larger cohort.

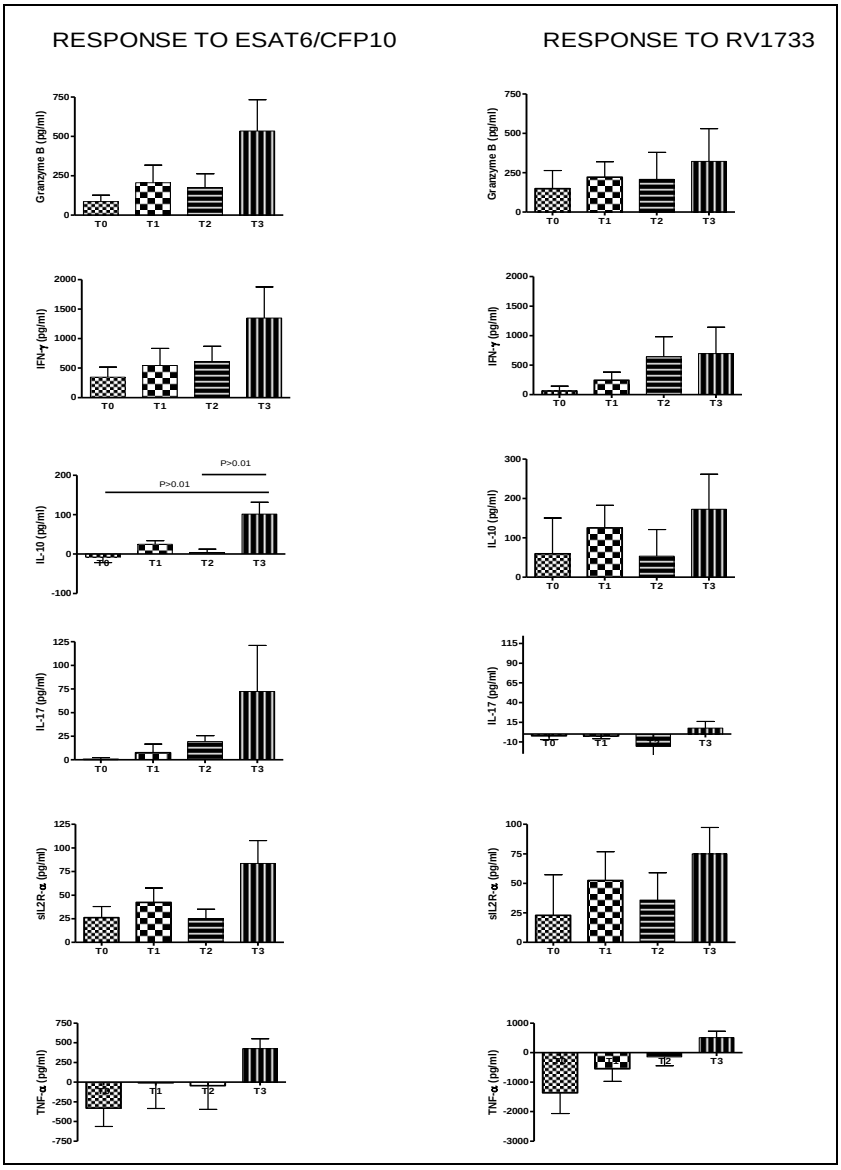

Figure 4.5: Changes in the levels of secreted cytokines to ESAT-6/CFP-10 and Rv1733 in a small subset of TB patients (n=5) from baseline till treatment completion

PBMC of the first 5 patients to complete TB treatment were stimulated with ESAT-6/CFP-10 and Rv1733 for 6 days and supernatant assayed for the six analytes (IFN-γ, Granzyme B, IL-10, IL-17, sIL2r-α, TNF-α). Cytokine secretion (pg/ml) was calculated by subtracting the values of the un-stimulated cultures from the stimulated cultures. Median cytokine levels were compared at four time points (baseline, 2 weeks on treatment, 2 months on treatment and six months) using one way ANOVA. A post test to identify statistically significant ($P<0.05$) means was done was the Kruskal-Wallis test.

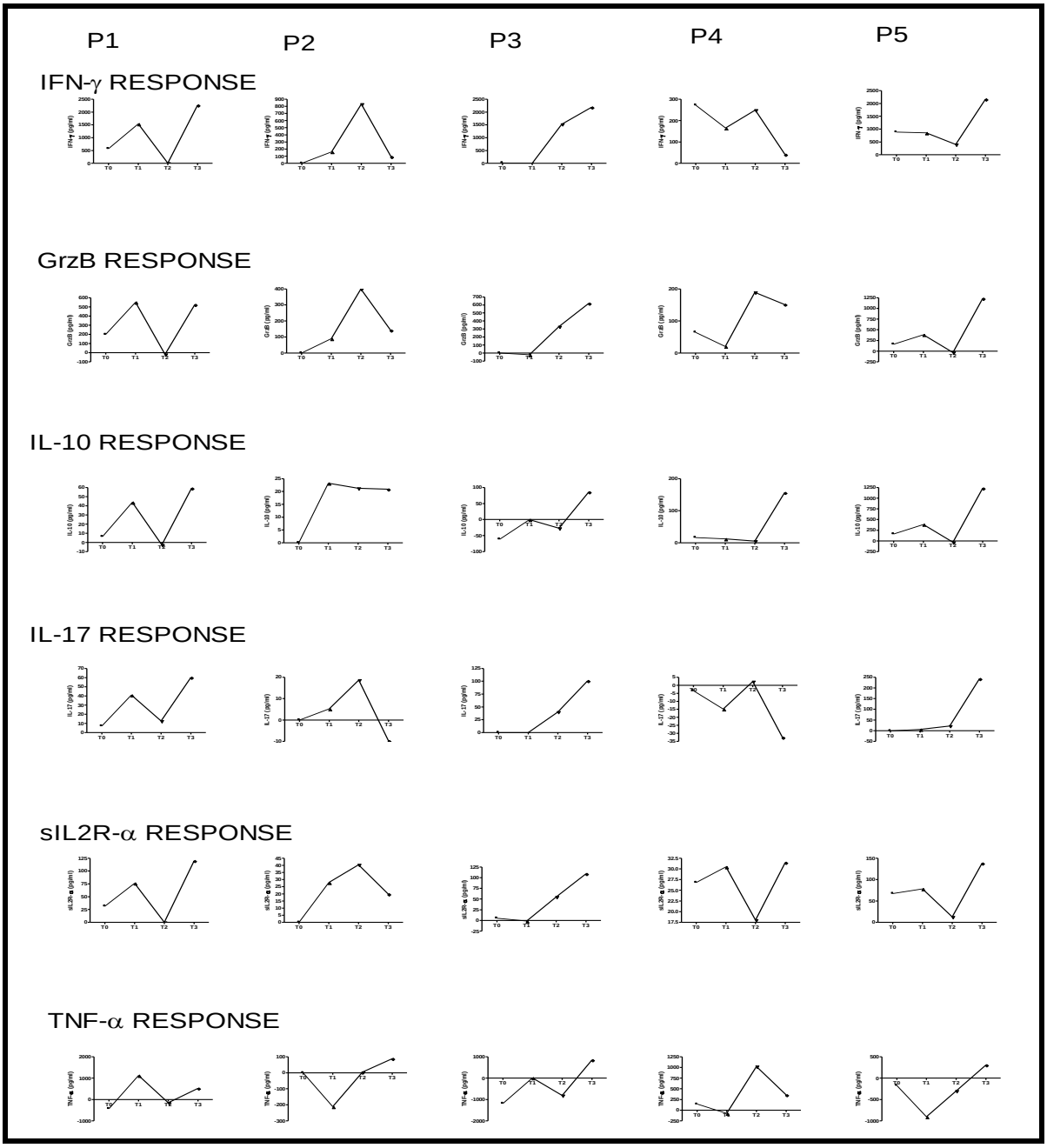

Figure 4.6: Cytokine dynamics per patient over the course of TB treatment

Shown are IFN-γ, Granzyme B, IL-10, IL-17, sIL2r-α, TNF-α secretion (pg/ml) in response to ESAT-6/CFP-10 at four time points; baseline (T0), week 2 (T1), month 2 (T3) and 6 months (T4) for Five (5) smear positive TB patients (P1, P2, P3, P4, P5) with all time points available.

