Evaluation of multiple cytokine levels to improve our understanding of protective immune responses against Tuberculosis and to develop novel diagnostic methods

By

Khutso Gemina Phalane

Thesis presented in partial fulfilment of the requirements for the degree Master of Science in Medical Science (Molecular Biology) in the Faculty of Medicine and Health Sciences at the University of Stellenbosch

Supervisor: Prof Gerhard Walzl
Co-supervisor: Dr Novel Chegou
Division of Molecular Biology and Human Genetics

March 2013

Declaration

I, Khutso Gemina Phalane, hereby declare that the work contained in this thesis is my own original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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"Without Him, I am nothing and can do nothing of value.
He is my only Source"

Summary

Important steps towards the global control of Tuberculosis include the improvement of diagnosis, the development of effective vaccines and the identification of correlates of protection/protective immunity to *Mycobacterium tuberculosis.*

This study has of three objectives:

1. To validate the findings of a previous study that showed increased levels of IL-1β and decreased levels of IL-17 in children who are exposed to tuberculosis but remain uninfected compared to those who are exposed/infected and unexposed/uninfected.
2. To define the protective immunological phenotype in children with negative IGRA's and TST following exposure to *Mycobacterium tuberculosis*.
3. To evaluate a number of cytokines in both serum and saliva samples of identified tuberculosis cases and controls for their diagnostic potential and to evaluate saliva as a possible new diagnostic sample type.

The study designs were as follows:

Objectives1, and 2: Children with documented tuberculosis exposure and with *Mycobacterium tuberculosis* infection as assessed through interferon gamma release assays, children with exposure but no infection and a control group with no exposure nor infection were investigated. These participants were selected according to their exposure and infection phenotypes from a larger TB household contact study that was conducted in communities in Cape Town. Whole blood was stimulated in QuantiFeron tubes overnight and ten cytokines were measured in antigen stimulated and unstimulated supernatants by Luminex multiplex Immunoassay. Differential production of cytokines in the three groups was evaluated.

Objective 3. Saliva and serum samples were collected from thirty eight adults with suspected tuberculosis who were recruited from a community health centre in Cape Town, after which the levels of thirty three host markers were evaluated in the samples using the Luminex platform.

The main findings of the studies included:

1. Increased levels of IL-1β and decreased levels of IL-17 in children who are tuberculosis exposed but remain uninfected compared to those who are exposed/infected and unexposed/uninfected could not be confirmed.
2. Immune responses other than IFN-γ are different in children with different exposure and infection phenotypes. Higher IL-23 and IL-33 levels in children with tuberculosis exposure without subsequent *Mycobacterium tuberculosis* infection compared to children with no exposure were shown.
3. In both the tuberculosis cases and controls, the levels of most markers were above the minimum detectable limit in both serum and saliva, but marker levels were not consistently higher in one sample type. The levels of fractalkine , IL-17, IL-6, IL-9, MIP-1β, CRP, VEGF and IL-5 in saliva, and those of IL-6, IL-2, SAP and SAA in serum, were significantly higher in tuberculosis patients, in comparison to the levels obtained in those without active tuberculosis ($p<0.05$). The area under the ROC curve was ≥ 0.70 for most of these markers, thereby confirming their diagnostic potential for TB disease.

The work presented in this thesis has identified markers that may grant an improved understanding on the mechanisms that are associated with protection against *Mycobacterium tuberculosis* in children. The preliminary results presented show that the identification of host markers in saliva is possible and the utility of saliva for the development of rapid immune-based tests for active tuberculosis is promising.

Opsomming

Noemenswaardige vooruitgang in die globale beheer van Tuberkulose is onderworpe aan verbeterde diagnose, die ontwikkeling van doeltreffende vaksienes en die identifikasie van aanwysers van immuniteit teen *Mycobacterium tuberculosis.*

Die doel van hierdie studie is:

1. Om die bevindinge van ‘n vorige studie te bevestig, waar verhoogde vlakke van IL-1β en verlaagde vlakke van IL-17 waargeneem is in kinders wat aan tuberkulose blootgestel is, maar nie geïnfekteer is nie. Hierdie bevindinge was in vergelyking met geïnfekteerde en nie-blootgestelde kinders.
2. Om ‘n beskermende immunologiese fenotipe te definieer in kinders met negatiewe IGRA’s en TST, na blootstelling aan *Mycobacterium tuberculosis.*
3. Om sekere sitokines, in beide serum en speeksel monsters van tuberkulose gevalle en kontroles, te evalueer as potensiële diagnosemiddels, asook die moontlikheid dat speeksel kan dien as ‘n nuwe diagnostiese monstertipe.

Die studieraamwerk was as volg:

Doel 1 &2:Die volgende groepe was onder meer ondersoek – Kinders blootgestel aan tuberkulose en wat gevolglik geïnfekteer is*,* soos vasgestel deur interferon gamma vrystellingstoetse; kinders wat wel blootgestel is maar nie geïnfekteer is nie en ‘n kontrolegroep wat geen blootstelling aan *Mycobacterium tuberculosis* gehad het nie. Hierdie individue is geselekteer volgens hul blootstellingsprofiel en infeksiefenotipes, uit ‘n groter blootstellingstudie op Kaapse huishoudings. Heelbloed is oornag gestimuleer en tien sitokiene is gemeet in antigeen-gestimuleerde en ongestimuleerde supernatante, deur middel van Luminex multipleks Immunotoetse. Differensiële produksie van sitokienes in hierdie groepe is gevolglik geëvalueer

Doel 3: Speeksel en serummonsters van 38 volwassenes met vermeende tuberkulose, is versamel en die vlakke van drie en dertig gasheermerkers is gemeet deur middel van die Luminex platvorm.

Die hoof bevindinge van hierdie studie sluit in:

1.Vehoogde vlakke van IL-1β en verlaagde vlakke van IL-17 kon nie bevestig word in die verskeie kindergroepe (Sien doel 1) nie.

2. Die immuunrespons, uitsluitend die IFN- γ respons, is veskillend in kinders met uiteenlopende blootstelling en infeksiefenotipes. Hoër vlakke van IL-23 en IL-33 is gevind in kinders wat blootgestel is aan tuberkulose, maar nie geïnfekteer is nie, in teenstelling met nie-blootgestelde kinders..

3. In beide die pasiënte en kontroles was die meeste sitokienvlakke hoër as die minimum meetbare limiet in beide speeksel en serummonsters, hoewel merkervlakke nie konstant hoër was in enige van die twee monstertipes nie. Die vlakke van fractalkine, IL-17, IL-6, IL-9, MIP-1β, CRP, VEGF en IL-5 in speeksel en IL-6, IL-2, SAP en SAA in serum, was merkbaar hoër in tuberkulosepasiënte, in vergelyking met vasgestelde vlakke in individue sonder aktiewe tuberkulose. ($p<0.05$). Die oppervlak onder die ROC kurwe was ≥ 0.70 vir die meerderheid van die merkers. Dit is ‘n sterk aanduiding dat hierdie merkers potensiaal het as diagnostiese merkers vir tuberkulose.

Hierdie navorsing het merkers geïdentifiseer wat die begrip van die megansime waarmee beskerming teen *Mycobacterium tuberculosis* gebied word in kinders, verbreed. Hierdie voorlopige resultate dui aan dat die identifikasie van gasheermerkers in speeksel moontlik is en dat speeksel moontlik kan dien as ‘n proefkonyn vir die ontwikkeling van immuungebaseerde sneltoetse vir die diagnose van aktiewe tuberkulose.

Acknowledgements

I wish to extend my sincere gratitude to the following people, without whom the completion of this thesis would have not been possible:

My principal supervisor, Prof Gerhard Walzl; and co-supervisor Dr Novel Chegou, I would like to thank them for their continued support, guidance and encouragement throughout the course of this project.

Prof Anneke Hesseling for her guidance and the Desmond Tutu TB Centre staff for provision of samples.

Shirley and nursing assistants/ SUN-IRG lab staff for their mentorship, guidance, provision of samples and training on various techniques.

My fellow colleagues and friends: Lance, Chandre, Marna, Angela, Belinda, Irene, Pauline, Andrea, Lani and Lizaan for their support, encouragement and empathy during the course of the study and writing of the thesis.

The EDCTP through the African European Tuberculosis Consortium (AE-TBC, grant number IP_2009_32040) and the Trials of Excellence in Southern Africa (TESA, project code CG_cb_07_41700) for financial support.

To my dad (may his sweet soul rest in peace) to whom this thesis is dedicated and my entire family for their encouragement, support and understanding during the course of this study.

List of abbreviations

AETBC	:	African European Tuberculosis Consortium
ASC	:	Apoptosis associated speck-like protein
ATP	:	Adenosine triphosphate
AUC	:	Area under the curve
BAL	:	Bronchoalveolar lavage
BCG	:	Bacillus Calmette-Guérin
BD	:	Becton Dickinson
°C	:	Celsius
CARD	:	Caspase recruitment domain
CDC	:	Centres for Disease Control and Prevention
CFP	:	C-reactive protein
CMI	:	Cell mediated immunity
COPD	:	Chronic obstructive pulmonary disease
CSF	:	Cerebro spinal fluid
CT	:	Computerised tomography
DC	:	Dendritic Cell
DNA	:	Deoxyribonucleic acid
DOTS	:	Directly Observed Treatment Short Course
EGF	:	Epidermal growth factor
ELISA	:	Enzyme linked Immunosorbent assay
ELISPOT	:	Enzyme linked Immunospot
ESAT	:	Early secretory antigenic target
FDA	:	Food and Drug Administration
FOXP	:	X-linked forkhead box transcription factor
G-CSF	:	Granulocyte colony stimulating factor
GDA	:	General discriminant analysis
GM-CSF	:	Granulocyte monocyte stimulating factor
HB	:	Hemoglobin
HIV	:	Human immunodeficiency virus
IFN-γ	:	Interferon gamma
IGRA	:	Interferon gamma release assay
INH	:	Isoniazid
IP	:	Inducible protein
IL	:	Interleukin
LAM	:	Lipoarabinomanan
LPS	:	Lipopolysaccharide

LTBI	:	Latent tuberculosis infection
MCP	:	Monocyte chemotactic protein
MDC	:	Minimum detectable concentration
MDR	:	Multi-drug resistant
MFI	:	Median fluorescent intensity
MGIT	:	Mycobacteria Growth Indicator Tube
MHC	:	Major Histocompatibility complex
MIP	:	Macrophage inflammatory protein
M. tb	:	Mycobacterium tuberculosis
NAATs	:	Nucleic acid amplification tests
NK	:	Natural Killer
NLR	:	Nod-like receptor
NOS	:	Nitric oxide synthase
NTM	:	Non tuberculous mycobacteria
OD	:	Optical Density
OLP	:	Oral Lichen Planus
PBMC	:	Peripheral blood mono nuclear cells
PCR	:	Polymerase chain reaction
PHA	:	Phytohaemagglutinin
PO_4	:	Phosphate
PPD	:	Purified protein derivative.
PRRs	:	Pattern recognition receptors
QFT	:	Quantiferon
QFT IT	:	QuantiFERON TB Gold In Tube
RCF	:	Relative Centrifugal Force
RD	:	Region of difference
RLRs	:	Retinoic acid-inducible gene I-like receptors
RNA	:	Ribonucleic acid
RNI	:	Reactive nitrogen intermediates
ROC	:	Receiver operator characteristics
ROI	:	Reactive oxygen intermediates
sCD40L	:	Soluble CD40 ligand
sIL-2Ra	:	Soluble interleukin-2 receptor alpha
SAA	:	Serum amyloid A
SAP	:	Serum amyloid P
T_{EM}	:	Effector memory T
T_{CM}	:	Central memory T
TB	:	Tuberculosis

TGF	:	Transforming growth factor
TH1/2	:	T helper ½
TIR	:	Toll-interleukin (IL)-1 receptor domain
TLKs	:	Toll-like receptors
TNF	:	Tumour necrosis factor
TST	:	Tuberculin skin test
TTP	:	Time to positivity
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization
WT	:	Wild type
xMAP	:	x(number of) Multiple analyte profiling
ZN	:	Ziehl Nielsen

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Chapter 1
Introduction

1.1 An Introduction to the History of Tuberculosis

Pulmonary tuberculosis (TB) dates back to the industrial revolution as one of the main causes of death (1). However it is believed that TB has been present in humans for thousands of years. Skeletal remains show that prehistoric humans (4000 BC) had TB , and tubercular decay has been reported in the spines of Egyptian mummies (3000-2400 BC) (2). In 1838-1839, more than 60 000 people in England and Wales were killed by TB (3). In 1679 Sylvius wrote his Opera Medica, in which he was the first to identify actual tubercles as a consistent and characteristic change in the lungs and other areas of patients with consumption (4). Due to the many different symptoms TB was not identified as one disease until the 1820s, and was not named TB until 1839 by J.L Schonlein. Other physicians such as Benjamin Marten came up with the concept of tiny living creatures being possibly responsible for TB and gave insight into the possibility of human to human spread through direct contact (5). Daniel (2006) asserts that the understanding of the origin and development of the disease started with the work of Theophile Laennec at the beginning of the 19th century and was advanced by the demonstration of the transmissibility of *Mycobacterium tuberculosis (M.tb)* infection by Jean-Antoine Villemin in 1865 (6). Ahasan et al (2009) report that the discovery and isolation of *M.tb* was done by Robert Koch in 1882 when he invented a technique which enabled the visualization of the 'culprit' organism (4). A public health response against TB is then argued to have begun after the discovery and isolation of the bacillus.

1.2 Tuberculosis

A few years ago (TB) was thought to be a disease of the past, particularly in the developed world. However the disease continues to be one of the largest infectious causes of death worldwide even in the 20th century and is now often described as a global epidemic (7). According to the World Health Organization (WHO) estimates, 8 million people develop active tuberculosis and nearly two million die worldwide. The WHO estimates that 36 million people will die of TB by 2020 if it is not controlled (8). *M.tb* infection is acquired by the inhalation of infected aerosols droplets which are generated by people with active pulmonary disease (9). This infectious disease is the most common cause of death in poverty-stricken countries in Africa and Asia**.** An encounter with *M.tb* gives rise to three possible outcomes: 1] a few members in the population develop primary active TB disease presenting clinical symptoms, 2] the majority of infected persons show no disease symptoms but develop an effective acquired response and are referred to as having latent infection, 3] a portion of latently infected persons will reactivate and develop post-primary active TB

(10). Studies have reported a group of individuals with TST-negative results who have transiently positive ELISpot results (11). This raises the interesting possibility that some TST-negative contacts may acquire, and spontaneously clear, a transient *M.tb* infection giving rise to another possible outcome of an encounter with *M.tb* (12).

The large number of people who gets infected remains latently infected with the pathogen and only 10 % progress to active TB within their lifetime (13). The body has the ability to form a fibrotic band/capsule around the TB bacteria which helps in keeping the infection in an inactive state (13).This further emphasises the crucial need to understand what constitutes protective immunity to TB as a step towards the development of improved diagnostics, treatment protocols and vaccines and the need for a rapid, point–of-care test that allows early detection of active TB at health clinics.

1.3 Immune response to Tuberculosis

M.tb is known to invade the alveolar space of the lung infecting the macrophages that are on the pulmonary epithelium (14). After infection the innate immune system may destroy the bacteria or the bacteria may multiply. Neutrophils migrate to the site of infection, followed by the monocytes, which then mature into macrophages; these then give rise to the tuberculous granuloma (15). At 2-4 weeks post-infection cell-mediated immunity arises and recruits new cells to the site of infection. These cells include lymphocytes, macrophages and fibroblasts. The recruitment of cells results in the chronic inflammation and caseation of the granuloma, which develops a necrotic acellular core surrounded by macrophages, epithelioid cells and Langhans giant cells, accompanied by an outer layer of fibroblasts and lymphocytes (16). The granuloma may however not be able to restrain the multiplication of the bacteria, which will then disseminate to other areas of the lung (17). The human immune system thus has the ability to either clear or contain infection by *M.tb* infection. The clearance mechanism is however far from being understood.

1.3.1 Innate immunity

A rapid immune response is an important factor that defines life and death for the host. The immune system relies on innate immunity, which is known as the first line of defense against microbial infection that engages adaptive immunity (18). Innate immunity is a rapid, non-specific response which does not generate memory. The cells of the innate immune system include natural killer (NK) cells, mast cells, dendritic cells and phagocytes (neutrophils and macrophages). The innate immune system is also associated with several receptor families (19). These receptor families include Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs). TLRs detect microbial pathogens

that include viruses, bacteria, protozoa, and fungi. The recognition of pathogen-associated molecular patterns is done through extracellular leucine-rich repeat motifs that transmit signals through the cytoplasmic Toll-interleukin (IL)-1 receptor (TIR) domain (20). RLRs differ from the TLR pathway; they recognize viral RNA which is present within the cytoplasm (21). RLRs proteins have a RNA-binding helicase domain and two amino (N)-terminal caspase recruitment domains (CARDs) which are important for the propagation to the interferon-regulatory factor and NF-κB signaling pathways (22).

NLRs are intracellular sensors that have an important role in innate immunity and inflammation (23). A group of NLR family members form multiprotein complexes which are known as inflammasomes, they also have the capability to activate the cysteine protease caspase-1 in response to a wide range of stimuli including both microbial and self-molecules (18). NLRs are responsible for inducing the recruitment of the apoptosis associated speck-like protein (ASC) containing CARD which leads to the activation and processing of pro-IL1β and IL-18 through caspase-1 (24). In this review we will discuss the mechanisms by which the inflammasome is activated in cells emphasising the role of the inflammasome in host defense.