4.3.4 Frequency of IFN-γ+ T cell responses to ESAT-6/CFP-10 in *M. africanum* and *M. tuberculosis* infected subjects

Studies conducted mostly in Gambia, West Africa, where there is a preponderance (60%) of *M. africanum* subspecies 2 (MAF2), and no subspecies 1 (MAF1), have reported an attenuated IFN-γ response to ESAT-6/CFP-10 in Maf- infected TB patients (de Jong et al., 2010). Studies in Ghana have on the other hand reported a Maf prevalence of 20 -30% with only about 9% being MAF2. In this study, of the 10 participants infected with Maf, only one was genotyped as MAF2, with the 9 being MAF1. To determine whether there is a similar attenuated response to ESAT-6/CFP-10 in our predominantly MAF 1 population, we compared ESAT-6/CFP-10 responses at Baseline in Maf patients (n=10) to that of the *Mtb* (n=10) population matched for age and sex. There was no difference in frequencies of IFN-γ+ CD4 or CD8 T cells between Maf and *Mtb* subjects in response to ESAT-6/CFP-10 **(Figure 4.8).**

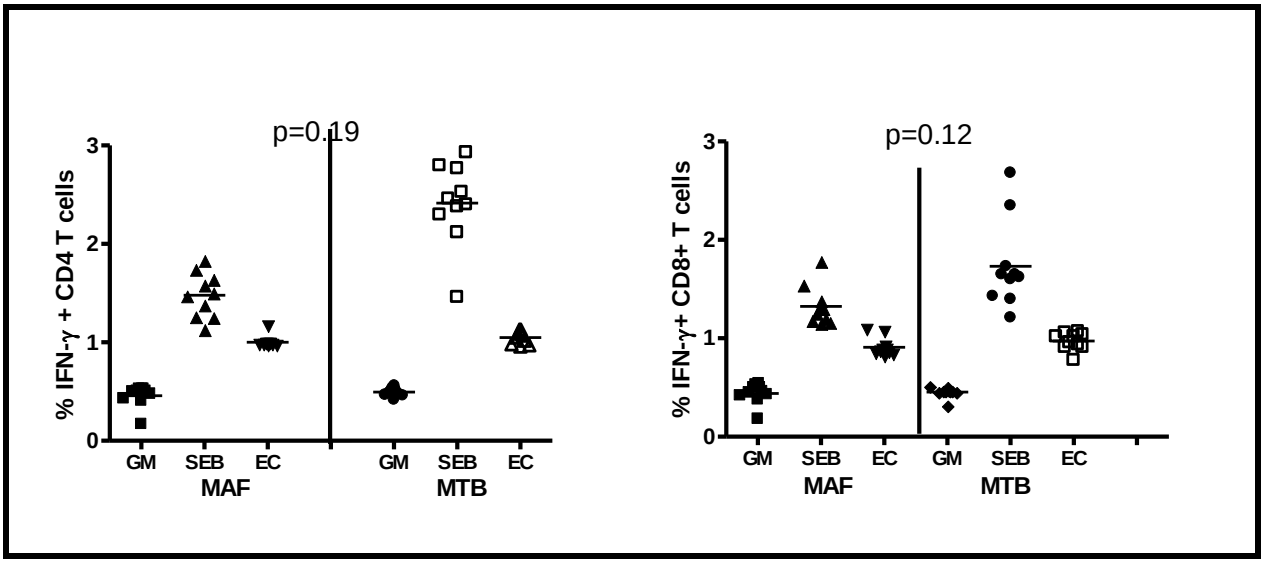

Figure 1Figure 4.7: No difference in frequency of IFN-γ+ CD4 or CD8 T cells in response to ESAT-6/CFP-10 fusion protein between Maf and Mtb- infected subjects.

The frequency of IFN-γ+ CD4+ and CD8+ T cells in response to ESAT-6/CFP-10 (EC) was compared using the Mann-Whitney U test between the 10 Maf subjects and 10 *Mtb*- infected subjects matched by age (34.4 verse 35.13) and sex (M (7): F (3). Y axes show the percentage IFN-γ + per each cell population and the X axes shows the *Mtb* strain. Also shown are response to growth medium/negative control (GM) and positive control/*Stapyloccucus enterotoxin* B (SEB).

4.4 Discussion

Longitudinal changes in CD4/CD8 T cell subset after long term stimulation with *Mtb*- specific antigens

The critical role CD4 T cells play in controlling TB infection is demonstrated by the uncontrolled mycobacterial growth seen in transgenic mouse strains unable to mount CD4 T cell responses or Th1 immune responses (Mogues et al., 2001; Carusso et al., 1998; Copper et al., 1997; Ladel et al., 1995). Both CD4 and CD8 T cells are thought to contribute to protection against TB (Hoang et al., 2009). We earlier evaluated the functional response of antigen- specific T cells by comparing the frequencies of IFN-γ+ CD4 and CD8 T cells upon exposure to ESAT-6/CFP-10, Rv1733, Rv2029 and Rv2628 antigens in freshly isolated PBMC of the first 20 participants recruited at baseline and week two of treatment. Of the three DosR proteins, the T cell response to Rv1733 in terms of frequency of IFN-γ+ CD4 cells were higher compared to Rv2029 and Rv2628.

To determine longitudinal changes in CD4 and CD8 T cell responses to *Mtb*-specific antigens, cyro-preserved cells from four time points (baseline, week two, month two, month six) were thawed and cultured under similar conditions as the fresh cells using ESAT-6/CFP-10 and Rv1733.

As previously observed, before treatment, the T cell profile consisted of more IFN-γ+ CD8 T cells than CD4 T cells and this trend was reversed during the second week with more IFN-γ+ CD4 than CD8 T cells.

Aside sequestration, other factors that have been implicated in decreased numbers of antigen specific T cells in the periphery include, T cell exhaustion or aberrant immune regulation during disease. During active disease aberrant immune regulation, mediated by regulatory T cells (Schuck et al., 2009), anti-inflammatory cytokines such as IL-10 and TGF-β (Hirsch et al., 1999) and Th2 cytokines like IL-4 and IL-13 may be up-regulated in chronic infections such as HIV and CMV. Such persistently high antigenic load drives specific T cell exhaustion and dysfunction (Barber et al., 2006; Day et al., 2006). These cells up-regulate markers like PD-1, and are more

prone to apoptosis (van Grevenynghe et al., 2008). A recent observation that T cells from patients with TB disease were strikingly less likely to survive in a 6-day culture, compared with T cells from persons with LTBI, suggests that T cell exhaustion may also be responsible for lower responses in TB patients at baseline. It was found that PD-1 expression is increased on *Mtb*-specific CD4+ T cells in TB diseased patients, compared to persons with LTBI (C. Day, unpublished observations). Essentially, T cell exhaustion leads to a loss of function in particular for antigens like ESAT-6/CFP-10 that predominate during the early stages of infection, and where continued exposure may lead to exhaustion (Barber et al., 2006). We could speculate that ESAT-6/CFP-10 - specific CD4 T cells are recruited earlier during the immune response to TB and may have gotten exhausted during the course of infection leaving the CD8+ T cells to predominate. An alternative explanation could be that because CD8 T cells are restricted to the recognition of antigens from the intracellular environment, they are better predictors of bacterial load, hence their abundance during the acute phase of infection when the bacterial load is high.

The subsequent increase in the frequency of IFN-γ+ CD4 cells in peripheral blood during week two could signal the end of the cytotoxic activity as it was also accompanied by a decline in the frequency of IFN-γ+ CD8 T cells. It is largely believed that most of the actively replicating bacteria are eliminated during the first 2 weeks of TB treatment and that follow up treatment is targeted at the persistent and the latent foci.

Interestingly, during the second month follow up, there was a further increase in median frequency of IFN-γ+ CD4 T cells and a decline in that of CD8 T cells. This is in contrast to many studies that have reported a decline in IFN-γ expression in CD T cells at month two treatment. This decline has been attributed to bacterial clearance at month 2. It has been suggested that a higher IFN-γ response at 2 months could be an independent indicator of the likelihood of remaining sputum culture-positive at the end of the intensive phase of anti-tuberculosis treatment (Katiyah et al., 2008), similarly a delayed drop in TB IFN-γ release could be an indicator of adverse outcome and poor response to treatment (Carrara et al., 2004). However, the majority of these studies reporting a dip in IFN-γ expression during the second month have

been based on the use of IGRA's which uses short term incubation. Short term incubation is thought to detect effector memory responses, which are expected to decline as bacterial load decreases with effective chemotherapy (Lalvani, 2004).

In contrast, this present study employed long term stimulation, which is thought to detect effector responses of central memory T cells (Sprent et al., 2002; Ketch et al., 2002). Central memory T cells are long-lived, even in the absence of persistent antigen, and home to secondary lymphoid organs, require long term stimulation assays and signify previous immunological sensitization to the pathogen (Walzl et al., 2011). In several infection models including TB, it has been shown that effector T-cells are expanded during active replication, whereas only memory cells are detectable after control or eradication (Butera et al., 2009; Jafari et al., 2009; Jafari et al., 2011). However, data on characterization of the memory phenotype of *Mtb*-specific cells during active disease in response to *Mtb*-specific antigens is limited. Measurements of memory T cell subpopulations and other biomarkers for pathogen persistence have so far not been adequately investigated for their ability to predict treatment outcome, and the field relies on clinical evidence of mycobacterial activity (Walzl et al., 2011). This study represents one of a few to monitor the response of memory cells to *Mtb*-specific antigens during chemotherapy using long term stimulation ostensibly to capture central memory responses.

The increased frequency of IFN-γ+ CD4 T cells at month 2 which continued through to month 6 could indicate that as treatment progress and bacterial load decreases, the frequency of CD4 specific- ESAT-6/CFP-10 memory responses increases. Based on the 0.2% cut-off for positive responders to antigen, in response to ESAT-6/CFP-10 all participants had positive responses throughout treatment, but with varying frequencies of IFN-γ+ CD4/CD8 T cells. All patients in this subset were successfully treated and deemed clinically cured of TB. Clinical cure is characterized by negative bacteriological examination for *Mtb* and by resolution or improvement of symptoms. These results indicate that immunologically, clinical cure is associated with higher frequency of ESAT-6/CFP-10-specific memory IFN-γ+ CD4 T cells and low

frequencies of IFN-γ+ CD8 T cells. This profile could be behind the improvement in cellular responses observed.

Two reasons have been adduced for the cellular improvement with anti-TB therapy, with regards to CD4 T cell responses; firstly an increase in the number of peripheral CD4 T cells that produce IFN-γ, owing to the fact that CD4 T cells responding to a vast array of *Mtb* epitopes are sequestered or compartmentalized at the site of the disease, and appear in the peripheral blood after effective chemotherapy, thus reversing the state of anergy seen in TB patients prior to therapy (Wilkenson et al., 1998; Dieli et al., 1999). Secondly, a shift in cytokine production by PBMC, from cytokines that down regulates the activation of Th1 cells and their cytokines such as IL-10 have been implicated. The levels of these regulatory cytokines are high in active TB patients and decrease upon treatment with anti-TB drugs (Hirsh et al., 1999; Garcia et al., 2002).

The decline of IFN-γ+ CD8 T cells following successful TB treatment suggests that frequencies of IFN-γ+ CD8 T cells during treatment will be useful as a surrogate marker of treatment response. In addition, unlike the conflicting results with regards to CD4 T cell responses during treatment which has been attributed to assay characteristics, recent studies using ELISPOT (Nyendak et al., 2013) and intracellular cytokine staining (Day et al., 2011) have also reported a decline in IFN-γ+ CD8 T cells following successful TB treatment, indicating consistency across different assays. Given that CD8 T cells have a high affinity for cells heavily infected with *Mtb* (Lewinsohn et al., 2006) and the finding that young children with TB exhibit a strong IFN-γ+ CD8 T cell response to TB antigens (Lancioni et al., 2012), similar findings in our adult cohort is not surprising.

There were no significant differences in the frequency of IFN-γ+ CD4 and CD8 T cells in response to Rv1733. There was, however, a trend towards increased frequency of Rv1733 specific-IFN-γ+ CD4 and CD8 at week two which declined at month two in both subsets. At the end of treatment there was a trend of increased frequency in Rv1733 specific -CD8 T cells and a decline in Rv1733 specific-CD4 T cells.

In general, the presence or absence of a specific memory response at the end of treatment on its own may not be indicative of long lasting protection in all individuals. Active tuberculosis can recur, either through re-infection with a new bacterial strain (in patients whose TB treatment resulted in sterilizing cure) or through relapse with the original bacterial strain (in patients whose infection returned to a quiescent phase after treatment (Walzl et al., 2010).

In the search for a TB vaccine, the potential candidate antigen is expected to evoke a high frequency of antigen specific cytokines after a long period of incubation, indicative of a central memory response. The limitation of this sub-study is that we did not use any specific markers that have been associated with central memory responses. We cannot therefore be categorical that the responses we have measured are truly central memory responses even though effector cells are less likely to survive in 6 day stimulation assays. Future studies employing such memory markers would give a more definitive insight into the nature of these CD4 and CD8 T cells persisting in 6 day cultures.

Longitudinal assessment of cytokine profile from baseline to treatment completion

Compared to baseline values, there was an increased concentration of all cytokines at two weeks of treatment. However, in the exploratory cohort (n=5), assessed at longitudinally at four time points, it was observed that all cytokine levels depressed at month two (intensive phase) after the increase during week 2, before increasing again at month 6 upon treatment completion. The only exception was IFN-γ and TNF-α. The decrease in cytokine levels at month two may correlate with the resolution of inflammation after the bacterial load is reduced. However, in the case of IFN-γ and TNF-α, the steady increase from week 2 to 6 months (treatment completion) after low baseline levels could be due to sequestration at the site of infection at the early phase of the disease (Schwander et al., 1998) leading to a reduction in peripheral blood.

Specifically the increased levels of pro-inflammatory cytokines during week two may reflect the contraction of *Mtb*-specific pools as the bacterial load drops and tissue inflammation resolves leading to infiltration of the two into the periphery. Low IFN-γ induction in PBMC of TB patients

prior to treatment may also be the result of a chronic depletion of antigen responsive T cells as suggested by studies of Hirsch and others (Hirsch et al., 1999; Hircsh et al., 2001). Also Sahiratmadja et al., 2007 evaluated the cytokine profiles for 93 TB patients before and after curative treatment and found that IFN-γ was strongly depressed in patients with active TB before treatment but increased after treatment. However, in a similar study by Su et al, there were elevated IFN-γ levels pre-treatment in TB patients compared to controls and a significant decline in IFN-γ levels after the intensive 2-month anti-TB therapy, the latter of which was also observed in this four time point cohort. It appears that generally IFN-γ levels are low pre-treatment and high post treatment, but between these two time periods the levels fluctuate. In the case of TNF-α although there was a steady increase from baseline to end of treatment, the background was so high that most of the values for the first three time points were negative after subtracting it from the stimulated cultures. Previous studies have observed high background level of TNF-α in un-stimulated samples from patients whether infected with *Mtb* or not (Lighter-Fisher et al., 2010; Chegou et al., 2009) and this could explain the high background values observed.

ESAT-6/CFP-10 -specific IFN-γ response to *M. africanum*

So far no study has investigated the immune response of *M. africanum* 1 (MAF1) infected patients to ESAT-6, so we compared the frequency of ESAT-6/CFP-10- specific IFNγ+ CD4 and CD8 T cell responses in our MAF1 cohort with that of an *Mtb* cohort (matched for age and sex) but found no significant difference between the two **(Figure 4.7).**

The epidemiological and clinical differences between *M. tuberculosis* (*Mtb*) and *M. africanum* (*Maf*) are still unraveling, however the observation that individuals infected with *Maf* have a reduced response to ESAT-6 (de Jong et al., 2006) compared to *Mtb*-infected individuals generated a lot of interest, especially because ESAT-6 is one of the major antigens in the IGRA, currently in use for TB diagnosis (Pai et al., 2006). The implication was that for most individuals,

particularly in West Africa, infected with *Maf*, diagnosis using the IGRA"s would yield inaccurate results. Even more worrying was the fact that ESAT-6 is also a potential vaccine candidate.

The two subspecies of *Maf*; MAF1 and MAF2 are predominantly found around the Gulf of Guinea and Western parts of West Africa respectively. The study, which reported the reduced ESAT-6 responses was conducted in the Gambia with Gambian patients so the reduced response was with respect to MAF2 as MAF1 is not prevalent in the Gambia.

Given the phylogenetic difference between MAF1 and MAF2; MAF1 is closer to *Mtb* whilst MAF2 is closer to *M. bovis* (Gagneux et al., 2007; Brosch et al., 2002*)* we could hypothesize that T cell response to MAF1 and *Mtb* are similar. Another explanation for our finding could be due to the fact that we used a fusion protein comprising of ESAT-6 and CFP-10 which could have masked the effect that using ESAT-6 alone (as was used in the former study) would have had. Co-secreted, these two most immunodominant proteins of the RD1 region of *Mtb* genome are thought to induce stronger responses together compared to individually. However a recent study has reported that, contrary to the lower ESAT-6 responses based on ELISPOT result, T cell responses were no different between mice experimentally infected with *Maf* and *Mtb* (Bold et al., 2012). The mouse study in question used MAF2 strains which implies that in terms of T cell response, MAF2 is similar to *Mtb*. Factors such as in vivo attenuation of *Maf* compared to *Mtb*, difference in ESAT-6 secretion and mutation in the Rv3879c gene in *Maf* have been investigated in an attempt to explain the lower ELISPOT response found in MAF2 infected compared to *Mtb* infected individuals, but none of these could successfully explain this finding (Bold et al., 2012). The reduced ELISPOT responses could thus only be attributable to variation in host response, especially as not all the *Maf*-infected individuals exhibited such reduced responses (de Jong et al., 2006).