1.3.2 The inflammasome

The complex formed by NLR molecules, caspase-1 and the adaptor molecule ASC is termed the inflammasome (25). The central effector molecule of the inflammasome is the cysteine protease caspase-1 that upon activation cleaves pro-IL-1β, pro-IL-18 to their active forms. Recent studies have shown that the NLRP1, NLRP3 and NLRC4 inflammasomes have an important role in host defense (18). The inflammasome is an important innate immune pathway that regulates two host responses protective against infections. Different types of inflammasomes have been identified: they are multiprotein complexes which contain pattern recognition receptors belonging to the Nod-like receptor (NLR) family or the PYHIN family and the protease caspase-1 (26). The one pathway involves the secretion of proinflammatory cytokines IL-1β and IL-18 and the other involves the induction of a pyroptosis, which is a form of cell death. Production of IL-1β and IL-18 has been shown to be protecting against many infectious agents including *M.tb* (27).

1.3.3 Inflammasome Activation Requirements

The responses of dendritic cells, macrophages and monocytes to microbial threats are very important to host defense (28). However differences in the inflammasome activation of these cells have been reported. Monocytes have constitutively activated caspase-1; the activation of caspase-1 in dendritic cells and macrophages has to be induced. ATP is an important molecule required for inflammasome activation. Monocytes release endogenous ATP (29) whereas macrophages and

dendritic cells lack endogenous ATP rendering them incapable of IL-1β secretion with one single stimulus (30). This unique characteristic of monocytes enables them to have a single TLR stimulus for IL-1β secretion whereas dendritic cells and macrophages require a double stimulation TLR For example LPS and NLR (ATP) stimuli (30).

The reason for this differential regulation is thought to be due to the different cells' adaptation to their respective environments. Dendritic cells and macrophages are constantly exposed to microbial pathogens and thus require a mechanism that provides a second checkpoint to avoid deleterious inflammation. Monocytes on the other hand function in a pathogen-free environment and thus must respond rapidly to a microbial threat (18). Recent studies have shown that the field of NLRs and inflammasome is an important area of innate immunity and inflammation (18, 25). A better understanding of the inflammasome and IL-1β production as well as inflammasome activation in cells other than macrophages and dendritic cells may prove useful for treatment of infectious diseases.

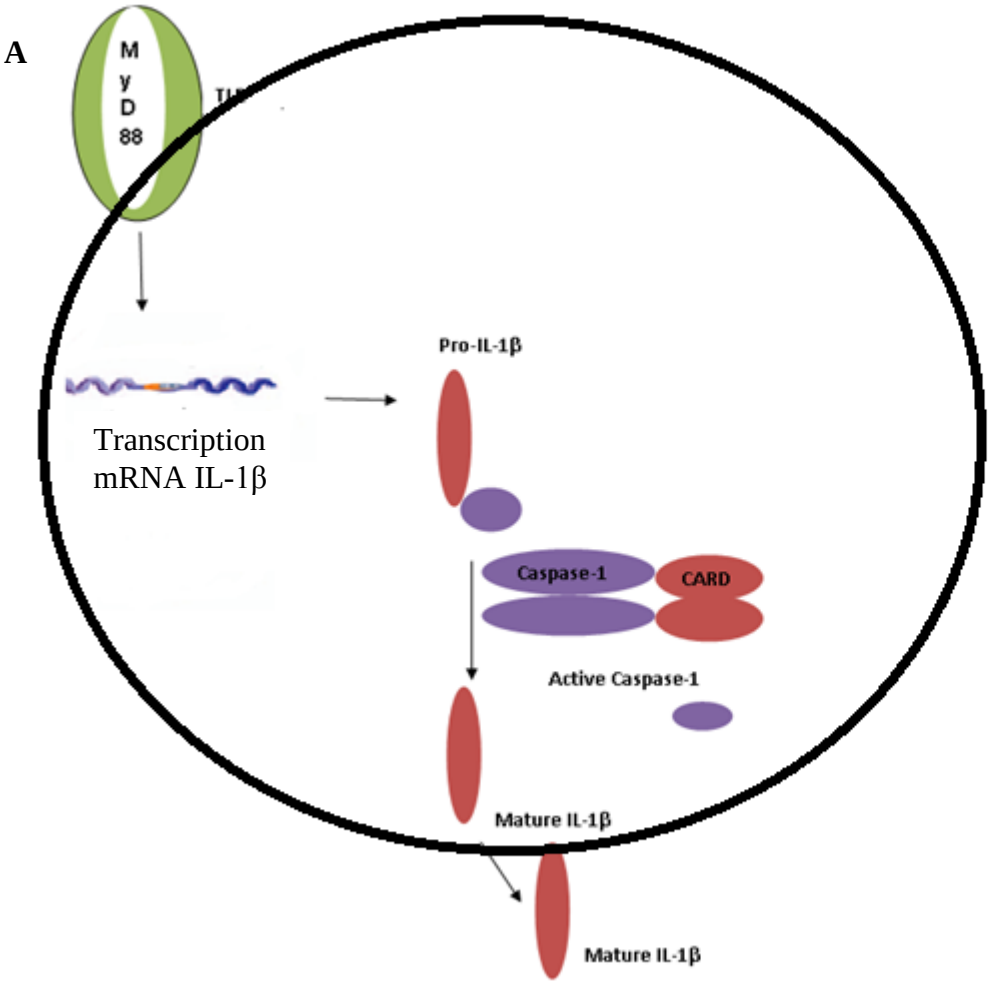

Figure 1.1. Diagram representing the differential caspase-1/IL-1b activation pathways in monocytes. Caspase-1 is constitutively activated in monocytes, and these cells release mature IL-1b after single stimulation with TLR ligands. IL-1b secretion is induced by endogenously released ATP. Figure re-produced with permission from.doi:10.1371/journal.ppat.1000661.g002(30).

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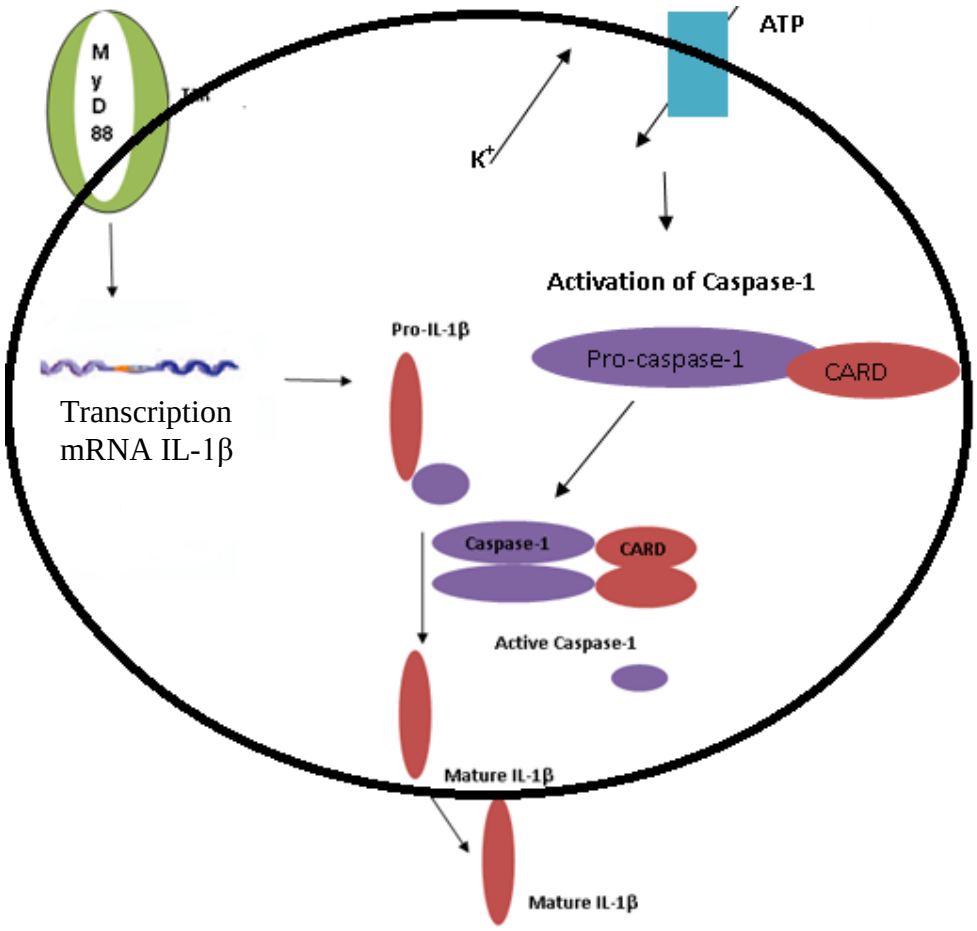

Figure 1.2. Diagram representing the differential caspase-1/IL-1b activation pathways in macrophages. In contrast to monocytes, macrophages need a double stimulation: one stimulus (TLR-ligands) induces transcription, and a second stimulus (ATP) induces IL-1b secretion. Figure re-produced with permission from.doi:10.1371/journal.ppat.1000661.g002(30).

1.3.4 The role of the inflammasome in tuberculosis infection

One of the most important characteristics of the inflammasome remains its ability to activate IL-1β, a very powerful proinflammatory cytokine that affects virtually every organ. Studies have shown that it has a protective function in several bacterial, viral and fungal infection models. Several studies in humans have also shown that inhibiting the process responsible for IL-1 using the IL-1R antagonist IL-1ra (Kineret) is associated with increased susceptibility to bacterial infection. IL-1 exerts its protective action against infections by activating several responses including the rapid recruitment of neutrophils to inflammatory sites (31).

IL-1β and IL-18 secretion by macrophages infected with *M.tb* has been reported to be dependent on NLRP3 and ASC but not NLRC4 (32). Inflammasome activation in this instance requires mycobacterial secretion of ESX-1 (33). Mice with IL1r1−/− and IL-18−/− are very susceptible to *M.tb* infection (34). Casp1−/− and Asc−/− mice are also more susceptible than WT mice due to defective granuloma formation (35). However, the resistance of NLRP3−/− mice to *M.tb* infection is not significantly different from that of WT mice, suggesting the existence of other pathways for inflammasome activation during *M.tb* infection. The production of IL-1β during *M.tb* infection was reported to occur also in a caspase-1-independent fashion (36). The discovery of the inflammasome and all the components that it is composed of has raised a lot of interest in cytokines such as IL-1β and the possible role it plays in host defence. The greatest challenge for the future remains the determination of the effector mechanisms in the pathogenesis of infectious diseases and the discovery of clinical interventions to prevent deleterious responses whilst enhancing the protective ones.

1.3.5 Macrophages

Monocytes and macrophages are phagocytes that act in both innate immunity (non-specific defences) and they assist with the initiation of defence mechanisms during adaptive immunity. Once *M.tb* is inhaled into the lungs the organisms are typically engulfed by alveolar macrophages, which will then secrete proteolytic enzymes and cytokines that exhibit antimycobacterial effects (37). These cytokines include IL-1, IL-6, IL-10, TNF-α and TGF-β. Once the macrophages have ingested the bacteria, dendritic cells will assist in the phagocyctic process (38). The bacterial uptake involves receptors (complement receptor 1, complement receptor 3, mannose receptor and type A scavenger receptor) on the surface of the phagocytes which recognise and bind the bacteria or the surface proteins (39).Some of the mechanisms by which macrophages eliminate *M.tb* include reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and phagosome-lysosome fusion.

Oxidative burts, phagosome-lysosome fusion, production of reactive nitrogen intermediates and cytokine production including IFN-gamma are involved in the macrophage defense against mycobacterium (40). TNF-alpha and IFN-gamma appear to have a synergistic effect on mycobacteria from macrophages in murine cell cultures. These two cytokines initiate the production of reactive nitrogen intermediates (RNI) by activating the inducible form of nitric oxide synthase (NOS2) which is highly expressed in patients with active TB (40).In addition, IL-6 and IL-4 also induce antimycobacterial antibody in macrophages (41). The macrophages also combat mycobacterial reproduction by fusion of the vacuole containing the mycobacteria with the lysosome or by decreasing the pH . If the macrophage can decrease the pH to 5.8, bacterial growth is inhibited, and at a pH of 5.3 bacterial growth is arrested (42)

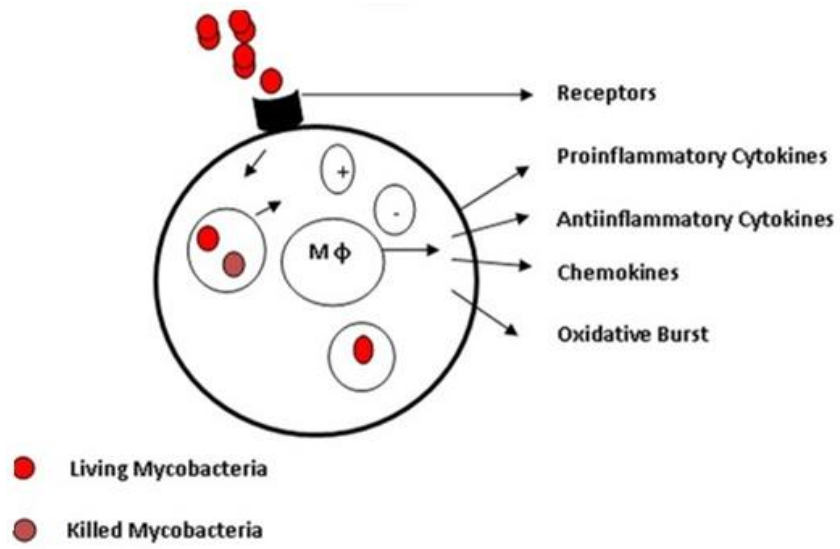

Figure 1.3. Macrophage response to encounter with mycobacteria (40).

1.3.6 Cytokines

Cytokines are proteins that are produced by cells. Cytokines play a role in the interaction with cells of the immune system to regulate the body's response to disease and infection (43). Cytokines also mediate normal cellular processes in the body. These cytokines are diverse and have different functions. The body produces colony stimulating factors which stimulate production of blood cells, growth and differentiation factors that function primarily in development and immunoregulatory and proinflammatory cytokines such as interferon, interleukins, and TNF-alpha that function in the immune system (44). The roles of cytokines are classified based on their secretion pattern either by Th1, Th2, Th17 and T regulatory cells amongst others. Th1 cells secrete IFN- y, IL-2 and Lymphotoxin and are known to drive protective immune response in TB while Th2 cells produce IL-4,-5.-6.-9,-10 and -13 (45).

1.4 Adaptive immune phase

The second line of defence is the adaptive immune system which is more specific and generates memory, It comprises of B and T lymphocytes (46). During the adaptive phase in response to *M.tb* infection antigen-presenting cells engage T cells which generate effector memory T (T_{EM}) and central memory T (T_{CM}) cells. B cells are also activated and *M.tb* specific antibodies are produced. Fortunately some individuals have the ability to clear the infection prior to progression into the adaptive phase. The majority of exposed individuals enter into the quiescent phase whereby the bacterium is contained in granulomas limiting its ability to replicate and disseminate. It is important to note that the bacterium is not eradicated in this phase. This immune phase is characterised by

Th2-type responses which are associated predominantly with IL-4 production and regulatory T cell phenotypes that limit immunopathology (47). The bacteria can escape immune control and enter into the replicating phase which is symptomatic. In the replicating phase the granulomas are disrupted, the acute-phase response is activated and the levels of pro-inflammatory markers are increased. Th cell balance is disrupted and as a result increased immunosuppression becomes evident (48).

1.5 Latent infection

Latent infection with *M.tb* is described as the presence of *M.tb* within the host whilst the infected individual remains asymptomatic. A more evolving concept is that the definition of latent TB encompasses a diverse range of individual states ranging from those who have completely cleared the infection to those who are incubating actively replicating bacteria in the absence of clinical symptoms (49). Although latent infection may last a lifetime in a host it can be detected using the TST and the IGRAs. In latent TB the hosts generate an immune response that is potent and stops the bacillus from growing and then enters into a stationary phase, eventually becoming non-replicating while retaining the ability to resume growth under favourable circumstances within the granuloma (50).