In reality, until we can discover antigens that can distinguish between *Maf* and *Mtb* in latently infected individuals, it will be difficult to fully characterize differences in the immune response to *Maf* and *Mtb* in LTBI. In the de Jong et al., 2006 study, latently infected individuals were characterized as *Mtb* or *Maf*- infected only on the basis that those individuals were household

contacts of active TB patients infected with *Mtb* or *Maf* respectively. While we can distinguish between *Maf* and *Mtb* active TB patients through isolating and identifying the infecting strain, we are yet to discover the tools for identifying infecting strain in latently infected TB patients. So far only one study (de Jong et al., 2010) has attempted this by using TbD1 which is present in *Maf* but absent in *Mtb,* but the immunogenicity was low and also it could not discriminate between *Maf* and *Mtb* infected patients. *Maf* is an important cause of TB in West Africa and further studies to discover other antigens Unique to *Maf* and even to MAF1 and MAF2 are needed to properly characterize the immune response associated with these infections. Studies involving a larger cohort of *Mtb* and *Maf* infected patients to *Mtb* antigens are needed to fully explore the differences, if any, in the immune response to these antigens as it will have implications for utility for *Mtb*-based diagnostics, vaccines and biomarkers in *Maf*-infected individuals.

CHAPTER FIVE

Comparison of immune profile of TB patients (after treatment) with LTBI and non-infected contacts (Baseline measurement)

5.1 Background

Only 10% of people infected with TB will progress to active disease; the vast majority will remain latently infected for life because they are capable of mounting an adequate immune response. LTBI (latent TB infection) is thought to be associated with a dormant/non-replicating state of low metabolic activity of the pathogen controlled by the dormancy survival regulon (DosR). Antigens predominantly expressed by dormant *M. tuberculosis* during LTBI are promising candidate immune markers of protection (Leyton et al., 2006)

The requirements for a protective immune response are yet to be fully elucidated, but include changes in the host immune system together with changes in the virulence and pathogenesis of the Mycobacterium (Young et al., 2010). It is widely known that IFN-γ producing CD4+ T cells provide the major effector response to TB, but while IFN-γ is required for protection against disease progression in TB, it is not sufficient on its own. TNFα as well as polyfunctional T cells have also been recognized as playing protective roles in tuberculosis infection (Stenger, 2005; however, other studies have reported varying results depending on the cytokines of interest, the antigenic stimuli, the age of the subjects and their genetic background (Scriba et al., 2008; Mueller et al., 2008). For a particular immune profile to be associated with TB disease, abrogation or reversal needs to be shown following standard treatment regimes for TB.

Effective treatment of active TB patients with anti-TB drugs have been shown to improve cellular responses (antigen-induced proliferation and IFN-γ secretion) of PBMC to various complex mycobacterial antigens and some immunodominant single antigens, e.g. ESAT-6, 16-kDa and 38-kDa proteins (Wilkinson et al., 1998; Dieli et al., 1999; Ulrichs et al., 2000). However, it is not known if the improvement in cellular responses is limited to these proteins or

is a generalized improvement of cellular responses to a large number of antigenic proteins of *Mtb.* Furthermore, there is conflicting information on the nature of the immune response after anti-TB treatment in comparison with latently infected and uninfected contacts. This information is critical to understanding immune protection in tuberculosis infection and will help identify immune correlates of TB cure.

For a particular immune profile to be associated with TB disease, abrogation or reversal needs to be shown following standard treatment regimes for TB (Young et al., 2010). As such, we compared the immune cell profiles of TB cases after treatment to that seen in latently infected (QFT+) and healthy (QFT-) household contacts (HHC) following stimulation with *Mtb* antigens.

5.2 Methodology

5.2.1 Recruitment of household contacts of TB index cases

All household contacts of TB index cases aged 6 months or over were invited to the clinic where the index cases were recruited and introduced to the study. Those who agreed to participate signed an informed consent form and were interviewed for demographic information. For minors, parents/guardians signed and responded on their behalf.

5.2.1 QuantiFERON® TB Gold -in- Tube Test (QFT-TB)

All participants (TB contacts) were screened for tuberculosis infection using the QuantiFERON® TB Gold -In- Tube (QFT) assay (Cellestis Ltd, Carnegie, Victoria, Australia). Briefly Iml of blood was drawn into each of two tubes; one tube pre-coated with synthetic peptide antigens (ESAT-6, CFP-10, TB7.7) and a second tube without antigens (negative control sample/NIL tube). The tubes were swirled several times to allow the blood to come into contact with the inner walls of the tubes in order to ensure complete mixing of the blood and the antigens along the wall. Samples were transported to the lab for analysis the for incubation within a few hours.

The tubes were incubated upright at 37^0C overnight and then centrifuged at 2500 rpm for 10 minutes. The supernatant was harvested and stored at -20^0C until ready for the IFN-γ Enzyme-

Linked Immunosorbent Assay (ELISA). IFN-γ ELISA was done according to manufactures' instructions using IFN-γ standard for quantification. The quality of all laboratory analysis and calculation of the results was controlled by using the accompanying QFT analysis software (v2.62).

A sample was considered positive if it exceeded the standard cut-off value at 0.35 IU IFN-γ/ ml. All positive results were confirmed by re-analysis of the same plasma sample before reporting it as positive. Samples with irreproducible positive results or indeterminate results after repeat run were not included in subsequent in vitro experiments.

5.2.3 PBMC of TB contacts

In addition to the 2 ml of blood used for the QFT testing, blood samples were also collected for in vitro stimulation assays. Depending on the age of participants, 5 to 30 ml of blood was drawn from each participant. PBMC separation and cryopreservation were carried out as previously described in **3.2.1.**

5.2.4 In vitro stimulation assays

All procedures previously described for TB patients **(3.2.2 to 3.2.5)** were used for the TB contacts as well. Briefly cryopreserved cells were thawed, stimulated with same recombinant proteins as that of the TB cases, supernatant was harvested and stimulated cells were stained for intracellular IFN-γ using fluorescently labelled monoclonal antibodies. Cells were acquired on a FACS Calibur and analyzed using Flowjo® software v7.6.5 (Treestar Inc, USA). For a comparative analysis of the results of the in vitro stimulation assays, individuals who lived in the same house as the TB index case, but had a negative QFT result were classified as Internal controls (uninfected) while individuals with positive QFT result were classified as latently infected (LTBI) contacts.

5.2.5 Data analysis

Data were entered into Excel and transported to IBM SPSS version 20 for statistical analysis. Different uni- and multivariate unconditional logistic regression analyses were performed in order to identify predictors for positive QFT. The outcome variable was QFT result (Positive or negative), Age (in 3 categories), sex, relation (spouse, parent, child, sibling) were included as independent variables in a preliminary multivariable regression analysis. The independent categorical variables were expressed as dummy variables. We subtracted one variable at a time using the likelihood ratio test as an elimination criterion ($p < 0.05$). The same approach was used to test the significance of the two-way interaction terms between the independent variables in the final model. The odds ratios calculated from the estimated coefficients in the final models were used to measure the strength of association.

Prism® software version 4.0 (GraphPad, Inc.) was used in the final analysis to compare cytokine responses in TB patients after treatment with LTBI and non-infected contacts. The Mann-Whitney U test was used for comparing two groups whilst the Wilcoxon matched pairs test was used for comparing paired groups. P values of <0.05 were considered significant.

5.3 Results

5.3.1 Enrollment and participant characteristics

A total of 112 household contacts of sputum smear positive TB patients were enrolled in the study, out of which 8 (7.14%) were excluded from the subsequent in vitro assays due to either their unavailability to provide blood for the QFT test or indeterminate QFT result after a repeat run and subsequent unavailability to provide a second sample for QFT testing.

There were more females (56.25%) than males (43.75%) **Table 5. 1.** Median age was 26 years (range: 2-86 years) with 9 unknown ages. Of the 103 whose ages were known, 10 (9.7%) were children under five; 25 (24.3%) were above 5 years, but under 15 years (children) while the remaining 68 (66%) were 15 years or older (adults). Participants were related to TB index cases as; Parents (14), Siblings (27), Spouse (25), Child (30) other relatives (6), Unknown (10). Hence TB contacts recruited were mostly children of TB index cases, followed by siblings, spouses and

parents. Recruitment was passive in the sense that the TB index cases were encouraged to bring their contacts to the clinic for screening, hence it could not be determined whether characteristics of those who declined to participate in the study were different from those who participated as the former were unknown to the study.

Table 5.1: Characteristics of TB contacts enrolled in the study

	No. of Participants (%)
Total	**112**
Sex	
Male	49 (43.75)
Female	63 (56.25)
Relating to Index case	
Parent (Mother/Father)	25 (22.32)
Child (Daughter/Son)	30 (26.50)
Siblings (Sister/Brother)	27 (24.79)
Others (other relations	6 (5.36)
Unknown	10 (8.93)

5.3.2 Quantiferon® TB Gold-In-Tube Test results

Of the 107 TB contacts screened for tuberculosis infection with the QFT, 68 (63.6%) were positive, 36 (33.6%) were negative and 3 (2.8%) had indeterminate results even after repeat run. More males (63.3%) tested positive compared to females (58.7%). **Table 5.2**.

Positive QFT results were more often detected among parents of an index case (78.6%) compared to spouses (60%), likewise positive QFT results in children (56.7%) of index cases were slightly more than within siblings (51.9%). **Figure 5.1**

Based on the 3 age categories chosen for analysis; 70% (7/10) of the "under fives" were QFT-positive compared to 48% (12/25) for the ''under 15 years'' and 67% (44/68) of adults.

Table 5.2: QFT results of household contacts of TB patients enrolled into the study

	No. (%)	No. (%)	No. (%)
Sex	Male	Female	Total
QFT Positive	31 (63.3)	37 (54.4)	68 (63.6)
QFT Negative	16 (32.7)	20 (29.4)	36 (33.6)
Indeterminate	2 (4.0)	1 (1.6)	3 (2.8)
Total	49	38	107

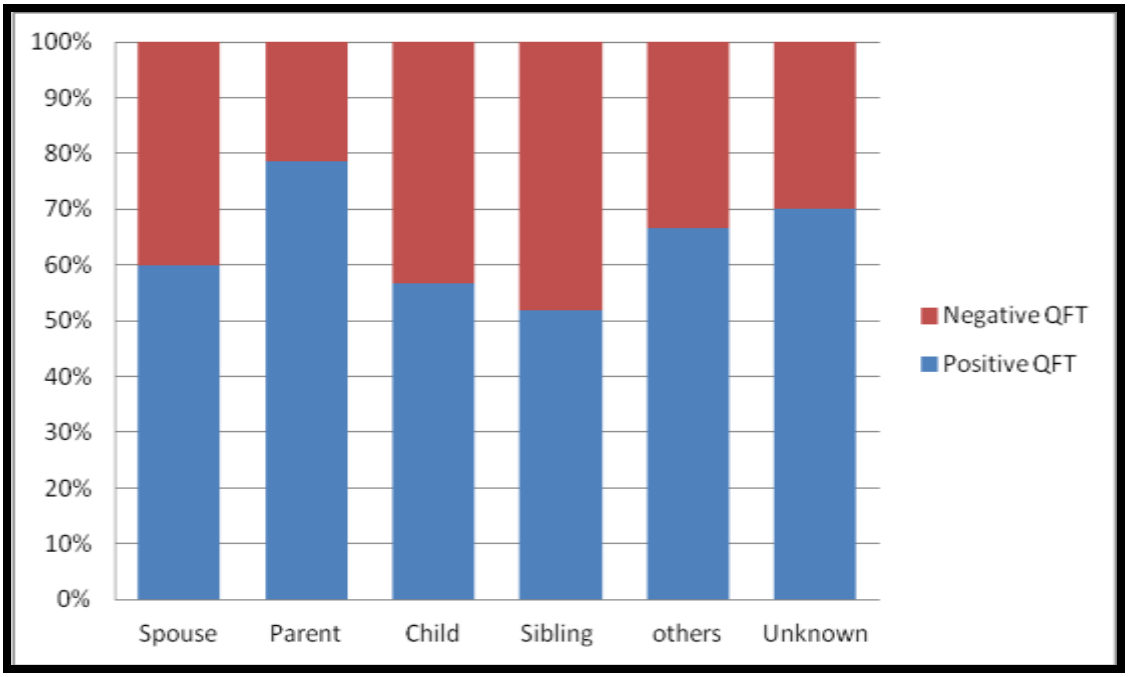

Figure 5.1: Distribution of QFT results according to relation of Household contact to TB index case

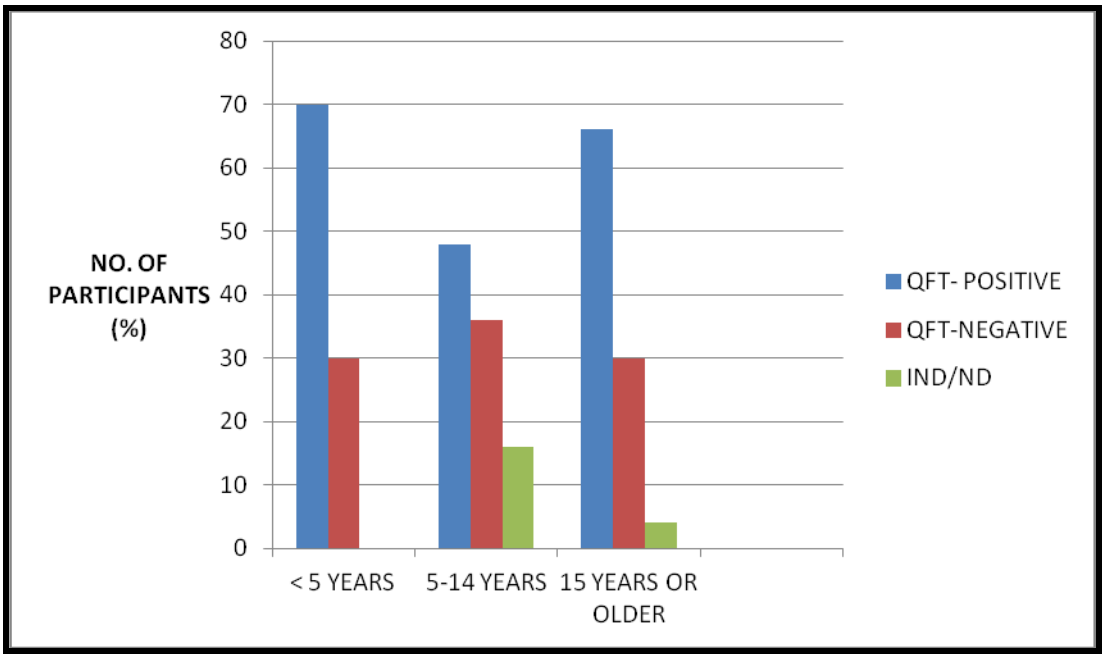

Figure 5.2: Prevalence of tuberculosis infection (QFT-Positive) within the age categories

5.3.3 Predictors of positive QFT result

Predictors of positive QFT could not be determined from the logistic regression (multivariate regression analysis) with the QFT result as an outcome or dependent variable. All the covariates were not significant at alpha =0. 05. A Pearson chi-square test for association found no association between gender (P value =0.911>>0.05), age group (P value=0.645>>0.05), or relationship to index case (P value=0. 372>>0.05) to QFT result. However, it could be inferred from the P values that ''relationship to index case'', followed by ''age group'' were more likely to be associated with type of QFT result than ''gender''. On the basis of this analysis, it was assumed that all the participants had equal chances of being QFT positive or negative and thus PBMC of any of the contacts could be included in the in-vitro assays without further randomization.

5.3.4 Frequency of IFN-γ+ T cell responses to ESAT-6/CFP-10 and latency associated antigens in QFT positive and negative TB contacts.

The Frequency of IFN-γ+ T cell responses to ESAT-6/CFP-10 fusion protein and latency associated Rv1733, Rv2029, Rv2628 as well as resuscitation associated Rv1115 and DosR which is an antigen pool comprised of the 3 latency associated proteins (Rv1733, Rv2029, Rv2628) were determined in QFT-positive (n=19) and QFT-negative (n=23) household contacts of TB patients previously described. Frequency of IFN-γ+ CD4+ and CD8+ T cells were determined for each antigen after long term stimulation (6 days). Increased T cell-derived IFN-γ responses after prolonged in vitro incubation have been previously described (Leyton et al., 2006; Leyton et al., 2007; Cebovin et al., 2007).

Table 5.3: Positive T cell responses against *Mtb*- stage specific antigens in QFT positive and QFT negative household contacts of sputum smear positive TB patients.