The large number of latently infected individuals world-wide poses a major risk for TB reactivation and subsequent transmission (51), which is why TB biomarker studies should focus mostly on this group. The low number of people who progress to active disease suggests/supports the existence of natural immunity to *M.tb* (52)**.**

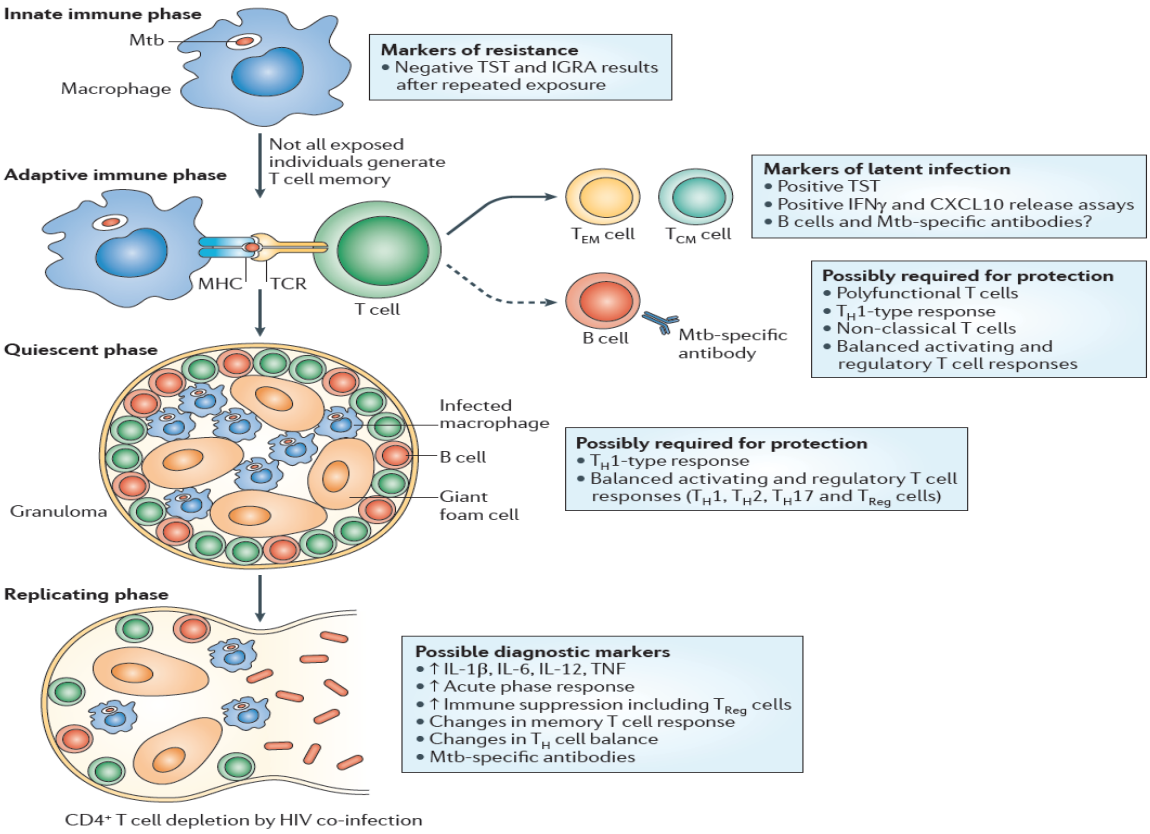

Figure 1.4: Immune response and potential host biomarkers of Mycobacterium tuberculosis exposure and infection. Figure adapted from *www.nature.com/reviews/immunol 2011*(48).

1.6 Host biomarkers

The identification of biomarkers is essential for better diagnosis, prevention and treatment of TB (53). There is currently a lack of an effective TB vaccine which further emphasizes the need for correlates of protective host immune response. The growing number of individuals with M.tb which is resistant to current TB drugs necessitates the development of newer and more efficient drugs. Thus biomarkers that indicate disease status could be helpful in boosting the development of better drugs, vaccines and lead to quicker diagnosis of disease (54). Literature in recent years has reported progress in the quest for TB biomarkers (48,54).

1.7 Tuberculosis diagnosis

In 2003 the World Health Organisation (WHO) reported that the Directly Observed Treatment Short Course (DOTS) programs managed to successfully treat 84% of new smear positive patients. However, these programs were only able to detect 28% of the estimated TB patients in the world (8). Thus, it is unlikely that the goal of reaching the target of 70% case detection by 2013 will be met unless interventions are made to increase the case-detection rate. Early diagnosis and effective therapy form the key elements of the TB control program. A delay in the diagnosis will

result in increased transmission as it is said that an untreated smear-positive patient could possibly infect ten contacts annually (55), Delayed diagnosis may also lead to a more advanced disease state which contributes to the increased mortality rate. This further emphasises the urgent need for biomarker identification and rapid diagnostic tools.

1.7.1Smear Microscopy

The diagnosis of TB is based on the identification of acid-fast bacilli, which utilises sputum smear microscopy (56). Smear microscopy is the simplest and most cost effective diagnostic test. Studies evaluating the cost effectiveness of diagnostic techniques have identified that reagents and equipment utilised for smear microscopy as more affordable compared to those required for other techniques such as the GeneXpert (57). WHO estimates that the test only identifies 35% of patients with active TB (86). Literature has shown that this method has low sensitivity especially in children, in patients with extra pulmonary TB and patients with TB–HIV (human immonodefiency virus) co-infection (58). Despite the shortcomings of the test it still serves as the front line tool for active TB diagnosis. This is due to the fact that more definitive culture techniques take longer and because it has the ability to identify the most infectious patients. Other techniques are used to identify active TB such as nucleic acid amplification tests (NAATs), culture, radiological examinations and Ziehl-Neelsen acid fast staining (59).

1.7.2 Radiological examinations

Radiological examinations include chest x-rays and computerised tomography (CT) scans. Chest x-rays are used to check for lung abnormalities in people with symptoms of TB disease (60). This technique is not specific as many other diseases can produce similar features in the lung. Hence the test is unable to confirm that a person has TB disease and is thus imperfect as a ‘rule in’ test. Other limitations to this test include its inability to distinguish between past, cured TB from current active disease since scarring in the lung remains after a previous TB infection (61). The chest x-ray has been reported to have poor sensitivity during the early stages of disease as the damage to the lungs may not be significant enough to detect so a large number of people with active TB are missed and in 40% of patients with extra or non-pulmonary TB the chest x-ray is not helpful (61). In some hospitals CT scans have proved useful for imaging TB lesions however this is particularly in the brain and spine. CT scans are therefore often used to identify non-pulmonary TB (62).

1.7.3 Ziehl-Neelsen acid fast staining

The Ziehl-Neelsen (ZN) staining technique is used to demonstrate acid-fast bacteria which belong to the genus of mycobacterium and this includes the causative agent for TB (63). This technique combines staining with microscopy after culturing. The presence of *M.tb* in the sample is identified by small red rod shaped organisms under a microscope (64). This technique was used to discriminate TB cases from controls in this study and is explained in detail in chapter 2.

1.7.4 Culture

Culture techniques are the gold standard for diagnosing active TB. Research has shown that this technique is highly sensitive (65). *M.tb* can be cultured from a variety of specimens (sputum, cerebro spinal fluid (CSF), pleural effusions and bronchoalveolar lavage (BAL) and can thus be used to detect pulmonary as well as non-pulmonary disease (66). The ability of researchers and scientists to assess the effect of antibiotics on the cultured bacilli allows for the identification of antibiotic susceptibility of the particular strain of *M.tb* infecting the patient. This important characteristic allows the technique to identify multi-drug resistant (MDR) TB. The test has high specificity and with the aid of polymerase chain reaction (PCR) assays after culture one can distinguish *M.tb* from other mycobacteria. An important drawback of this test is the time it takes to obtain results which could be anything from 2 to 6 weeks (67). The delay in time to results contributes to the increased number of new infections annually (68).

1.7.5 NAAT

Nucleic acid amplification tests (NAATs) such as polymerase chain reaction (PCR), are recently used for active TB testing. Although NAATs have the ability to magnify the smallest amounts of genetic material, the sample used for the test has to contain a certain number of TB bacilli which is not always possible to obtain. This is often experienced in non-pulmonary TB where sensitivity has been reported to be as low as 60% (69). To improve on the sensitivity of the test the laboratory has to culture the sample and allow the bacilli to multiply before carrying out the PCR test which can take several days or weeks. NAATs are mainly used to rule out infections caused by atypical mycobacteria in a sputum smear positive patient prior to obtaining culture results. This helps treatment to be initiated quickly, with the therapy then being tailored to the patient based on the culture results obtained six weeks later. Other studies have reported the use of NAATs to identify MDR TB. This was made possible by identifying mutations in the DNA of *M.tb*. These methods appear to be quicker than culture but they generally only identify resistance to rifampicin and isoniazid (70).

1.8 Diagnosis of latent *M.tb* infection

The diagnosis of latent tuberculosis (LTBI) has proven to be challenging in the field of medicine, this is mainly due to the difficulty in identifying 'latent bacilli' with the current evolving technology (71). Immunodiagnostic techniques such as the tuberculin skin test (TST) and interferon gamma release assays (IGRAs) are widely used to successfully diagnose LTBI (72).

1.8.1 Interferon gamma release assays

The first IGRA was developed /approved in 2001 and was based on the use of purified protein derivatives (PPDs).PPD from mammalian tuberculin and PPD from *M. avium* were used as the test antigens and with phytohaemaglutinin (PHA) as the positive control. This PPD-based QFT test was approved by the United States Food and Drug Administration (FDA) in 2001 and guidelines regarding its use as an aid in the diagnosis of LTBI subsequently published by the Centres for Disease Control and Prevention (CDC) in 2003 (73). This PPD based QFT test was shown to be useful in the diagnosis of LTBI. However, this QFT version was later replaced by the region of difference 1 (RD1) (ESAT-6 and CFP-10) antigen-based test called the 'QuantiFeron® -TB Gold' (QFT G) test, which was approved by the FDA in May 2005 and guidelines regarding its use published by the CDC in December 2005 (74). RD1 is a genomic segment that has been reported to be found in *M.tb* complex but absent from all other strains of *M.bovis* BCG and almost all environmental mycobacteria (75). RD1 gene products have been reported to have the potential to provide a platform for the development of new diagnostic tests that might differentiate *M.tb* infection from BCG vaccination and exposure to environmental mycobacteria (76). In 2008 the FDA approved the QuantiFeron-TB Gold In Tube (IT) test (QFT IT), which was used to test for LTBI in this thesis (75); the procedure is further explained in chapter 2.

Interferon gamma release assays are based on the principle that individuals who have been exposed to *M.tb* at any point in their lifetime have circulating pre-activated T cells which rapidly respond by secreting IFN-γ upon re-encounter of *M.tb* antigens (77). IGRAs measure cell mediated immune responses to *M.tb* infection by detecting IFN-γ released by sensitized lymphocytes *in vitro* (77). Different types of IGRAs are commercially available ranging from those that employ whole blood such as QFT tests and those that employ peripheral blood mononuclear cells (PBMCs) such as the T-Spot *TB*. In the QFT test *M.tb* specific antigens are used to stimulate whole blood from which the supernatants obtained after overnight incubation is harvested and the IFN-γ released by the sensitized lymphocytes is quantified by Elisa. The T-Spot TB test employs PBMCs which are seeded into test panels and stimulated with RD1 antigens (ESAT-6, CFP-10) after which IFN-γ secreting T cells are enumerated using Elispot.

1.8.2 The tuberculin skin test

The purified protein derivative (PPD) was originally administered by Robert Koch in 1890 as a possible therapeutic reagent for TB (78) it is a crude mixture of mycobacterial antigens, some of which are found in the Bacille Calmette Guerine (BCG) vaccine strains and many non-tuberculous mycobacteria (NTMs). PPD is used in the tuberculin skin test, which has since become the oldest diagnostic test to date (79).

The tuberculin skin test (TST) measures cell mediated immune (CMI) responses to *M.tb* in the form of a delayed type hypersensitivity reaction (80). Results are read 48 to 72 hours following PPD administration. Based on the route of administration and the manner of interpreting/reading results three different forms of the tuberculin skin test are available. The Mantoux test was developed in 1908 when a French physician Charles Mantoux administered diluted solutions of PPD intracutaneously and the induration of the reaction is measured transversely to the long axis of the forearm (48 to 72 hours after test application) and the results recorded in millimeters (80). The Heaf test uses undiluted PPD, which is injected subcutaneously using a multipicture device containing six needles. The Tine test differs from the Heaf test in that it uses a four-pronged disposable puncture device (80). This test was used to identify TB infection and the procedure is explained in detail in chapter 2.

1.8.2.1 Limitations of the tuberculin skin test

The test has been reported to have poor specificity in populations with high BCG vaccination coverage, and in populations where there is high exposure to NTMs, as a result of cross reactivity between shared antigens (79). The sensitivity of the test has been shown to be poor in immnunocompromised subjects including HIV infected individuals (possibly due to anergy), and in patients on immunosuppressive therapy and children (81).

The above mentioned shortcomings of the diagnostic tests highlight the need for better diagnostic tools. The development of new diagnostic technologies is limited by the availability of well-characterised, easily accessible clinical specimens from patients with and without TB (82). Diagnostic studies should consider obtaining samples from specimen banks were a large number of different body fluids are collected from participants for the identification of biomarkers. This could aid in early diagnosis and contribute to the fight against TB (82).

1.9 Tuberculosis Biomarkers

Biological markers have a long history in research (83) and the quest to identify the best biomarkers in TB still continues in the 21st century. The need for biomarkers in TB is very important and specifically so in patients who are latently infected. Biomarkers could help indicate the risk of reactivation and identify potential protection by vaccines. The identification of biomarkers would assist in patients with active disease to predict the success of the treatment and to identify the chances of relapse in people on treatment (9). In doing so the rate of development to MDR may be reduced as patients will be placed on the correct treatment regime earlier. The identification of biomarkers could shorten the lengthy periods of clinical trials and thus increase the pharmaceutical industry's capability to develop more efficient anti-TB drugs.

Biomarkers can be used to differentiate between active or latent disease, predict treatment response and may serve as correlates of risk or protection post vaccination. One of the major challenges facing biomarker studies is the detection of latent tuberculosis; this is mainly due to the absence of a gold standard test for latent infection (9). The concept of a spectrum of latency underlines the challenge of developing a single biomarker that would differentiate active or latent TB. Instead, biomarkers that provide a position on the spectrum will need to be developed so that the relative risk of reactivation for an individual can be assessed (10).

1.9.1 Potential biomarkers for active TB

A recent study investigating potential host biomarkers in blood or blood cells reported IL-10, IL-6 and IP-10 to be among the most promising candidates for the diagnosis of active TB as reported by a number of articles investigating both unstimulated and stimulated samples (53). Studies have further shown that IGRAs alone are not able to distinguish between active TB disease and latent infection; they are currently used to assist active TB diagnosis together with sputum smear microscopy and radiological examinations (72,84). The capability of the above mentioned markers to differentiate between active TB and latent infection needs to be evaluated in the future.

1.9.2 Potential biomarkers for correlates of protection

The majority of people who are infected with *M.tb* remain asymptomatic. The protective host immune responses to TB that help to contain the pathogen are not fully understood yet. Recent studies have identified FOXP3, IL-4 and IL-12 as the most promising biomarkers which are differentially expressed in active TB in comparison to latently infected individuals (53). The identification of protective immune responses in such healthy infected individuals would define

correlates of protective immunity in TB (85). Identifying the correlates of protective immunity could be useful for vaccine efficacy studies. The search for biomarkers with such discriminative ability was undertaken in this thesis (chapter 3). The focus was on the validation of IL-1β and IL-17 as potential host biomarkers for protective immunity in exposed uninfected children.

1.10 Study Hypotheses and Objectives

1.10.1 Hypothesis 1

Children with negative IGRA's and TST following exposure to *M.tb* represent a highly protected immunological phenotype that will be distinct from responses in children without prior exposure.

1.10.1.1 Objective1

To validate the increased levels of IL-1β and decreased IL-17 in children who are tuberculosis exposed but remain uninfected compared to those who are exposed/infected and unexposed/uninfected.

1.10.1.2 Objective 2

To identify the protective immunological phenotype in children with negative IGRA's and TST following exposure to *M.tb.*

1.10.2 Hypothesis 2

Levels of host immune markers expressed in saliva and serum will be highly correlated and both sample types can be used to discriminate between TB cases and controls. Thus saliva could potentially represent a new sample type for TB diagnosis.

1.10.2.1 Objective 3

To evaluate a number of cytokines in both serum and saliva samples of identified TB cases and controls and evaluate saliva as a possible new diagnostic sample type.

CHAPTER 2

Methodology

CYTOKINE PROFILES IN CHILDREN WITH DOCUMENTED *MYCOBACTERIUM* TUBERCULOSIS EXPOSURE AND INFECTION, EXPOSURE AND NO INFECTION COMPARED TO NO EXPOSURE NOR INFECTION

2.1 Study Setting

In 2009 the TB incidence in South Africa was 948 per 100 000 (86), the Western Cape Province was among the provinces with the highest rates of cases reported. The annual rate of TB infection was reported to be 3.5% in the period 1998 to 1999 in the Ravensmead/Uitsig community and these numbers increased significantly to 4.1% in 2005(86). Newborns in this area receive BCG vaccination and a TST is routinely performed in the study area in children younger than 5 years of age and in HIV-infected adults to guide preventative TB therapy.

2.2 Participants

The study intended to investigate children ≤5 years who are HIV negative and who were exposed to an adult, smear positive TB case in their households within 2 months prior to recruitment into the study. Children with a positive TST and a positive QuantiFeron Gold In tube (QFT IT) test (infection-susceptible phenotype) were compared to children who were also exposed but who tested negative in all these tests (infection-protected phenotype). An unexposed control group with negative tests of *M.tb* infection were included as controls. These *M.tb* infection parameters had to remain unchanged for at least 6 months.

Table 1. Classification of participants

Marker	**Exposed Infected (N=36)**	**Exposed Uninfected (N=47)**	**Unexposed (N=37)**
TST	Positive	Negative	Negative
QFT IT	Positive	Negative	Negative

The children were compared at baseline and again at month six as per scheduled study visits in an ongoing large community-based TB household contact diagnostic study. Children with an *M.tb*

exposure gradient >4 were considered as significantly exposed, while those with a score of ≤3 were considered unexposed. The *M.tb* contact score (see section 2.5) incorporated measures of the proximity, intensity and duration of the child's exposure to an adult with TB and correlated well with measures of *M.tb* infection in children in our setting (87).

2.3 Inclusion criteria

Children who are participating in a larger immunological and diagnostic community-based contact study in communities in Cape Town with high burden of TB and low HIV-prevalence (Ravensmead, Uitsig and Site C) were eligible for this immunological sub study. Children with complete data regarding *M.tb* contact score (this value quantifies the extent of *M.tb* exposure and therefore was used as proxy for infection) of ≥4 for exposed and <4 for controls were included. Both infection and exposure status had to remain unchanged for the next 6 months (in other words no new exposure and baseline TST and QFT IT result had to remain unchanged throughout the six month follow up).

2.4 Exclusion criteria

Children who developed disease at any time point during the study or with HIV infection were excluded. As this was a validation study of a study conducted in our department which identified the cytokines IL-1β and IL-17 to be differentially expressed between children who were exposed to an adult TB case in their household within the past 3 months but who remained uninfected and children without TB exposure in their household who were uninfected according to IGRA tests. All participants that were used in the initial study were excluded. It is important to note that INH therapy was not an exclusion criterion since all children <5 years with a TB contact require INH according to national guidelines.

2.5 Calculation of exposure gradient

Caregivers of children were interviewed to determine each participant's extent of contact with a TB index case during a typical 7-day week. A model developed by Hesseling et al (87) was modified and used for calculation of the resulting gradient of exposure. The calculation is based on the assumption that the grade of *M. tb* exposure (contact score) = infectivity of the index case + duration of exposure to the index case + proximity of the exposure + relationship of the contact to the index case. The score has been developed to provide participants without any known contact to a TB case a value of zero. The full definition and components of the TB contact score is shown in table 2.

Table 2: Formula for calculating *M.tb* contact score

Variable	Weight assigned
Relationship to Tuberculosis index	
No known Tuberculosis contact	**0**
Non-household Tuberculosis contact	**1**
Relative/other contact in household with Tuberculosis	**2**
Secondary caregiver (care provider during day) in household with Tuberculosis	**3**
Primary caregiver in household with Tuberculosis	**4**
Infectivity of TUBERCULOSIS index case	
No known Tuberculosis contact	**0**
Sputum acid-fast negative	**2**
Sputum acid-fast positive	**4**
Type of exposure to Tuberculosis index case	
No known Tuberculosis exposure	**0**
Lives and sleeps in different house	**1**
Lives and sleeps in same house	**2**
Sleeps in same room	**3**
Duration (total hours) average contact per day with Tuberculosis index case	
No known Tuberculosis contact	**0**
0-3 hours	**1**
4-7 hours	**2**
8-11 hours	**3**
≥ 12 hours	**4**
Total contact score (maximum = 15)	

Table reproduced from Hesseling et al. (2009).

2.6 QuantiFeron-TB Gold In Tube (IT) test (QFT)

The QFT test is a diagnostic test that uses a peptide cocktail based on Esat-6, CFP-10 and TB 7.7 (p4) proteins to stimulate cells in heparinised whole blood. It is an indirect test for *M.tb* infection. At TST was done according to published methods (88). A QFT test was performed according to manufacturer's instructions (Cellestis, Carnegie, Victoria, Australia). The QFT test was performed

in two stages, the first stage involved the collection of 1ml of whole blood into each of the Quantiferon-TB Gold tubes including a nil tube which served as the control tube, a TB antigen tube which is coated with the peptide cocktail from ESAT-6, CFP-10 and TB7.7 and a mitogen tube as a positive control. The tubes were then incubated at 37^{O}C for 16-24 hours (overnight) with 5% CO_2.

In the second stage the tubes were centrifuged at 2000-3000 RCF for 15 minutes before the plasma was harvested and assayed for IFN-γ production by Enzyme-Linked Immunosobent Assay (Elisa). The Elisa plate was coated with the antibody-enzyme conjugate and samples, controls and standards were added to the appropriate wells. The antibody part of the conjugate binds to IFN-γ which is produced in response to the proteins ESAT-6, CFP-10 and TB7.7. The plates were then incubated at room temperature for two hours to allow the reaction to take place and thereafter it was washed with wash buffer which was prepared as per manufacturer's instructions. Enzyme substrate solution was then added to each well and mixed thoroughly using a microplate shaker.

The plate was then incubated for 30 minutes at room temperature in the dark to avoid direct exposure to light. Enzyme stopping solution was added to each well in the same order as the substrate and mixed again using a microplate shaker. The Optical Density (OD) of each well was measured using a microplate reader fitted with a 450nm filter and with a 620nm-650nm reference filter. The Quantiferon-TB Gold IT Analysis Software was used to analyse the raw data and calculate the results. A standard curve was generated and test results for each participant were obtained. The rest of the sample was aliquoted into 0.5ml tubes and stored at -80°C for further use in Luminex assays. T-SPOT®.TB test was performed on the participants as an additional test to identify infection. However,as not all participants had results for this test it was excluded from analysis.

2.7 Luminex multiplex immunoassay

The Luminex Assays were divided into two kits one of which investigated interleukin (IL)-17, IL-1β, IL-6, IL-2, IL-10, IL-1α and interferon inducible protein 10 (IP-10) using the standard antibody covered beads and the second set of kits investigated IL-23, IL-33 and IL-21 using magnetic beads. The immunoassays were performed on the QFT supernatants according to manufacturer's instructions (Milliplex, cat no.MPXHCYTO-60K and HCYP2MAG-62K, Millipore, Billerica, MA, USA). The 96-well filter plate was prewetted with assay buffer and shaken on a plate shaker for 10 minutes at room temperature. The assay buffer was then removed by vacuum. Quality controls and standards which were provided with each kit were added to the appropriate wells to measure the precision of the selected cytokines. An additional interplate control from a healthy volunteer was added into each of the plates.

The samples were then added to the appropriate wells in duplicate on the same plate as indicated by the template prepared prior to the experiment. The antibody covered beads were sonicated separately and then mixed together. Thereafter 25ul of the mixture was added into each well. The plates were then sealed and incubated with agitation on a plate shaker for 1 hour for the standard beads and 2 hours for the magnetic beads. The fluid was removed by vacuum and then the plates were washed twice. The magnetic plates were washed using a plate washer (Bio-Tek ELx405). The plate was allowed to soak on a magnet for 60 seconds to allow complete settling of the beads. All contents were removed by aspiration.

The wash protocol was as follows: soak for 60 seconds, aspirate, dispense, soak, aspirate, dispense, soak, aspirate. Detection antibodies were added to each well of the plates and incubated with agitation for 30 minutes for the standard beads and for one hour for the magnetic beads after incubation Streptavidin-Phycoerythrin was added to each well and incubated for 30 minutes on a shaker, the plates were washed and sheath fluid was added to all the wells and placed on the shaker for 5 minutes. The beads were analysed on the Bio-plex array reader (Bio-rad, Hercules, CA, USA). The median fluorescent intensity (MFI) was determined using a spline curve-fitting (standard curve) method for calculating cytokine/chemokine concentrations in samples.

DIFFERENCES IN THE LEVELS OF HOST MARKERS DETECTED IN SALIVA AND SERUM AND THEIR POTENTIAL FOR DIAGNOSING TB DISEASE

2.8 Study Setting

The aim of the work in this section (chapter 4) was to assess the levels of cytokines detected in saliva of TB cases and controls in comparison to the levels detected in serum and to evaluate if any of the markers detected in serum and saliva discriminates between the TB cases and controls and therefore warrants further investigation as serum or saliva diagnostic markers for active TB. This was done by measuring the cytokine profiles in saliva and serum samples of community participants. Saliva as sample type for TB diagnosis would have several advantages over blood, including the non-invasive collection, decrease of biohazard risk to health care workers and ease of collection.

2.9 Participants

TB suspects were recruited from the Fisantekraal community in the outskirts of Cape Town, South Africa, as part of the ongoing EDCTP funded African European Tuberculosis Consortium (AE-TBC) biomarker study (www.ae-tbc.eu). Recruitment of the study participants began in October 2010 but was intentionally delayed in the first year, in keeping with the objectives of the study (conduction of pilot studies on limited participant numbers to guide the main trial). At the time this study was conducted, 43 TB suspects had been enrolled and TB disease was already confirmed in 11 of these participants.

Participants were eligible for the study if they presented to the health care facility with symptoms suggestive of active TB. Briefly, all study participants presented with persistent cough lasting ≥2 weeks and least one of fever, malaise, recent weight loss, night sweats, knowledge of contact with a TB patient, shortness of breath, hemoptysis, chest pain or loss of appetite. Participants were eligible for the study if they were aged 18 yrs or older, willing to give written consent to take part in the study and to have their HIV status tested or be willing to have their HIV infection status disclosed to the study field workers. Patients were excluded from the study if they had not been residing in the study area for more than 3 months, of they were severely anaemic (HB<10g/l), if they were already on anti-TB treatment, had received anti-TB treatment in the previous 90 days and if they were on quinolone or aminoglicozide antibiotics in the past 60 days. At enrollment, a case report form was completed for each participant after which blood was collected into serum tubes in addition to other samples including sputum that were required for the main trial, and saliva into salivette tubes (Sarstedt) as described below. The study was approved by the Committee for Human Research of the University of Stellenbosch and City Health, City of Cape Town.

2.10 Sample collection and diagnostic tests

Blood was collected into 4ml plain BD vacutainer® tubes (BD) and transported at ambient conditions to the laboratory. The tubes were then centrifuged at 2500rpm for 10 minutes after which serum was collected, harvested, aliquoted and frozen (-80˚C) until use. Saliva was collected from all participants into Salivette tubes (Sarstedt) according to the instructions of the manufacturer. Saliva samples were then transported on ice (4^{O}C) to the laboratory after which the tube was centrifuged for 2 minutes at 1000xg and the saliva harvested and aliqouted into four labelled tubes 1ml into each tube and kept at -80°C until analysis. 25 µl of saliva was used in the Luminex experiment and the remaining aliqouts were stored.

Sputum samples collected from all participants were cultured by the MGIT method. Confirmation of acid fast bacilli in the positive MGIT cultures was done by the Ziehl-Neelsen method, after which PCR tests were performed to confirm the presence of *M.tb* complex organisms. Participants with

positive *M.tb* complex sputum cultures were classified as TB cases. Participants with positive MGIT cultures were excluded from the study when the time to positivity for the culture result was less than 4 days, which would suggest culture contamination. All sputum and saliva samples were processed in a biological safety level 3 (P3) laboratory.

2.11 The MGIT method

One sputum sample that contained no preservatives was obtained from all participants and cultured for the detection and recovery of mycobacteria using the Mycobacteria Growth Indicator Tube (MGIT). The MGIT tube contains modified Middlebrook 7H9 broth base. This complete medium, which also contains OADC enrichment and PANTA (polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin) antibiotic mixture is reportedly the most commonly, used liquid media for the cultivation of mycobacteria. OADC is a mixture of Oleic Acid, Albumin Fraction V, Bovine, Dextrose, catalase powder and sodium chloride it is added to middlebrook 7H10 and 7H11 base media as a supplement to enhance the growth of mycobacteria. Oleic acid is a long-chain fatty acid which is utilized by the mycobacterium species in their metabolism. The dextrose is a source of energy. The albumin binds toxins to protect the bacilli from toxic agents and the catalase catalyzes the reaction of iron with molecular oxygen to stimulate revival of damaged bacilli (PML Microbiologicals, technical data sheet #870 Rev.2).A fluorescent compound is embedded in silicone on the bottom of the tube. This fluorescent compound is particularly sensitive to the presence of oxygen which is dissolved in the broth. Initially the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. As the duration of the incubation increases, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected giving a positive result if growth has occurred. One of the advantages of MGIT culturing is the fact that all types of clinical specimens, from pulmonary as well as extra-pulmonary cases, can be processed using conventional methods. Once the processed specimen is inoculated MGIT tubes must be continuously monitored either manually or by the automated BACTEC MGIT 960 system until positive or until the end of the testing period (42 days) in this experiment.

2.12 Microbiological processing of sputum specimen

The processing of the sputum samples was carried out in a biological safety level 3 (P3) laboratory. In short, 1ml of sputum was transferred to a 50ml tube and an equal amount of Mycoprep solution was added; this amount was slightly increased for more purulent samples. The samples were then placed in a shaker for 15 minutes to allow degradation to take place. Phosphate buffer (PO_4) was then added to the mixture to neutralize the reaction, mixed by inversion and centrifuged at 4˚C at 3000 x g for 15 minutes. 800µl of Panta solution was added to

the MGIT tubes- this contains a lyophilized mixture of antimicrobial agents and was added and mixed by inversion. After the sputum mixture was spun the supernatant was discarded and 1 ml of the phosphate buffer was added to the pellet. 0.5 ml of this mixture was added to these tubes, which were placed into the BACTEC MGIT 960 instrument and monitored automatically on an hourly basis for up to 42 days. MGIT tubes that were flagged as positive were removed from the instrument and smears prepared for ZN staining. A time to positivity (TTP) was recorded by the instrument and samples that had a TTP lower than 4 days were regarded as contaminated. To rule out contamination a negative control MGIT tube, which contained all the reagents was placed in the machine as negative control. If the negative control was positive at any point samples were recultured using another sputum aliquot, which had been stored at -80˚C.

2.13 Ziehl-Neelsen (ZN) staining

The ZN staining technique is used to demonstrate acid-fast bacteria, which belong to the genus of mycobacterium and this includes the causative agent for TB. This technique is based on the principle that acid-fast organisms take up carbolfuchin and resist decolorization with a dilute acid rinse. The penetration of aqueous based staining solution is prevented by the high molecular weight of the lipoid capsule of the mycobacteria; this allows it to be waxy at room temperature and thus detectable under the microscope. In short, smears from patients with positive MGIT cultures were stained for acid fast bacteria. 1ml of the positive culture was smeared onto slides; the slides were then placed on a staining rack and flooded with carbol fuchsin. The slides were then heated to steaming point using a Bunsen flame and left to stand for 5 minutes. Water was used to clean the slides after which they were flooded with acid-alcohol and left to stand for 2 minutes, washed with water, flooded with methylene blue and left to stand for 2 minutes. The slides were then washed and air dried after which the smears were examined using a light microscope. Bacteria were counted and the smears were given a positive (scanty, +, ++ or +++) or negative score.

2.14 Luminex multiplex immunoassays

After defrosting, saliva and serum samples were analysed within 1-2 hours. Levels of the 33 analytes were determined in the selected supernatants using customized Milliplex kits (Merck Millipore, St. Charles, Missouri, USA), on the Bio Plex platform (Bio PlexTM, Bio Rad Laboratories). Analytes included C-reactive protein (CRP), Serum amyloid A (SAA), Serum amyloid P (SAP), epidermal growth factor (EGF), IL-1α, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1β, soluble CD40 ligand (sCD40L), transforming growth factor (TGF)α, vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor G-CSF, GRO, interferon gamma (IFN-γ), IL-1β, IL-2, IL-5, IL-8, IL-12p70, IL-13, IP-10, IL-15, soluble interleukin-2 receptor alpha (sIL-2Ra), tumour necrosis factor (TNF)α),Fractalkine, IL-4, IL-6,IL-7,

IL-9, IL-10,IL-17 and granulocyte monocyte stimulating factor (GM-CSF). The assay was done according to the manufacturer's instructions (Merck Millipore). All analyte levels in the quality control reagents of the kits were within the expected ranges. The standard curve for all analytes ranged from 3.2–10000 pg/ml. Bio-Plex Manager Software, version 4.1.1 was used for the analysis of bead median fluorescence intensity. A similar procedure was followed as mentioned above (2.7) however these samples were added in singlet in a laminar flow hood.

2.15 Statistical analysis

CYTOKINE PROFILES IN CHILDREN WITH DOCUMENTED *MYCOBACTERIUM* TUBERCULOSIS EXPOSURE AND INFECTION, EXPOSURE AND NO INFECTION COMPARED TO NO EXPOSURE NOR INFECTION

All statistical analyses were conducted using Statistica version 10 (StatSoft, Tulsa, OK, USA). All results were transformed using the logarithm base-10 to reduce variance in distributions. The nonparametric Wilcoxon matched pairs test was used to determine differences between stimulated and unstimulated values. For all further analysis unstimulated (nil) values have been subtracted from the antigen stimulated values. Groups (exposed infected compared to exposed uninfected and exposed uninfected compared to unexposed uninfected) were analysed by nonparametric ANOVA with the Dunn's post-test. The degree of association between cytokines was calculated using Spearman's nonparametric correlation coefficient. $P \leq 0.05$ was considered significant.

DIFFERENCES IN THE LEVELS OF HOST MARKERS DETECTED IN SALIVA AND SERUM AND THEIR POTENTIAL FOR DIAGNOSING TB DISEASE

Statistical differences in analyte levels were evaluated by the Mann Whitney U test for non-parametric data analysis. Cut-off levels for differentiating between TB disease and no-TB were ascertained by receiver operating characteristics (ROC) analysis based on the highest likelihood ratio. Values lower than 0.05 ($p<0.05$) were considered as statistically significant. Data were analyzed using the GraphPad prism, version 5.00 for Windows(GraphPad Software, San Diego, California, USA) and Statistica software (Statsoft, Ohio, USA).