	QFT POSITIVE (LTBI)		**QFT NEGATIVE (CONTROL)**	
	Positive T cell responses [^{1}n/^{2}N (%)]			
Stimulation	**%IFN-γ+CD4+**	**%IFN-γ+CD8+**	**%IFN-γ+CD4+**	**%IFN-γ+CD8+**
SEB	18/19 (94.7)	17/19 (89.5)	22/23 (95.7)	19/23 (82.6)
ESAT-6/CFP-10	18/18 (100)	4/17 (82.3)	3/22 (13.6)	2/22 (9.1)
Rv1733	16/18 (88.9)	6/17 (35.3)	8/20 (40.0)	9/20 (45.0)
Rv2029	12/18 (66.7)	15/17 (88.2)	4/17 (23.5)	2/14 (14.3)
Rv2628	9/18 (83.3)	16/17 (94.1)	2/19 (10.53)	12/19 (63.2)
Rv1115	15/18 (83.3)	16/17 (94.1)	2/19 (10.53)	12/19 (63.2)
DosR	7/10 (70.0)	7/10 (70.0)	9/22 (40.9)	12/22 (54.5)

[1]Number of participants with more than or 0.2% IFN-γ producing CD4+/C8+ T cells

[2]Number of cultures assessed (Uncontaminated and SEB positive)

All the QFT+ samples gave positive IFN-γ responses to ESAT-6/CFP-10 with 100% of CD4+ T cells and a lower frequency of 82.3% in CD8+ T cells**. (Table 5.3).** Surprisingly, IFN-γ positive responses to ESAT-6/CFP-10 were detected in 3 (CD4+) and 2 (CD8+) QFT negative samples, indicating a past exposure that could not be detected in the short term stimulation used in the IGRA. Rv1733 induced expression of IFN-γ in CD4+ T cells was higher than the other antigens, making Rv1733, the most recognized of the 3 latency associated proteins with respect to T cell response. There were positive IFN-γ responses in the QFT negative group to all other DosR antigens as well. Resuscitation associated protein, Rv1115 induced IFN-γ positive responses in >80% of QFT positive subjects and 63% of QFT negative subjects while, DosR, an antigen consisting of a mixture of the three DosR proteins induced responses in 70% and 54.5% of CD4+ T cells in QFT positive and QFT negative subjects respectively. The data further revealed that some of the latency associated antigens might be strong inducers of CD8+ responses, as with the exception of Rv1733, the CD8 T cell response in Rv2029, Rv2628 and Rv1115 were stronger compared to CD4+ responses.

5.3.5 Magnitude of T cell responses against *Mtb*- specific antigens in QFT+ and QFT- household contacts of TB patients.

The magnitude of the T cell responses to *Mtb* antigens in the two groups was determined by comparing the median T cell expression of IFN-γ in response to Mtb- specific antigens in QFT positive **(A and B)** and QFT negative subjects (**C and D).** There was a significantly higher frequency of IFN-γ+ expression to Rv1733 compared to Rv2628 (1.38 vs 0.14 %, $P<0.05$) on CD4+ T cells and to Rv2029 (0.0 vs 1.2 %, P<005) in CD8+ T cells.

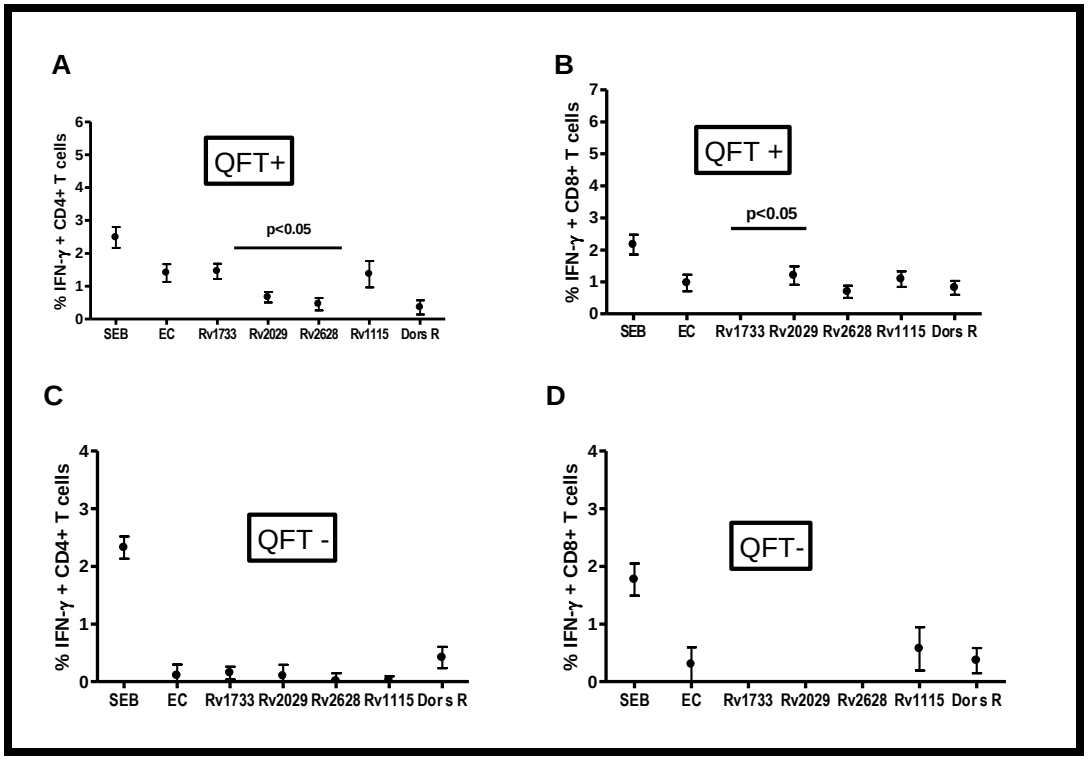

Figure 5.3: Magnitude of T cell response against *Mtb*- specific antigens in QFT + and QFT - household contacts of TB patients.

T cell responses were measured by intracellular flow cytometry after 6 days of in vitro stimulation in PBMC from QFT + (n=19) and QFT- subjects (n=22). Scatter plots indicate mean and standard deviation. Background values of non-stimulated controls were subtracted and negative values were converted to zero for plotting. Percentages of IFN-γ expressing CD4+/CD8+ T cells in QFT + **(A and B)** and QFT - **(C and D)** are indicated on the y-axis for stimulation with SEB, ESAT-6/CFP-10 (EC) fusion protein, latency associated Rv1733, Rv2029, Rv2628, resuscitation associated Rv1115 and a pool of the three DosR proteins (DosR) from *M. tuberculosis* (x- axes). Data were analyzed using a Kruskal-Wallis ANOVA followed by Dunn's post-test comparison and p-values indicated.

5.3.5 Comparison of the cytokine expression profile of the three groups

To determine whether at the end of treatment, T cell response resembles that of latently infected individuals or non infected controls, the frequency ESAT-6/CFP-10 and Rv1733 specific IFN-γ+ CD4 and CD8 T cells was compared between, QFT+ (n=19), QFT- (n=23), TB cases at baseline (n=21) to TB cases after treatment completion (n=21).

The median frequency of ESAT-6/CFP-10- specific IFN-γ+ CD4 T cells was significantly higher in TB patients ''post-treatment'' compared to ''pre treatment'' (3.45 vs 1.14 %, P<0.01), however, between QFT positive and QFT negative individuals, there was no difference in ESAT-6/CFP-10-specific IFN-γ+ CD4+ T cells (0.65 vs -0.015 %, P>0.05). This highlights the difference in incubation times between the two assays as the QFT uses a shorter incubation time (16-24 hrs) compared to the 6 days in vitro stimulation, which is known to induce long-lived central memory T cells, less likely to release IFN-γ during the short period of exposure to antigens in the QFT assay **Figure 5.4.** The median frequency of ESAT-6/CFP-10- specific IFN-γ+ CD4 T cells at the end of TB treatment was significantly higher compared to QFT positive (3.45 vs 0.65 %, P<0.01) and QFT negative individuals (3.45 vs 0.015 %, P<0.001). Also the frequency of ESAT-6/CFP-10- specific IFN-γ+ CD4 T cells in TB cases pre-treatment was higher than in QFT negative individuals (1.140 vrs -0.015 %, P<0.05).

The frequency of ESAT-6/CFP-10- specific IFN-γ+ CD8 T cells was equally higher in TB cases pre treatment compared to QFT negative controls (1.850 vs 0.260 %, P<0.001) and significantly lower in TB cases post treatment (1.850 vs 0.227 %, P<0.001). Also the median frequency of ESAT-6/CFP-10- specific IFN-γ+ CD8 T cells in QFT negative individuals was insignificantly lower than that in QFT positive individuals (0.26 vs 0.81 %). These results indicate that with respect to ESAT-6/CFP-10, the cellular response (T cell) profile of TB cases after treatment is not the same as that in latently infected individuals (LTBI). We speculate that successful TB treatment is associated with accumulation of central memory CD 4 T cells, which can differentiate to generate effector responses that are much greater than what pertains in latently infected individuals while the reverse is true for CD8 T cells which decline after treatment to frequencies lower than that seen in LTBI.

After in vitro re-stimulation with the latency associated protein Rv1733, the frequency of IFN-γ+ CD4 T cells was higher in QFT positive compared to QFT negative individuals (1.350 vs 0.030 %, P<0.01). There was also a significant difference between the median frequency of Rv1733 specific IFN-γ+ CD4+ T cells in QFT positive individuals compared and TB cases after treatment (1.350 vs 0.16 %, P <0.01). The CD8+ IFN-γ+ expression levels in response to Rv1733 was

significantly higher in TB cases, pre-treatment compared to QFT negative individuals (1.965 vs 0.045 %, P<0.01) and TB cases after treatment (1.964 vs 0.06 %, P<0.01).