CHAPTER 3

CYTOKINE PROFILES IN CHILDREN WITH DOCUMENTED *MYCOBACTERIUM TUBERCULOSIS* EXPOSURE AND INFECTION, EXPOSURE AND NO INFECTION OR NO EXPOSURE NOR INFECTION

3.1 Background

A number of researchers have recognized that some individuals exposed to infectious TB resist developing a positive TST for longer periods than their peers despite similar exposure levels (89). These findings raise the possibility that the innate immune response has the ability to clear an infection without the induction of an acquired response. The spontaneous clearance of a transient *M.tb* infection gives rise to another possible outcome of an encounter with (12) and the possibility of the existence of a “protective phenotype”. The investigation of factors which may be linked with innate resistance to *M.tb* infection was hampered by the poor sensitivity and specificity of the TST (90). The development of more sensitive and specific blood based methods to evaluate the T cell response to TB known as the IFN-γ release assays (IGRA’s) have provided an important advancement and have been used to determine the factors associated with the resistance to *M.tb* infection in children (91).

A study conducted by Martineau et al 2007 on adults who had come into contact with patients diagnosed with active TB in London and the United Kingdom showed that the risk of TB infection was inversely and independently associated with the peripheral blood neutrophils count in contacts of patients diagnosed with pulmonary TB (92). Their study findings lead to the conclusion that neutrophils contribute to innate resistance to TB infection, an activity which is associated with their antimicrobial peptides (92). Despite numerous studies protective immunity to *M.tb* is not completely understood as yet as it depends on a wide range of innate and also adaptive immune mechanisms. Studies have reported on the ability of $CD4^+$ T cells to produce IFN-γ, which activates phagocytes to contain the intracellular pathogen, as being central to protection. In addition IL-17 producing $CD4^+$ T cells were reported to mediate the recruitment of protective TH1 cells to the lung upon *M.tb* challenge (93).

In countries with a high prevalence of TB, childhood TB has become a growing problem and thus it is important to identify the risk factors of infection and mechanisms of protection against TB especially in children because of the vulnerability of their immune system. Once infected they are at an increased risk of progressing to active TB disease (94). The participants in our study have received BCG vaccination. A study conducted by Soysal et al in 2005 studying a large population

of tuberculosis-exposed children with an accurate test for *M .tb* infection has identified a protective effect of BCG vaccination against acquisition of infection (91). These findings have important implications for the development and assessment of improved tuberculosis vaccines. Despite the fact that all the children in our study had received BCG vaccination and had exposure to household contacts with active TB disease some of the children progress to disease and others do not. This has given rise to the hypothesis that there is a protective phenotype that distinguishes the exposed uninfected children from the unexposed uninfected. Identification of this protective phenotype may facilitate rapid screening of new TB vaccine candidates and may lead to targeted intervention to prevent TB disease.

3.2 Rationale for this study

This section of the work was done to validate the differences in cytokine profiles of children with known tuberculosis (TB) exposure and infection and those with no known exposure and infection in a high TB incidence setting. A previous study conducted in our department identified the cytokines IL-1β and IL-17 to be differentially expressed between children who were exposed to an adult TB case in their household within the past 3 months but who remained uninfected and children without TB exposure in their household who were uninfected according to IGRA tests. In this chapter we tried to validate the earlier findings by doing multiplex immunoassays (described in chapter 2) using the same cytokines (IL-1β and IL-17) as well as to expand the cytokine panel to include IL-6, IL-10, IP-10, IL-2, IL-33, IL-23, IL-1α and IL-21.

Furthermore we hypothesised that children with negative IGRA's and TST following exposure to *M.tb* represent a highly protected immunological phenotype that will be distinct from responses in children without prior exposure. This study aimed to identify the protective immunological phenotype in children with negative IGRA's and TST following exposure to *M.tb*. To ensure that no new variables were introduced to this validation study we ensured that the participants were obtained from the same study cohort and that similar inclusion and exclusion criteria were used. The QuantiFeron (QFT) and the Tuberculin Skin test (TST) were used as measures of infection as was done in the previous study.

Interleukin-1 beta (IL-1β) is a member of the cytokine 1 family. It is a proinflammatory cytokine which is produced by activated macrophages as a proprotein. IL-1β is inactive and requires processing by the cysteine protease, caspase-1 for maturation and secretion. Its maturation is controlled by multiprotein, caspase-1-activating platforms called inflammasomes (95). Caspase 1 is the prototypic member of a family of inflammatory caspases (including human caspase-4 and -5 and mouse caspase-11 and 12) which all contain the N-terminal caspase recruitment domain (CARD) (96). This cytokine is involved in cell proliferation, differentiation and apoptosis, thus

making it an important mediator of the inflammatory response (95). The important functions associated with this cytokine and its potential involvement in protective immunity further emphasised the importance of validating the findings and studying it further.

3.3 Participants

The study intended to investigate children ≤5 years who are HIV negative and who were exposed to an adult, smear positive TB case in their households within 2 months prior to recruitment into the study. Children with a positive tuberculin skin test (TST) and a positive QuantiFeron Gold In tube (QFT) test (infection –susceptible phenotype) were compared to children who were also exposed but who tested negative in all this tests (infection- protected phenotype). An unexposed control group with negative tests of *M.tb* infection were included as controls. These *M.tb* infection parameters had to remain unchanged for at least 6 months.

The investigation was approved by the Ethics Review Committee of the Faculty of Health Sciences, University of Stellenbosch, South Africa (N05/07/129). All participants in this study or their legal guardians provided written informed consent for participation in the study.

3.4 Eligibility

Children who are participating in a larger immunological and diagnostic community-based contact study in communities in Cape Town with high burden of TB and low HIV-prevalence (Ravensmead, Uitsig and Site C) were eligible for this immunological sub study. Site C is a high HIV prevalence area. Children with complete data regarding *M.tb* contact score (Chapter 2; this value quantifies the extent of *M.tb* exposure and therefore was used as proxy for infection) of >4 for exposed and <4 for controls were included as well as children whose infection and exposure status remained unchanged (e.g. if a child had a positive TST and QFT result at baseline it had to remain that way throughout the six month follow up) for the next 6 months as measured by the TST and QFT tests.

3.5 Calculation of exposure gradient

Participants (and caregivers in case of children) were interviewed to determine each participant's extent of contact with a TB index case during a typical 7-day week. A model developed by Hesseling et.al was modified and used for calculation of the resulting gradient of exposure. The calculation is based on the assumption that the grade of *M. tb* exposure (contact score) = infectivity of the index case + duration of exposure to the index case + proximity of the exposure + relationship of the contact to the index case. The score has been developed to provide participants

without any known contact to a TB case a value of zero (87). The full definition and components of the TB contact score is shown in table 2 (chapter 2)

3.6 Results

3.6.1 Demographics

The median age of the participants was 3 years. The study included 36% males and 64% females. The exposed infected group consisted of 36 QFT and TST positive children, the exposed uninfected group were 47 QFT and TST negative participants and the 37 unexposed uninfected group were all QFT and TST negative.

Table 3: Demographic and clinical characteristics of study participants

	Exposed Infected (n=36)	Exposed Uninfected (n=47)	Unexposed Uninfected (n=37)
Median age (years)[1]	1.64(0.44-5.0)	3.08 (0.87-5.0)	2.6 (0.40-5.0)
Median TST induration at baseline (mm)[1]	15.8 (9-27)	0 (0)	0 (0)
Median contact score[2]	8 (4-15)	9 (4-13)	0 (0)
Female[3]	20 (56%)	36 (72%)	21 (57%)

[1]Median is shown with range in brackets; [2]High contact score defined as ≥4 and a low score defined by <4; [3]Numbers of subjects, followed by the percentages of subjects relative to the total number of subjects in each age group in brackets, are shown. TST = tuberculin skin test.

Cytokine results of the unstimulated samples

In the unstimulated (QFT nil) samples, most cytokines were found above the minimum detectable concentration (MDC) as published by the kit manufacturer's (Merk Millipore) except for IL-21. The unstimulated concentration of IP-10 was the highest measured at 2198 pg/ml (exposed infected), 2401 pg/ml (exposed uninfected), 2158 pg/ml (unexposed uninfected) at baseline. Regardless of the group IP-10 (exposed infected, p = 0.00; exposed uninfected, p = 0.17; unexposed uninfected, p = 0.70) and IL-1β (exposed infected p=0.19; exposed uninfected p= 0.51; unexposed uninfected p= 0.06) increased after stimulation with the mycobacterial antigens similar to IP-10 and are in agreement with the results obtained in the previous study Loebenberg PhD 2011 (97). IL-1α (exposed infected, p = 0.02; exposed uninfected, p = 0.39; unexposed uninfected, p= 0.15) decreased in all groups after stimulation. IL-6 decreased in the exposed infected group (p = 0.05)

and in the unexposed uninfected group (p= 0.85) after stimulation but increased in the exposed uninfected group (p= 0.06). In the exposed uninfected group and the unexposed uninfected group IL-23 (p = 0.01 and p= 0.01) was significantly decreased upon stimulation and increased in the exposed infected group (p= 0.80). IL-10 decreased after stimulation in all groups. In the exposed infected group IL-33 (p = 0.52) was increased upon stimulation and decreased in the exposed uninfected (p= 0.01) and unexposed uninfected (p= 0.04). IL-2 was increased in all groups after stimulation.

Table 3.1: Median responses in 10 cytokines as measured at recruitment in all participants with and without tuberculosis exposure and infection

	Exposed Infected (n=36)				Exposed Uninfected (n=47)				Unexposed Uninfected (n=37)			
	M.tb antigen stimulated			Unstimulated	M.tb antigen stimulated			Unstimulated	M.tb antigen stimulated			Unstimulated
Cytokine	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)	Median (pg/ml)	Median fold difference from unstimulated	p-value	Median (pg/ml)
IL-1β	705.01	1.26	0.19	556.80	710.12	1.58	0.51	448.40	680.90	1.24	0.06	548.10
IL-6	715.87	0.56	0.05	1274.00	581.20	0.91	0.06	638.30	693.12	0.87	0.85	837.90
IL-10	14.93	0.59	0.26	25.35	15.07	0.75	0.39	20.04	14.91	0.67	0.82	22.35
IL-2	2.12	2.99	<0.01	0.71	2.12	3.07	<0.01	0.69	2.12	5.17	0.10	0.41
IL-33	30.05	1.45	0.52	20.68	30.27	0.93	0.01	32.39	28.43	0.46	0.04	60.74
IL-23	530.21	1.08	0.80	491.20	544.22	0.84	0.01	648.50	526.36	0.60	0.03	873.3
IL-21	18.50	18.5	0.85	0.00	18.50	18.5	0.93	0.00	18.50	18.5	0.93	0.00
IL-17	3.16	2.75	0.21	1.15	3.00	1.02	0.55	2.93	3.30	0.93	0.56	3.55
IL-1α	56.25	0.84	0.02	66.68	57.17	0.96	0.39	59.60	55.43	0.77	0.15	72.05
IP-10	7929.68	3.61	<0.01	2198.00	7851.23	3.27	0.15	2401.00	7581.21	3.64	0.00	2158.00

Supernatants from unstimulated and stimulated whole blood were tested for 10 cytokines. Median fold differences and *P* values for significance testing by the Wilcoxon test are presented. Values in red are representative of significant decreases from unstimulated median. *M.tb* = *Mycobacterium tuberculosis*.

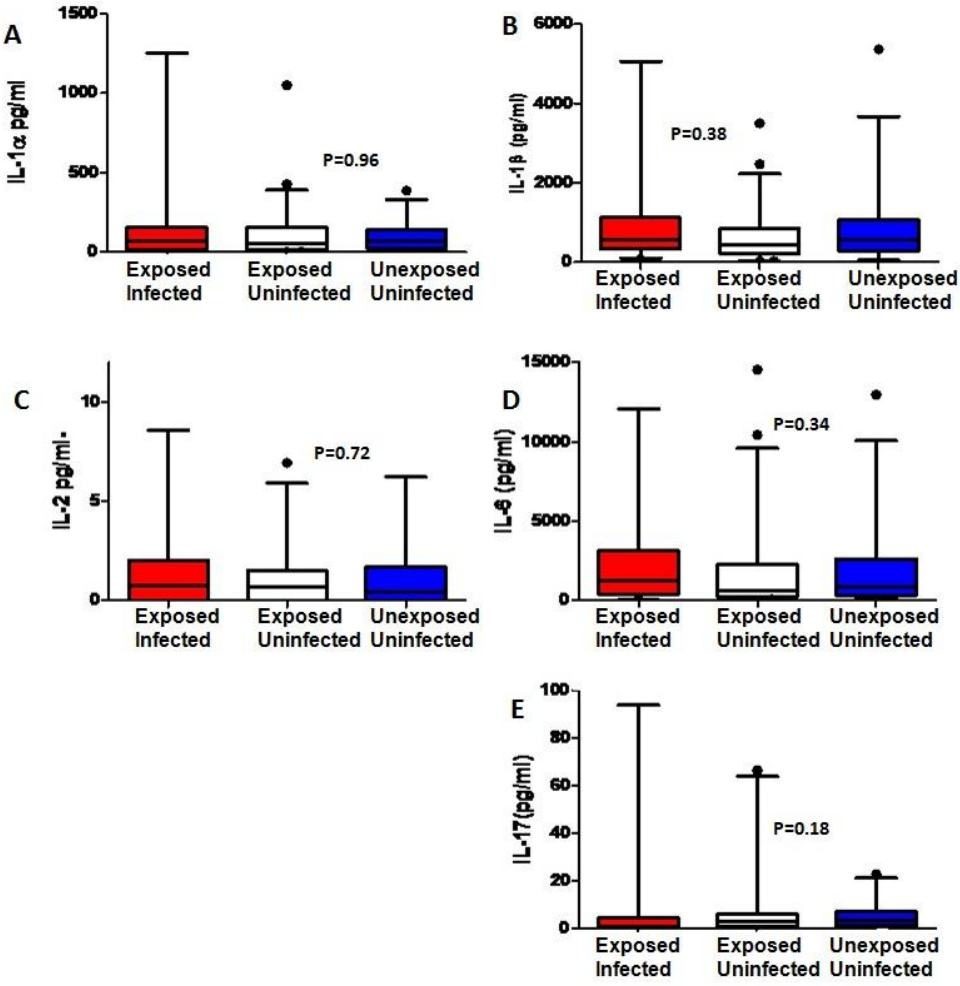

Figure3.1. Unstimulated Cytokine levels in all participants (n= 120). A= IL-1α, B= IL-1β, C= IL-2, D= IL-6, E= IL-17. Unstimulated QFT tube supernatants from children with or without household exposure to an adult TB case and with or without immunological evidence for *M.tb* infection (IGRA and TST) were evaluated for the levels of 10 cytokines by multiplex cytokine arrays. Red bars= exposed infected; white bars = exposed uninfected; blue bars = unexposed uninfected group. P- value denotes significant difference between the groups as determined by the Kruskal-Wallis test $p \leq 0.05$ is regarded as significant. Cytokines with p-value <0.1 are shown. ●= Outliers

3.6.2 Cytokine differences between exposed infected and exposed uninfected participants

The results shown below are those of the *M.tb* antigen stimulated samples with unstimulated values subtracted this was done to obtain the TB specific responses. IP-10 levels were higher in the exposed infected group compared to the exposed uninfected and the unexposed uninfected group p=0.00. A small, statistically not significant difference (p=0.15) was observed between the unexposed group and the exposed uninfected. IL-17 levels were higher in the exposed infected group compared to the other two groups (p=0.01). IL-1α and IL-2 were significantly higher in the exposed infected group compared to the exposed uninfected group (p=0.00 and 0.00) respectively.

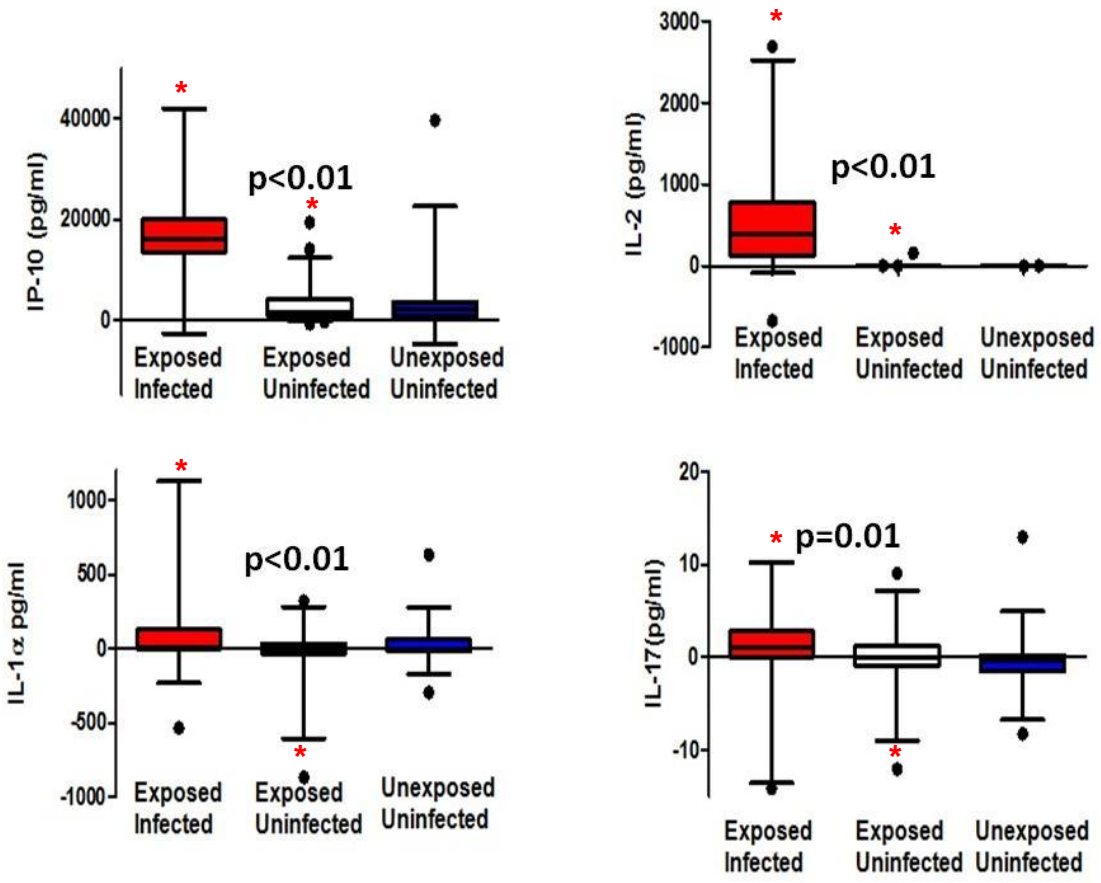

Figure 3.2: *M.tb* antigen stimulated samples with unstimulated values subtracted from children with or without household exposure to an adult TB case and with or without immunological evidence for MTB infection (IGRA and TST) were evaluated for the levels of 10 cytokines by multiplex cytokine arrays (n=120). Red bars= exposed infected; white bars = exposed uninfected; blue bars = unexposed uninfected group. P-value denotes significant difference between the groups as determined by the Kruskal-Wallis test $p \leq 0.05$ is regarded as significant. Cytokines with significant differences between the exposed infected and the exposed uninfected groups are shown. ●= Outliers. * denotes significant differences between groups (exposed infected and exposed uninfected).

3.6.3 Cytokine differences between exposed uninfected and unexposed uninfected participants

In the unstimulated (nil) samples IL-23 was higher in the unexposed uninfected group and lowest in the exposed infected group. In the antigen stimulated group IL-23 was higher in the exposed uninfected group (p=0.03). The stimulated IL-23 values (with unstimulated levels subtracted) in the unexposed uninfected group were significantly lower than in the exposed uninfected group (p=0.01). Similarly, IL-33 levels were significantly lower in unexposed uninfected individuals than in the other two groups (p=0.01). These differences are driven by the decreased expression of

cytokine in stimulated levels compared to unstimulated values that was only seen in the unexposed, uninfected group.

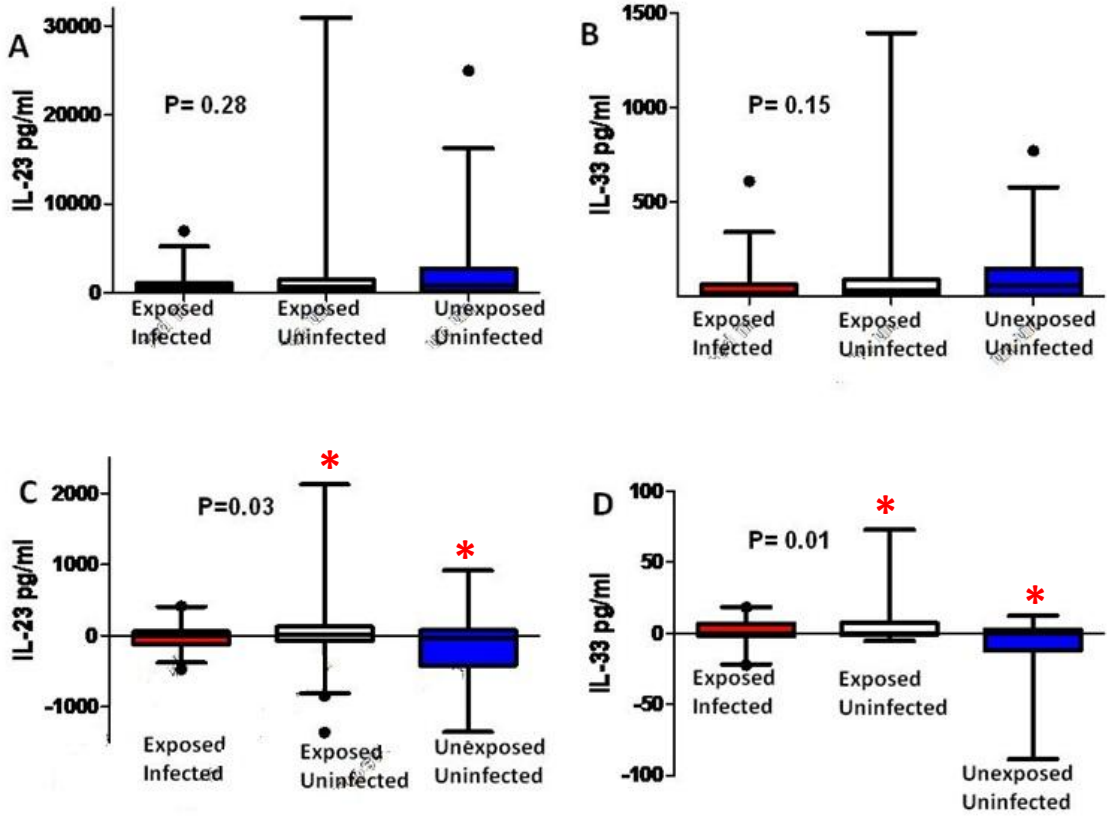

Figure 3.3: A and B unstimulated graphs of IL-23 and IL-33 Unstimulated Cytokine levels in all participants (n= 120). Unstimulated QFT tube supernatants from children with or without household exposure to an adult TB case and with or without immunological evidence for MTB infection (IGRA and TST) were evaluated for the levels of 10 cytokines by multiplex cytokine arrays. C and D *M.tb* antigen stimulated samples with unstimulated values subtracted from children with or without household exposure to an adult TB case and with or without immunological evidence for *M.tb* infection (IGRA and TST) were evaluated for the levels of 10 cytokines by multiplex cytokine arrays (n=120). Red bars= exposed infected; white bars = exposed uninfected; blue bars = unexposed uninfected group. P- value denotes significant difference between the groups as determined by the Kruskal-Wallis test $p \leq 0.05$ is regarded as significant. Cytokines with significant differences between the exposed Uninfected and the unexposed uninfected groups are shown. ● = Outliers. * denotes significant differences between groups (exposed uninfected and unexposed uninfected).

3.6.4 Combination of IL-1β observations between two studies

As it was a validation study the results obtained from IL-1β in all three groups were combined (results from the previous Loebenberg study and the current validation study) to see whether an increased number of participants would yield significant differences. The total number of

participants from the combined studies was 165. Using Prism one-way ANOVA was conducted and a nonparametric Kruskal-Wallis test was done. The Dunns post test was done to compare each group individually. There were no significant differences between the three groups (p=0.42). Although IL-1β was higher in the exposed uninfected group compared to the unexposed uninfected group the difference was not significant (p=0.28).

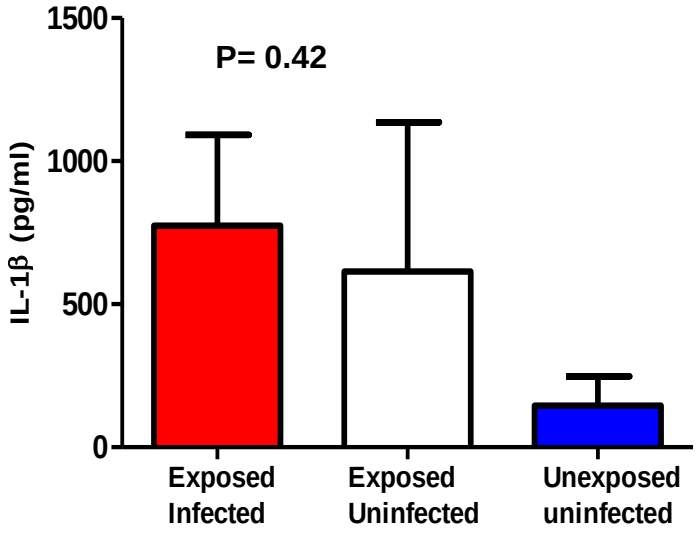

Figure 3.4 *M.tb* antigen stimulated samples with unstimulated values subtracted from children with or without household exposure to an adult TB case and with or without immunological evidence for *M.tb* infection (IGRA and TST) were evaluated for the levels of IL-1β by multiplex cytokine arrays (n=165). Red bars= exposed infected; white bars = exposed uninfected; blue bars = unexposed uninfected group. P- value denotes difference between the groups as determined by the Kruskal-Wallis test. The IL-1β data presented above was pooled from two separate studies. P=0.42 differences between all three groups (exposed infected, exposed uninfected and unexpected uninfected). P=0.28 differences between the exposed infected and exposed uninfected. P=0.06 differences between exposed uninfected and unexposed uninfected groups.

3.7 Discussion

This study was designed with the main aim to validate the differences in cytokine profiles of children with known TB exposure and infection, exposure and no infection compared to no exposure nor infection in a high TB incidence setting with particular interest on IL-1β and IL-17. The study also aimed to identify the protective immunological phenotype (if any) in children with negative IGRA's and TST following exposure to *M.tb* Infection.

A number of studies have reported the excellent results obtained in individuals latently infected with TB using the QFT assay (74, 77, 98). This assay detects sensitization to *M.tb* antigens by measuring IFN-$_\gamma$ production after whole blood stimulation with ESAT-6, CFP-10 and TB7.7. Therefore it was appropriate to use the QFT as a measure of infection in our study. Unfortunately, due to the large number of outstanding results the T-spot was not included as a measure of infection. This would have been an additional measure of infection. IGRAs have shown great specificity and have a closer correlation with the exposure gradient compared to the TST (99). Cytokine concentrations were determined in unstimulated and stimulated QFT plasma samples.

The effects of whole blood stimulation were investigated; stimulation did not have the same effect on all marker levels in relation to unstimulated levels in all groups. We observed that IL-1β and IP-10 were increased in all study groups upon stimulation. IP-10 results are in agreement with the previous study conducted Loebenberg PhD 2011 (97), however, the previous study Loebenberg PhD 2011, reported IL-1α to be increased in all study groups as opposed to IL-1β.In the present study IL-6 increased upon stimulation in the exposed uninfected group and IL-23 decreased upon stimulation in the exposed uninfected group whilst IL-1α decreased in all groups upon stimulation. IL-2 increased in all groups following stimulation. IL-21 was not detected across all study groups. IL-33 decreased upon stimulation in the exposed uninfected and unexposed uninfected groups. The results show that stimulation resulted in the increase of cytokines that are released by adaptive immune cells. The immune response against *M.tb* is believed to involve mainly TH1 cells, however, this study together with a previous study Loebenberg PhD 2011 suggest the upregulation of both TH1 (IL-2) and TH2 (IL-6) cells.

Cytokines that showed significant differences between the TB infected and uninfected groups were IP-10 (a chemokine), IL-17, IL1α, IL-6 and IL-2. A trend ($P>0.05<0.1$) was observed in IL-1β ($p=0.06$). These findings suggest that these markers work in concert with IFN-$_\gamma$ in an attempt to control *M.tb* infection. Our findings are in agreement with other studies e.g. Lighter et al 2009 (64) reported IP-10 to be a potentially useful biomarker in children to determine TB infection. In a study conducted by Chegou et al 2009, IL-1α was reported to be one of the markers with an ability to distinguish latent TB infection from active disease, although this was not in children (100). Similar findings were reported in the previous study by Loebenberg PhD 2011(97).

IL-1β is a pro-inflammatory cytokine, which was found to be present at higher levels in the exposed infected groups in the pilot study. These findings were not unexpected as IL-1β has been reported to be produced at the site of infection during TB (101). This protein induces chemokines such as MCP-1 and also induces the secretion of IFN-$_\gamma$ by natural killer cells, which results in further macrophage activation (102). A study by Garlanda et al 2007 reported increases in IL-1β in those infected with TB (103). Elevated levels of IL-1β in the exposed group indicates that early immune

activation has taken place and that the innate immune systems of these children is sensitized to *M.tb*. The antigen-specific increase in IL-1β may suggest that production of this cytokine by innate immune cells is being amplified by adaptive immune cells. A similar antigen-specific increase in IP-10 production (also produced by innate immune cells) is seen in *M.tb* sensitized individuals and is ascribed to T cells that activate macrophages (104). The negative QFT and TST in the exposed but uninfected group, yet differential production of cytokines originating from innate and adaptive immune cells show that IFN-$_{\gamma}$ does not serve as adequate marker for prior exposure to *M.tb* antigens. This data also shows that the evaluation of both adaptive and innate immune system effects is required to increase our understanding on protective immune responses against this pathogen. The levels of some markers (IL-6, IL-10, IL-1α) investigated in this study were lower in the TB antigen stimulated than in the unstimulated samples in the exposed infected group. A study by Chegou et.al suggests this may be due to the expression kinetics of the different markers after stimulation with the TB antigens (100). The actual reasons behind this observation are beyond the scope of this study and should be investigated in future studies.

When the IL-1β results were combined for both studies with a total sample size of 165 it showed that the exposed uninfected group had higher levels than the unexposed, uninfected group, however, these differences were not significant and the IL-1β in the exposed infected group remained higher than all the other groups. These findings show that both the exposed groups have elevated levels of IL-1β but the mechanism by which one group fails to generate IFN-$_{\gamma}$ remains unclear. The exposed uninfected group may clear the pathogen without generating an IFN-$_{\gamma}$ response but by innate immune mechanisms or by adaptive immune mechanisms that are independent of IFN-$_{\gamma}$. When comparing the exposed, uninfected group to the unexposed, uninfected group IL-1β is higher in the former group, which may suggest that this cytokine may play a protective role in these individuals, preventing demonstrable infection. Whether these children remain uninfected after additional exposure is unknown and longer follow up times should be implemented for future studies. It would also be interesting to measure the levels of IL-1β after a further six months after the first documented exposure. The proinflammatory cytokine IL-6 was found to be higher in the exposed, infected group.

These findings are in agreement with studies that have reported that significant amounts of IL-6 are produced in response to *M.tb* infection and human and murine macrophages secrete IL-6 in response to *M.tb in vitro* (105,106). IL-6, produced by macrophages, has also been shown to play a role in the initiation of T cell activation (107). The higher levels of IL-6 in exposed, infected participants may therefore result in activation of the adaptive immune system in this group whereas this may not take place in the exposed, unifected and unexposed groups. However some studies have reported the role of IL-6 in induction of a type 1 protective T-cell response during *M.tb* infection (108).

IL-17 is a pro-inflammatory cytokine that is produced by TH 17 cells in response to IL-23 but has also been shown to be produced by innate γδ T cells in response to IL-1β (109). This cytokine has been reported to be responsible for effective Th-1 responses and granuloma formation in mice (110). A critical role for IL-23 in induction of Th 17 cells in humans has recently been reported (111). IL-17 is one of the cytokines that showed differences between TB exposed, uninfected and the unexposed, uninfected groups in the pilot study by Loebenberg PhD (97), however in our present study there was no significant difference between groups although the median level was higher in the unexposed, uninfected group.

A number of cytokines have been reported to be responsible for the development of Th17 cells and among these are IL-1, IL-6, TGF-β, IL-21 and IL-23 (109). A study by Kader et al 2008 showed that both IL-17 and IL-23 have an important role in human anti-mycobacterial immune responses and protection against pathogens (112). High levels of IL-17 have been reported in TST negative adult participants in a study conducted by Babu *et al*. (2010) (113). These findings were in agreement with findings from a study conducted by Van de Veerdonk *et al*. (2010), which showed the presence of memory Th17 cells in TST negative people, who were not BCG vaccinated, and released IL-17 upon *M.tb* stimulation (114). In our present study this result could not be replicated. Upon exposure we see a slight increase in IL-17 concentrations although the values are very low. We would have expected to see increased levels in the exposed groups as IL-6 has been identified as one of the cytokines responsible for the expansion of Th 17 cells and as this cytokine was found to be increased in the exposed groups in the present study.

The reasons for the discrepancies in IL-17 findings in the different studies are not known but differences in study populations may play a role. In addition, large intra-group variation in expression levels was seen and the study may not have the necessary power due to relative small numbers of participants to show differences between groups. The role of this cytokine in *M.tb* infection should be further investigated as well as the pathways involved.

IL-23 is a heterodimeric cytokine with two disulfide-linked subunits: a p19 subunit that is unique to IL-23 and a p40 subunit that is shared with IL-12 (112) and shares similar roles with IL-12 in promoting T-helper 1-type responses. This cytokine however has unique roles in regulating immunity as it drives a T cell population that is characterised by the production of the IL-17-related cytokines IL-17A and IL-17F. As a result of the prominent production of IL-17 these cells are referred to as Th17 cells (115). IL-23 is predominately produced by activated (antigen –presenting) dendritic cells and phagocytic cells (116). In the present study this cytokine was found to be higher in the exposed, uninfected group. In the unexposed group negative values were obtained after

subtraction of unstimulated levels and little or no IL-23 expression was observed in most of the individuals within the exposed, infected groups.