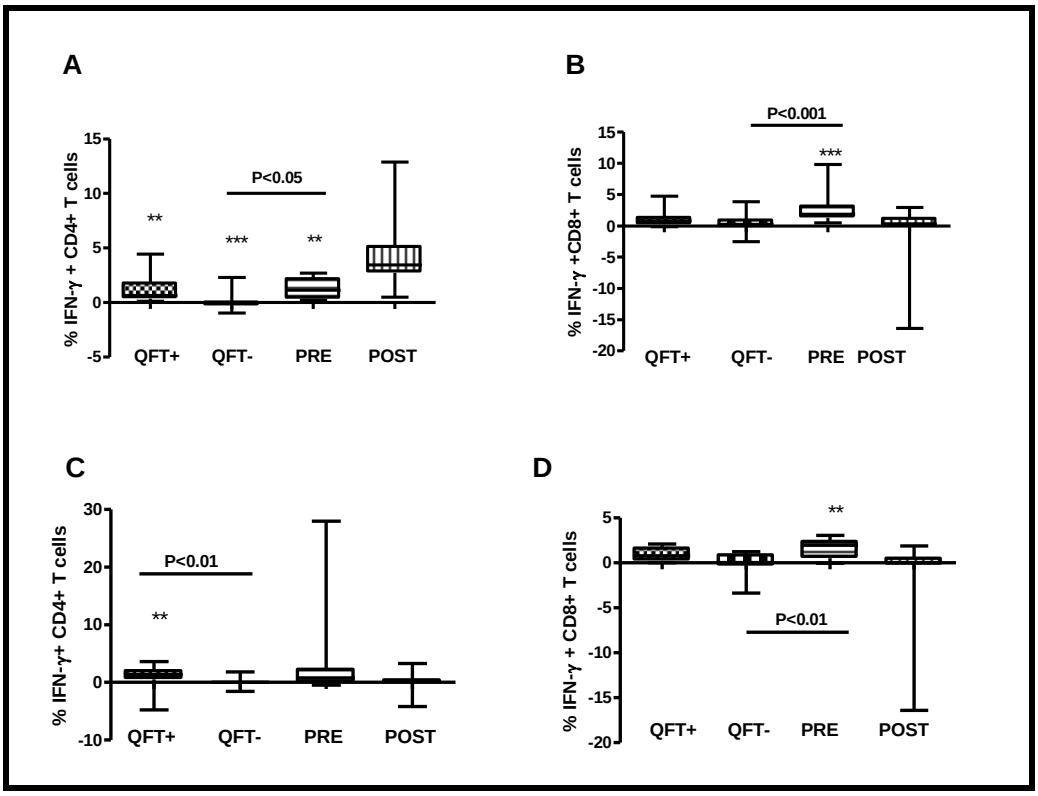

Figure 5. 4 Comparison of frequency of ESAT-6/CFP-10 and Rv1733 specific IFN-γ+ CD4 and CD8 T cells in TB cases post treatment to QFT+, QFT-, and TB cases pre- treatment

Bar indicates median frequency of antigen- specific IFN-γ positive CD4 or CD8+ T cells in QFT- (n=23), QFT+ (n=19), TB cases pre treatment (n=21) and TB cases post treatment in response to ESAT-6/CFP-10 (A and B) and latency associated Rv1733 (C and D). Background values of non-stimulated controls were subtracted for all data points with a positive SEB response. Data were analyzed using a Kruskal-Wallis ANOVA followed by Dunn's post-test comparison and p-values indicated as follows: P<0.05 (*), P<0.01 (**), P<0.001 (***) for ONLY comparisons with TB cases post-treatment. For all other comparisons, significant P values are indicated.

5.4 Discussion

During latent tuberculosis infection (LTBI), the tubercle bacilli contained within granulomas (Ulrichs et al, 2004) are thought to be subject to nutrient and oxygen deprivation (Tufariella et al., 2003, Wayne et al, 1998). As part of the *Mtb*-adaptive response to hypoxia, expression of the DosR regulon is observed. The functions of most DosR-regulon-encoded proteins (DosR or latency associated proteins) are still mostly unknown (Yaun et al., Park et al., 2003). However, it has been suggested that long lasting memory response to this subset of antigens will be useful in vaccine design.

The results indicate that there is a higher frequency of specific T-cells to both secreted RD1 associated ESAT-6/CFP-10 and latency-associated (Rv1733, Rv2029, Rv1628, Rv1115 and DosR) antigens in QFT positive household contacts of TB cases and very little to no response in QFT negative household contacts.

Various studies have reported that there is a higher IFN-γ response to ESAT-6/CFP-10 by latently-infected Individuals compared to active TB patients, however, in a comparison of TB cases (pre-treatment) and QFT positive (LTBI) individuals in this study (**Figure 5.4 A and B**), there was a higher median frequency of IFNγ+ CD4 and CD8 T cells in TB cases pre-treatment compared to LTBI but the difference was not statistically significant. The results in the literature regarding IFN-γ responses to the classical antigens in active-TB patients are inconsistent (Kassa et al., 2012). Differences in host genetic makeup (Jabado and Philippe, 2005), in *Mtb* strains (Tsenova et al., 2007), in study methodologies (Day et al., 2011), and in the extent of TB disease progression, with diminished IFN-γ production during advanced disease (Widek et al., 2008) have all been implicated in these inconsistencies. Most studies that reported lower IFN-γ response to ESAT-6/CFP-10 in active TB patients compared to LTBI used short term assays which will detect effector memory rather than central memory T cell responses (Sallustro et al., 1999).

Additionally, long-term assays have also been shown to enhance detection of LTBI and to distinguish between recently acquired and remote infections (Buteraa et al., 2009; Golleti et al., 2009). In this study, we used long- term stimulation (6 days) hence we can speculate that the QFT positive individuals were harbouring recent infections while most of the TB cases probably had previous exposures leading eventually to TB disease. This is interesting as it indicates that most people succumb to infection after numerous encounters leading to an enhanced central memory response. It could also indicate that the TB contacts had been actually recently exposed as a result of close contact with an active TB patient. In a West African cohort of TB patients and controls, using long term stimulation, good discrimination was shown between infection and disease following TB10.4 (a virulent part of *Mtb* genome) stimulation indicating that a longer-term stimulation is optimal for detection of active disease (Sutherland et al., 2010).

A number of studies have also reported higher frequencies of IFN-γ+ responses to Rv1733 compared to other tested DosR proteins (Leyten et al., 2006; Schuck et al., 2009; Commanduer et al., 2011 Kaasa et al., 2012) and this was consistent with our results which showed a higher frequency of IFN-γ positive T cell responses to Rv1733 compared to Rv2029 and Rv2628 ($P<0.05$, **Figure 5.3**). In the first attempt at determining the immunogenecity of the entire set of 48 antigens spanning the Dos R regulon, Black et al., 2009 reported that Rv1733 was among the top 3 most recognized antigens in Guinea, Gambia and South Africa population of latently infected individuals. While the recognition of the latency-associated antigens by cells from active TB patients could reflect the fact that most TB patients undergo a latent infection prior to TB disease (Schuck et al., 2009), it might also indicate the involvement of latency antigens in the pathogenesis of TB. It could also be that active TB actually coexists with latency hence an individual could harbour actively replicating bacteria and succumb to TB, but also harbour latent foci that will lead to a recognition of latency related antigens. To determine whether these antigens are differently recognized by cells from LTBI populations than active TB patients, we compared the response to Rv1733 in TB cases pre-treatment and QFT positive (LTBI)

individuals, we found no significant differences, although it has been reported that in BALB/mice persistently infected with *Mtb* there is preferential recognition of latency antigens than in acutely infected mice (Roupie et al., 2007). Not all lesions in the human lung are active during disease so consequently *Mtb* infection may be viewed as a continuous spectrum extending from sterilizing immunity to full blown TB (Barry et al., 2009; Lin et al., 2009), making the response to these "'Stage specific antigens "' not unusual.

Interestingly the resuscitation associated protein Rv1115 and the DosR pool induced some CD8+ T cell response in QFT negative subjects. Those individuals, even though had no detectable effector memory cells for TB, may more likely have been previously exposed to TB infection (past infection) which was not detectable using the QFT because the QFT detects only effector memory response (Lalvani et al., 2004) due to its short incubation period. A QFT negative result may rule out a recent infection or exposure, but does not necessarily imply that the individual has never been exposed to TB infection. Comparing the frequency of IFN-γ+ CD4+ and CD8+ T cells post treatment to QFT+, QFT- and TB patients pre-treatment revealed that there is a higher frequency of IFN-γ+ CD4+ T cells after treatment compared to LTBI subjects and a lower frequency of IFN-γ+ CD8 T cells. This offers further proof that the high frequency of CD8 T cells is associated with active TB while low frequency CD8 T cells is associated with cured TB as was observed in the longitudinal study. CD4 T cells have taken center stage when in comes to protective response in TB, but this study suggests that CD8 T cells should not be overlooked and that they in fact may be better indicators of the state and stage of TB infection. It has been suggested that IFN-γ assays targeted at CD8+ T cells may be able to distinguish between latently infected and actively infected patients (Day et al., 2011), and our data supports this strategy. It would be a great improvement on the current IGRA, s which cannot make that distinction between latent infection and active infection and which are also thought in its present form to measure IFN-γ responses in the periphery dominated by CD4+ T cells (McCoy et al., 1994.)