IL-23 has been shown to be important for the control of acute bacterial infections as it is among the cytokines produced early by alveolar macrophages upon exposure. Thus it is well placed to drive an early IL-17 response, which is said to be protective from innate cells within the lung (117). Studies conducted in mice have shown that high-dose exposure to *Mycobacterium bovis BCG* in the lung results in an effective IL-17 response that originates from the γδ T-cell population, which is required for a rapid inflammatory response for protection (118). The IL-17 response in *M.tb* infection is largely dependent on IL-23 and IL-1 (119). Similar studies have reported that compared to high–dose infection IL-17 does not appear to have a protective role in low-dose infection. It is unclear how this relates to the kind of exposures seen in our study. However it would be interesting to compare the role of IL-17 in high versus low-incidence areas as this could give a clear understanding on the role of IL-23 in inducing IL-17 production and thus its role in protection.

Literature has shown that both IL-23 and IL-17 are required for a protective response, which is characterized by a change from a mononuclear to a granulocyte driven inflammatory response in the mouse model with local expression of IL-23 at the inflamed site being responsible for the enhanced IL-17 response and change in chemokine profile that results in granulocytic influx (120). Our study was unable to show the IL17/IL-23 interaction and reasons as to why IL-17 was not expressed in most individuals and why IL-23 was higher in the exposed uninfected group are not clear. We think that the differentiation state in phagocytes in the lung affected the cytokine profile as human monocytes differentiated by granulocyte colony stimulating factor produce IL-23, whereas those differentiated in the presence of monocyte-colony stimulating factor produce IL-10 after mycobacterial exposure (121). It would be wise to measure the levels of colony stimulating factor vs monocytes-stimulating factor as this could explain why IL-17 was poorly expressed. It is thus too early to conclude whether IL-23 has a protective role in the exposed, uninfected group.

IL-33, the newly identified member of the IL-1-like cytokines, was found to be higher in the exposed, uninfected group. IL-33 is reported to induce helper T cells, mast cells, eosinophils and basophils to produce type 2 cytokines. The generation of IL-33 from its proprotein precursors is regulated by the processes that are dependent on the multiprotein complex referred to as the inflammasome. These processes are similar to those of IL-1β and IL-18 (122,123). IL-33 is being reported to have proinflammatory properties, however these properties are not known at this stage. Studies have reported that the treatment of mice with IL-33 resulted in the increase in IL-4, IL-5 and IL-13 production. The production suppresses Th1 differentiation and favors Th2 differentiation (124). Although IL-33 is a newly discoverd cytokine it is associated with a number of functions. It is reported to be involved in host protection against helminth infection and also known to reduce

atherosclerosis by promoting Th2-type immune responses (125). Therefore IL-33 can be a new target for therapeutic intervention across a range of diseases. The role of IL-33 in TB is not known, yet it has been reported to enhance the production of IL-4 and IFN-γ . It has also been reported to promote the development of dendritic cells (DCs) from bone marrow cells and is involved in the production of IL-6 and augments the expression of MHC class II (126).

Dendritic cells have been reported to play a central role in the initiation of the immune response against *M.tb* (127). Upon *M.tb* infection pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) recognize specific chemical signatures found on *M.tb* cells. Once DCs have a presentable antigen, they become activated into mature dendritic cells and begin to migrate to the lymph node (128). The initial interaction between DCs and naive T cells and the surrounding cytokine environment influence the quality of immune response. IL-6 is a Th1 related proinflammatory cytokines which has been shown to be involved in immunity to TB (129). A number of studies has identified IFN-γ to have a protective function against *M.tb*. As IL-33 is reported to be associated with Th2 responses it therefore would be expected to have a limited role in determining host resistance to infection. Studies in mice deficient in St2, the signaling chain of the IL-33R were found to be indistinguishable from wild type (WT) animals in terms of their bacterial loads and pulmonary histopathology (128,129). Even though we do not know much about IL-33 we know that it is responsible for the enhancement and production of cells and cytokines that are involved in promoting immunity to *M.tb* infection.

In summary, we have shown that IL-1β is expressed at increased levels in the exposed, infected group of children but no significant differences were observed for this cytokine between the exposed, infected and unexposed, uninfected group. These data do therefore not support the findings of the pilot study. Reasons for the discrepancy are not clear but large intra-group variation and limitations in accuracy of mutliplex cytokine array technology may all play a role. The increased levels of IL-1β in the infected group which were previously reported may suggest that it plays a role in early TB exposure and sensitization of the innate immune system. Elevated IL-33 and IL-23 levels upon *M.tb* antigen stimulation in exposed, apparently uninfected children compared to unexposed, uninfected children is indicative of recent exposure with infection, in spite of the absence of IFNγ in the former group as both these cytokines are among the first cytokines to be secreted upon immune activation.

In combination with IL-33, IL-17 and IL-23 should be investigated in future studies to investigate the full nature of a protective immune response against *M.tb* infection and disease. It is therefore too early to make conclusions as to the role of IL-23 and IL-33 in protection and its clinical implications. These findings should be further investigated in larger cohorts with attention directed specifically towards comparing individuals according to severity of exposure as infecting dose may

well affect the outcome of infection. Furthermore, the reasons for the discrepant results in the pilot, the current and other published studies need to be elucidated.

3.8 Conclusion

We can thus conclude that the expression of certain cytokines (IL-23 and IL-33) is associated with immune responses in children who are exposed to *M.tb* but who fail to develop infection phenotypes that are characterized by IFN-γ production in response to *M.tb* antigens. The study has therefore identified early data on an infection phenotype that is distinct from unexposed, uninfected individuals. We can also conclude that immune responses other than IFN-γ are protective in exposed uninfected children.

Chapter 4

Differences in the levels of host markers in saliva and serum and their potential for diagnosing TB disease

4.1 Abstract

Background

Despite the recent introduction of molecular beacon assays such as the GeneXpert into clinical practice, the diagnosis of tuberculosis (TB) disease remains challenging in individuals with difficulty in providing good quality sputum samples such as children. Host biosignatures of inflammatory markers could be valuable in such cases, especially if they are based on more easily obtainable samples such as saliva, and are developed into rapid, point-of-care tests. The aim of this study was to compare the levels of host markers detectable in the saliva and serum of patients with possible pulmonary TB, and to assess the diagnostic utility of these markers for TB disease.

Methods

Saliva and serum samples were collected from 38 TB suspects who were recruited from a community health centre in Cape Town, after which the levels of 33 host markers were evaluated in the samples using the Luminex platform.

Results

Of the 38 TB suspects enrolled, active TB disease was confirmed in 11 (28.9%) by sputum culture. In both the TB cases and non-cases, the levels of most markers were above the minimum detectable limit in both sample types, but there was no consistent pattern regarding serum/saliva ratio of marker levels. The levels of fractalkine, IL-17, IL-6, IL-9, MIP-1β, CRP, VEGF and IL-5 in saliva, and those of IL-6, IL-2, SAP and SAA in serum, were significantly higher in TB patients, compared to the levels obtained in those without active TB ($p<0.05$). The area under the ROC curve was ≥ 0.70 for most of these markers, thereby confirming their diagnostic potential for TB disease.

Conclusions

There are vast differences in the levels of host markers expressed in saliva in comparison to serum and some markers in both sample types have potential for the diagnosis of TB disease. These preliminary findings warrant further investigation.

4.2 Introduction

Mycobacterium tuberculosis *(M.tb)* infects nearly 2 billion people worldwide and is responsible for about 1.3 million deaths annually (86). In 2003 the World Health Organisation (WHO) reported that the Directly Observed Treatment Short Course (DOTS) programs managed to successfully treat 84% of new smear positive patients. However, these programs were only able to detect 28% of the estimated tuberculosis patients in the world (31), thus making the goal of reaching the target of 70% case detection by 2013 unlikely unless interventions are made to increase the case-detection rate (31). Failure or a delay in diagnosis of the disease results in treatment delay and ongoing transmission with potentially ten people infected annually per untreated case (130). The most commonly used diagnostic test for TB disease, especially in resource-limited settings is smear microscopy. The method is relatively inexpensive and rapid, but has poor sensitivity as it requires 10^4 bacilli per ml of sputum for a positive result (131). The gold standard test for the diagnosis of TB disease, sputum culture, is more accurate but culture facilities are not widely available in resource limited settings, in addition to the fact that culture results may only be available in a minimum of one week and up to 42 days (67). There have been many recent developments in the field of TB diagnostics (132), the most significant being the developments of the real-time molecular beacon assay, the XpertMTB/RIF assay (Cepheid Inc, CA, USA). The XpertMTB/RIF assay yields results within two hours which include the presence of the most common rifampicin resistance mutations, as marker for multi-drug resistance (133). However, the relatively high operating costs of the test and other requirements as highlighted by Trebucq et al (134) preclude its use in resource-limited settings, which are often also the areas with the highest TB burdens (135,136). Furthermore, diagnostic tests based on sputum are not suitable in individuals who have difficulty in providing good quality sputum samples such as young children who cannot expectorate (137). Immunodiagnostic techniques employing host biosignatures of inflammatory markers could be valuable in such cases (138,98), especially if they are based on more easily obtainable samples such as saliva and are developed into rapid, point-of-care tests.

Saliva is relatively easy-to-collect and is abundantly produced in individuals of all age groups, but has not been investigated to the same extent as other easily obtainable sample types like urine and serum (139, 140, 141, 142). Saliva is a watery mixture primarily secreted through the parotid, submandibular and sublingual glands and is composed of 98% water and other substances including electrolytes, mucus, antibacterial compounds and various enzymes (143). An average human reportedly secretes 0.3 to 7 ml of saliva per minute and always has about 1 ml saliva in the oral cavity (143). Collection of saliva is simple, non-invasive, and does not carry any of the inconveniences or risks of drawing blood (144). There has recently been an interest in exploring saliva for potentially useful inflammatory biomarkers (144) and diagnostic tests based on saliva, for example the HIV oral fluid rapid tests (145) are commercially available, this relatively easy-to-

obtain and abundant sample type has not been widely investigated in biomarker research studies, despite the large numbers of such studies that are being done (48,54). In the present study, we assessed the levels of 33 host markers in saliva of people presenting with symptoms suggestive of pulmonary TB and compared them to the levels detected in serum. We show vast differences in the amounts of markers expressed in saliva and serum and that some of the salivary markers have potential in the diagnosis of TB disease.

4.3 Materials and methods

Study participants

Patients with possible pulmonary TB (TB suspects) were recruited from the Fisantekraal community in the outskirts of Cape Town, South Africa, as part of the ongoing EDCTP funded African European Tuberculosis Consortium (AE-TBC) biomarker study (www.ae-tbc.eu). Recruitment of the study participants began in October 2010. At the time this study was conducted, 46 TB suspects had been enrolled and 38 included as TB disease was confirmed in 11 (24%) of these participants.

Participants were eligible for the study if they presented to the health care facility with symptomatic pulmonary disease and thought to have a high likelihood of having TB. Briefly, all study participants presented with persistent cough lasting $\geq$2 weeks and least one of fever, malaise, recent weight loss, night sweats, knowledge of close contact with a TB patient, hemoptysis, chest pain and loss of appetite. Participants were eligible for the study if they were aged 18 years or older, willing to give written consent to take part in the study and to have their HIV status tested or be willing to have their HIV infection status disclosed to the study field workers. Patients were excluded from the study if they had not been residing in the study area for more than 3 months, were severely anaemic (HB<10g/l), on anti-TB treatment, had received anti-TB treatment in the previous 90 days and if they were on quinolone or aminoglycoside antibiotics in the past 60 days. At enrollment, a case report form was completed for each participant before blood and saliva samples along with other samples, including urine and sputum as required for the main study were collected as described below. The study was approved by the Committee for Human Research of the University of Stellenbosch and the City of Cape Town, Ethics reference number N10/08/274 and Institutional Review Board number IRB0005239.

Sample collection and diagnostic tests

Blood was collected into 4ml plain BD vacutainer® tubes (BD) and transported at ambient conditions to the laboratory. The tubes were then centrifuged at 2500 rpm for 10 minutes after which serum was harvested, aliquoted and frozen (-80˚C) until use. Saliva was collected from all

participants into salivate tubes (Sarstedt) according to the instructions of the manufacturer. Saliva samples were then transported on ice (4^{o}C) to the laboratory after which the Sarstedt tubes were centrifuged for 2 minutes (1000xg) and the saliva harvested, aliqouted into labelled tubes, and kept at -80°C until analysis.

Sputum samples collected from all participants were cultured by the MGIT method. Positive MGIT samples were examined for AFB using the Ziehl-Neelsen method, after which PCR experiments were performed to confirm the isolation of *M.tb* complex organisms. Participants with positive *M.tb* complex sputum cultures were classified as TB cases. All sputum and saliva samples were processed in a BSL 3 laboratory.

Luminex Multiplex Immunoassay

The levels of following 33 host markers were evaluated in serum and saliva samples from all participants; interferon (IFN)-γ, interleukin (IL)-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, soluble IL-2 receptor alpha (sIL-2Ra), Interferon inducible protein (IP)-10, tumour necrosis factor (TNF)-α, fractalkine, granulocyte monocyte stimulating factor (GM-CSF), epidermal growth factor (EGF), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, soluble CD40 ligand (sCD40L), transforming growth factor (TGF)-α, vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (G-CSF), CXCL1 (GRO), C-reactive protein (CRP), Serum amyloid protein A (SAA) and Serum amyloid protein P (SAP). Customized Milliplex kits (Merck Millipore, St. Charles, Missouri, USA) were used on the Bio Plex platform (Bio Plex^{TM}, Bio Rad Laboratories). All samples were analysed undiluted in a blinded manner, according to the instructions of the manufacturer (Merck Millipore). All analyte levels in the quality control reagents of the kits were within the expected ranges. The Bio-Plex Manager Software, version 6 was used for the analysis of bead median fluorescence intensity.

Statistical analysis

Statistical differences in analyte levels between the TB patients and participants without TB disease , or between the marker levels detected in saliva and serum were evaluated by the Mann Whitney U test for non-parametric data analysis. Cut-off levels for differentiating between TB disease and no-TB disease were ascertained by receiver operating characteristics (ROC) analysis based on the highest likelihood ratio. Differences between groups were considered significant if the p-values were <0.05. Data were analyzed using the GraphPad prism, version 5.00 for Windows (GraphPad Software, San Diego, California, USA) and Statistica (Statsoft, Ohio, USA).

4.4 Results

Saliva and serum samples from 11 TB cases were investigated in this study alongside samples from 27 individuals without TB disease. The individuals without TB disease, investigated in this study as controls were all randomly selected from our study database. Of all the 38 participants included in the study, 71% were females. The median age of all study participants was 38.5 (Table 4.1).

Table 4.1: Demographic and clinical characteristics of study participants

	ALL	TB Cases	TB Controls
Number of participants	38	11	27
Mean Age (years)	38.5	47	34
Male/Female ratio	11/27	3/8	8/19
HIV positive n (%)	8 (21.0)	2(18.2)	6(22.2)
Quantiferon			
Positive	12/38	11/11	0.0
Negative	26/38	0.0	26/38

4.3.1 All study participants

An analysis of the markers in all the study participants was conducted regardless of disease status. This was done to evaluate the differences in marker expression between the two sample types (saliva and serum). The observations in table 4.2 and 4.3 below show that markers such as IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL12p70, IL-13, IL-15 ,IL-17, IFN-γ, G-CSF, GM-CSF, EGF, VEGF, Fractalkine and MMP-9 were significantly higher in saliva compared to serum (Table4.2). Markers such as sIL-2Rα, sCD40L, GRO, IP-10, MIP-1β, MCP-1, CRP, SAA, SAP and MMP-2 were found to be significantly higher in serum (Table4.3). TGF-α showed a trend for higher levels in saliva (p-value >0.05, <0.1). Other host markers, including IL-4 and IL-9, were almost completely undetectable/had low detection values in both sample types. TNF-α was slightly higher in serum, however not significantly different from saliva**.**

There was on average, a 4-fold increased expression of CRP, GRO, IP-10 and MIP-1β in serum of all study participants, in comparison to saliva, and on average a 6-fold higher expression of EGF, Fractalkine, GM-CSF, IFN-γ, IL-12(p70), IL-13, IL-15, IL-17 and IL-1α in saliva in comparison to serum. Figures 4.1 and 4.2 are representative plots showing the markers that were differentially

expressed in serum and saliva in the entire group of study participants, regardless of disease status.

Table 4.2: **Host markers highly expressed in saliva**. Median levels of biomarkers detected in saliva and serum of study participants (n=38), proportion of all samples above the minimum detectable concentration (MDC) and inter-quartile ranges (IQR) are shown.