Summary

Tuberculosis (TB) continues to be a major global health problem ranking as the second leading cause of death from an infectious disease worldwide after HIV, the human immunodeficiency virus (WHO, 2012). With an estimated two billion people living with latent *M. tuberculosis* infection (Corbett et al., 2003), the global control of tuberculosis can only be achieved through the development of effective vaccines, improved diagnostics, and novel and shortened therapy regimens and biomarkers (Abu Raddad et al., 2009).

Month two sputum culture conversion remains the only biomarker accepted by the IUTLD for monitoring TB treatment response (Mitchison, 1993). Use of classical microbiological methods like "sputum culture status'' as a biomarker for TB treatment response and cure has limited utility in children and extra-pulmonary cases, where appropriate quality sputum samples are difficult to obtain. Immunodiagnostic techniques could be valuable in such cases (Chegou et al., 2008; Munk et al., 2001) especially if they can be developed into rapid, point-of care tests. Also, if validated as a surrogate marker it will be useful in clinical trials (Walzl et al., 2008). Identification of immunological parameters in blood that correlates with culture sterilization may also provide important information about host factors most relevant to anti-TB therapy. Understanding the interplay between the host immune system and *Mtb* may provide a platform for the identification of suitable biomarkers, through both unbiased and targeted hypotheses-driven approaches (Walzl et al., 2011).

Urgently needed biomarkers include those that can detect early response to treatment. Such markers should be present at baseline (before treatment) and show measurable change that correlates with improved bacteriological or immunological outcomes. Due to the important role of IFN-γ, many studies have used it as a marker for treatment response, but the predictive ability has been low (Chee et al., 2010). It has been suggested that multiple cytokines will a give better predictive value (Walzl et al., 2008). We therefore assessed in addition to IFN-γ, five other pro- and anti inflammatory host factors for this criteria using the Luminax platform.

Freshly isolated PBMC of the first 20 subjects recruited into the study were stimulated for 6 days with ESAT-6/CFP-10 fusion protein and three Dormancy survival Regulon (DosR) proteins at baseline and 2 weeks of treatment. Week two was chosen because it is believed that most of the actively replicating bacteria are eliminated during first two weeks of treatment, leaving only the latent foci (NIHCE, 2006; Rouillon et al., 1976). Hence, during this period, improvement in immunological response would be expected to coincide with the decreasing bacteria burden. In response to all antigenic stimuli, however, we observed a cytokine profile which consisted of high levels of IFN-γ, followed by Granzyme B, TNF-α and IL-17 with lower levels of sIL2R and IL-10. This profile was in contrast to expectations that ESAT-6/CFP-10 being a virulence factor (Brodin et al., 2006; Guinn et al., 2004; Dwivedi et al., 2012) would induce secretion of both pro- and anti-inflammatory cytokines whiles Rv1733 only induces pro- inflammatory responses and relatively minor anti-inflammatory response. This observed profile suggests that cytokine responses are dependent on the stage of disease in the host (in this case active disease), not antigen type (associated with virulence or dormancy). At week two of treatment, we observed a trend of increased cytokine levels for IFN-γ, Granzyme B, TNF-α and IL-17 but only the increase in Rv1733-induced Granzyme B (P=0.013) was significant. Although cytokine quantity does not equate function, the high levels of Granzyme B warrants further studies to determine its utility as part of a multi cytokine marker signature for treatment response. Improvement in cellular response of TB patients was seen after two weeks of effective chemotherapy and was characterised by increased cytokine response to *Mtb*-specific antigens. However, due to the wide inter-individual variation observed, a wider pool of cytokines and *Mtb*- specific antigens would have to be investigated to discover the most effective cytokine signatures for monitoring TB treatment response.

We also investigated the functional differences in T cell response during treatment by characterizing IFN-γ+ CD4 and CD8 specific responses. Monitoring treatment response using frequency of IFN-γ sensitized cells is not new, but many of these IGRA based studies have been unsuccessful due to its low predictive ability (Chee et al., 2010). IGRA mainly detects effector

memory response, hence information on dynamics of central memory response which require long term incubation (Leyton et al., 2007; Sallutro et al., 1999) is lacking. In the initial attempt at characterizing T cell response, the PBMCs (from the first 20 patients) remaining after harvesting the supernatant from the 6 day cultures for the Luminex assay, were used in the intracellular cytokine assay for IFN-γ after overnight incubation with the protein transport inhibitor Brefeldin A (BFA). Similar to other studies (Kassa et al., 2012; Ravn et al., 1999; Black et al., 2009) frequency of ESAT-6/CFP-10 specific responses were high and in agreement with (Kassa et al., 2012; Black et al., 2009) among the DosR proteins, frequency of IFN-γ+ T cell responses were highest for Rv1733. The CD8+ T cell response to *Mtb* is normally of a lower magnitude than the CD4+ T cell response (Bruns et al., 2009; Stenger et al., 1998), however, we observed lower frequency of CD4 T cells at baseline and a compensatory increase in CD8, but by week two, the frequency of CD8+ T cells had declined (P=0. 0024)and that of CD4 cells had increased significantly (P=0.0008). Having established that memory T cell response can be detected upon long term stimulation, we proceeded to perform a longitudinal study using cyropreserved cells to determine the memory response to stage-specific antigens, ESAT-6/CFP-10 (associated with actively metabolizing bacteria) and Rv1733 (associated with latent bacteria) at four time points; before, during and upon completion of TB therapy (month 6).

We selected cryopreserved PBMC samples of 38 patients (out of the 104 recruited for the entire study) with all four time points available. Samples were thawed, rested overnight, stimulated for 6 days with ESAT-6/CFP-10 and Rv1733 and stained for intracellular production of IFN-γ. However the final analysis included data from 21 out of the 38. Similar to what was observed using fresh cells, the frequencies of ESAT-6/CFP-10 -specific IFN-γ+ CD4 increased from baseline to week 2 while that of CD8 T cells declined. The increase in frequency of IFN-γ+ CD4 T cells continued through to month two and month 6, when treatment completed while in CD8 T cells, there was a further decline in month 2 untill treatment completion. In response to Rv1733, however, they were no significant changes.

Lastly, we compared the immune profile of treated TB patients with their latently infected and uninfected household contacts. In the treated patients, the frequency of ESAT-6/CFP-10-specific

IFN-γ+ CD4 T cells were significantly increased in comparison to pretreatment levels ($P<0.01$) as well as levels in QFT+ ($P<0.01$) and QFT- ($P<0.001$) household contacts. Levels in Rv1733 were not significant.

In Conclusion, our data support the concept that studying the immunological profile of TB patients to *Mtb*-stage- specific antigens in the context of multiple cytokines and specific T cell subset responses will generate information that will be useful in biomarker design and discovery. We found that successful anti TB therapy is associated with improved ESAT-6/CFP-10 specific- IFN-γ+ CD4 T cell memory response and decreased ESAT-6/CFP-10- specific IFN-γ+ CD 8 T cell response. To use this CD4/CD8 T cell profile as a tool to monitor response to treatment, there is the need to quantify what levels of this response are actually associated with complete cure and even if complete cure is achieved, how long lasting this T cell memory response will be. To achieve this, future studies would have to follow up a cohort of successfully treated TB patients until up to two years to determine durability of this profile and its potential association with relapse, reinfection or relapse-free cure.

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Optimization of Intracellular flow cytometry, ELISPOT and Luminex assays for detection of cellular responses to antigenic stimuli in TB cases and healthy controls **(February - April 2011**)

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Esat-6/cfp-10 and Rv1733 induced functional changes in CD4 and CD8 T cells in TB patients after 2 weeks of treatment. *40th Keystone Symposia on Molecular and Cellular Biology (Drug Resistance and Persistence in Tuberculosis). Kampala, Uganda, 13th - 18th May 2012. Abstract No. 238*

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List of Publications

Mensah, GI, Addo, K. Tetteh, KA Sowah, SA Loescher, T, Geldmacher, C and Jackson-Sillah D (2014) Cytokine response to selected MTB antigens in Ghanaian TB patients, before and at 2 weeks of anti-TB therapy is characterized by high expression of IFN-gamma and Granzyme B and inter- individual variation. BMC Infectious Diseases- *accepted*

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