Marker	MDC (pg/ml)	Saliva		Serum		P value
		%> MDC	Median (IQR)	% > MDC	Median (IQR)	
IL-1α	1.5	100.00	4618.93 (1956.29-10000)	21.00	0.00 (0.00-0.00)	0.00
IL-1β	0.7	95.00	24.58 (12.25-54.73)	18.00	0.00 (0.00-0.16)	0.00
IL-2	0.4	97.00	6.45 (2.34-14.35)	32.00	0.00 (0.00-0.56)	0.00
IL-5	0.1	32.00	0.00 (0.00-1.12)	0.00	0.00 (0.00-0.00)	0.00
IL-6	0.4	45.00	0.00 (0.00-37.34)	32.00	0.00 (0.00-11.49)	0.12
IL-7	1.0	45.00	0.00 (0.00-19.01)	16.00	0.00 (0.00-0.00)	0.00
IL-8	0.3	100.00	145.15 (78.57-237.31)	97.00	13.64 (6.22-27.68)	0.00
IL-12p70	0.9	89.00	9.82 (3.71-16.92)	16.00	0.00 (0.00-0.25)	0.00
IL-13	0.3	92.00	20.67 (11.41-34.10)	0.00	0.00 (0.00-0.00)	0.00
IL-15	0.6	45.00	0.00 (0.00-8.35)	5.00	0.00 (0.00-0.00)	0.00
IL-17	0.4	97.00	13.00 (8.60-18.87)	16.00	0.00 (0.00-0.00)	0.00
IFN-γ	0.4	82.00	4.06 (0.56-10.44)	42.00	0.00 (0.00-4.97)	0.00
G-CSF	3.9	100.00	1347.96 (841.98-2263.20)	97.00	90.72 (45.30-114.37)	0.00
GM-CSF	2.3	100.00	100.53 (65.26-137.91)	8.00	0.00 (0.00-0.00)	0.00
TGF-α	1.4	100.00	9.46 (6.68-16.56)	92.00	6.87 (3.22-20.84)	0.08
EGF	5.3	100.00	5716.96 (3991.86-7964.38)	97.00	98.55 (45.47-185.14)	0.00
VEGF	10.1	100.00	618.16 (457.33-802.58)	95.00	303.53 (145.68-493.18)	0.00
Fractalkine	7.6	97.00	451.84 (137.87-699.92)	10.00	0.00 (0.00-0.00)	0.00
MMP-9	1.0	100.00	164631.40(105484.30-348292.90)	100.00	2673.04 (1795.93-3951.56)	0.00

Table 4.3: Host markers highly expressed in serum. Median levels of biomarkers detected in saliva and serum of study participants (n=38), proportion above the minimum detectable concentration (MDC) and inter-quartile ranges (IQR) are shown.

Marker	MDC (pg/ml)	Saliva		Serum		P value
		%> MDC	Median (IQR)	% > MDC	Median (IQR)	
sIL-2Rα	7.5	8.00	0.00 (0.00-0.00)	29.00	0.00 (0.00-10.35)	0.01
TNF-α	0.2	87.00	10.60 (6.86-20.62)	95.00	11.05 (6.50-12.95)	0.23
GRO	11.4	97.00	132.49 (74.08-204.02)	100.00	1209.05 (855.98-2099.57)	0.00
IP-10	1.3	100.00	102.55 (67.38-213.27)	100.00	407.99 (307.42-709.98)	0.00
MIP-1β	3.2	100.00	12.04 (8.44-17.02)	100.00	47.73 (22.18-81.29)	0.00
MCP-1	1.2	100.00	124.54 (29.50-203.98)	100.00	473.37 (314.28-644.56)	0.00
CRP	0.0012	71.00	88.22 (0.00-232.04)	100.00	27668.45 (9213.65-127253.15)	0.00
SAA	0.21	50.00	119.53 (0.00-848.83)	97.00	11408.91 (2519.07-95050.38)	0.00
SAP	0.055	21.00	0.00 (0.00-0.00)	100.00	46954.88 (37567.08-60894.78)	0.00
MMP-2	48	13.00	0.00 (0.00-0.00)	100.00	1148.82 (971.08-1332.97)	0.00

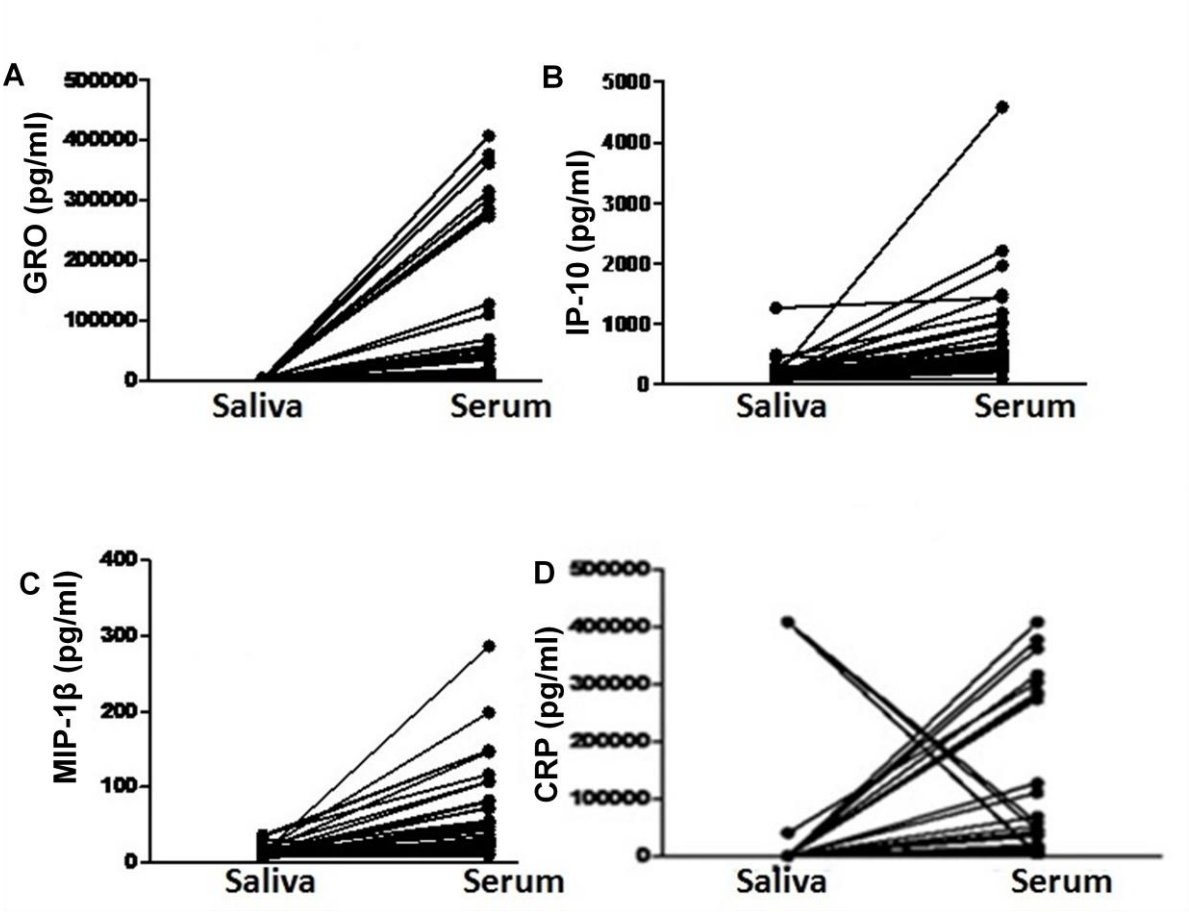

Figure 4.1: Representative plots showing the levels of markers highly abundant/expressed in serum in the study participants regardless of disease status (n=38).A=GRO, B=IP10,C=MIP-1β and D=CRP. The level of the host markers detected in serum was mapped to the levels detected in the saliva of the same study participant. Only the top four most differentially expressed markers in the two sample types (p-values <0.01) are shown.

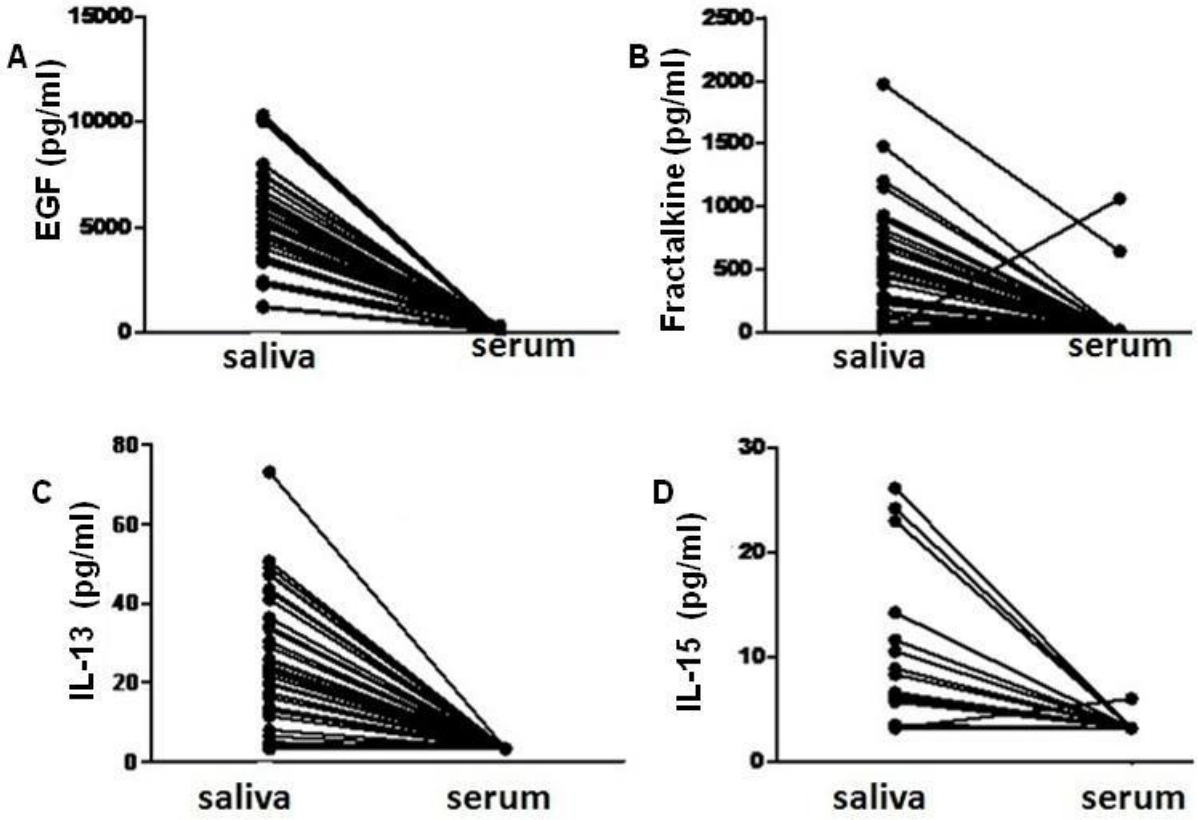

Figure 4.2: Representative plots showing the levels of markers highly abundant/expressed in saliva in study participants regardless of disease status (n=38). A=EGF, B=Fractalkine, C=IL-13 and D=IL-15. The level of the host markers detected in the saliva is mapped to the levels detected in the serum of the same participant. Only the top four most differentially expressed markers in the two sample types (p-values <0.01) are shown.

4.3.2 Pulmonary TB cases

To investigate the suitability of saliva as a preferable sample type to use for biomarker research employing Luminex technology (the platform employed in this study) we classified the participants according to disease status (TB cases and non-TB cases). The level of each host marker detected in saliva was calculated and compared to the minimum analyte detectable concentrations (MDC) as published by the kit manufacturer (Merk Millipore). These analyte amounts detected were then compared to the levels detectable in serum from the same study participants in both cases.

Of the 33 markers investigated in this study, the levels of 13 (IL-1α, IL-8, IL-17, sCD40L, G-CSF, GM-CSF, TGF-α, EGF, VEGF, IP-10, MIP-1β, MCP-1, Fractalkine and MMP-9), were above the MDC in the saliva of all the TB cases. In comparison the levels of 14 (IL-8, sCD40L, GRO, TGF-α, EGF, VEGF, IP-10, MIP-1β, MCP-1, CRP, SAA, SAP, MMP-2 and MMP-9) were above the MDC in serum samples of all these cases . When the levels of markers detected in saliva were compared to the levels detected in serum of these TB patients, the levels of IL-1α, IL-1β, IL-2, IL-5, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, G-CSF, GM-CSF, EGF, VEGF, Fractalkine and MMP-9 were significantly higher in saliva ($0.00 \leq p < 0.05$) while sCD40L, GRO, IP-10, MIP-1β, MCP-1, CRP, SAA, SAP and MMP-2 levels were significantly higher in serum ($0.00 \leq p < 0.05$). The median levels of biomarkers detected in saliva and serum of pulmonary TB cases and the proportion of participants with levels above the MDC are shown in appendix A, figure A.1.

4.3.3 Individuals without active TB disease

The levels of 15 of the 33 host markers evaluated in the study (IL-1α, IL-2, IL-8, IL-13, IFN-γ, sCD40L, G-CSF, GM-CSF, TGF-α, EGF, VEGF, IP-10, MIP-1β, MCP-1 and MMP-9) were above the MDC in the saliva of all 27 non TB cases evaluated in the study. Similarly, serum levels of 10 markers (sCD40L, GRO, G-CSF, IP-10, MIP-1β, MCP-1, CRP, SAP, MMP-2 and MMP-9) were above the MDC in the serum samples of the 27 non TB controls, whereas those of IL-8, TNF-α, EGF, VEGF and SAA were above the MDC in >90% of the controls. When marker levels obtained in saliva were compared to the levels obtained in serum in the non TB cases, the levels of IL-1α, IL-1β, IL-2, IL-8, IL-12(p70), IL-13, IL-17,IFN-γ,G-CSF, GM-CSF, EGF, VEGF, Fractalkine and MMP-9 were significantly higher (p values, all <0.01) in saliva of these non TB cases in comparison to serum, while those of sCD40L, GRO, IP-10, MIP-1β, MCP-1, CRP, SAA, SAP and MMP-2 were significantly higher in serum (p values, all <0.001) (Table 4.1 and 4.2). The median levels of biomarkers detected in saliva and serum of the 27 non TB cases and the proportion of participants that had values above the MDC for each marker are shown in appendix A, figure A.2.

4.3.4 Utility of markers detected in saliva and serum in the diagnosis of TB disease

To investigate if any of the markers detected in saliva and serum could be potentially useful in the diagnosis of TB disease, the levels of the markers detected in the saliva and serum samples of the 11 TB cases were compared to the levels obtained in the 27 non TB cases respectively.

4.3.5 Markers in saliva

When the levels of the markers detectable in the saliva of TB cases were compared to the levels obtained in the saliva of the non TB cases with the Mann Whitney U test, the levels of 8 of the 33 markers evaluated in this study (IL-6, CRP, IL-9, IL-5, MIP-1β, fractalkine, IL-17 and VEGF) were significantly different, or showed trends for differences between the TB cases and controls. With the exception of VEGF, the median levels of all 8 markers were higher in the TB cases (Table 4.4)

When the diagnostic utility of the markers was evaluated by receiver operating characteristics (ROC) analysis, only IL-6, CRP, MIP-1β and fractalkine discriminated between TB disease and no TB with AUC ≥ 0.70 (Table 4.4). Although none of the markers was able to diagnose TB disease with sensitivity above 64% at the cut-off value corresponding to the highest likelihood ratio, saliva levels of CRP, MIP-1β and fractalkine all had specificity ≥93% for TB disease (Table 4.4, Figure 4.3).

Table 4.4: Abilities of biomarkers detected in saliva to discriminate between the pulmonary TB cases (n=11) and individuals without active TB disease (n=27). Median levels (pg/ml) and Inter quartile range (25th to 75th percentile, in parenthesis) of markers and abilities to discriminate between TB cases and non TB cases are shown. Only markers for which Mann Whitney U p-values were significant or showed trends are shown. AUC = Area under the receiver operating characteristics (ROC) curve, 95% CI = 95% Confidence interval. The cut-off values are for the sensitivity and specificity for TB disease and were selected based on the highest likelihood ratio.

Marker	TB disease	No TB disease	P-value	AUC (95% CI)	Cut-off value	Sensitivity % (95% CI)	Specificity % (95% CI)
IL-6	37.34 (0.00-52.18)	0.00 (0.00-13.16)	0.019	0.72 (0.54-0.91)	> 25.8	63.6 (30.8-89.0)	81.5 (61.9-93.7)
CRP	246.50 (21.99-353.91)	45.92 (0.00-122.04)	0.024	0.74 (0.53-0.94)	> 271.7	45.5 (16.8-76.6)	92.6 (75.7-99.0)
IL-9	0.00 (0.00-11.02)	0.00 (0.00-0.00)	0.027	0.65 (0.44-0.86)	> 10.3	27.3 (6.0-60.9)	96.3 (81.0-99.9)
IL-5	0.92 (0.00-9.17)	0.00 (0.00-0.00)	0.033	0.68 (0.48-0.88)	> 7.8	27.3 (6.0-61.0)	96.3 (81.0-99.9)
MIP-1beta	17.02 (11.32-22.18)	11.32 (8.44-15.60)	0.039	0.72 (0.54-0.90)	> 18.7	45.5 (16.8-76.6)	92.6 (75.7-99.1)
Fractalkine	772.85 (225.79-1148.32)	338.23 (104.29-565.54)	0.041	0.72 (0.52-0.91)	> 912.2	36.4 (10.9-69.2)	96.3 (81.0-99.9)
IL-17	18.87 (7.58-36.97)	12.56 (8.60-16.62)	0.085	0.68 (0.46-0.90)	> 29.0	45.5 (16.8-76.6)	96.3 (81.0-99.9)
VEGF	457.33 (307.73-754.92)	680.00 (512.44-802.58)	0.085	0.68 (0.47-0.90)	< 370.5	45.5 (16.8-76.6)	92.6 (75.7-99.1)

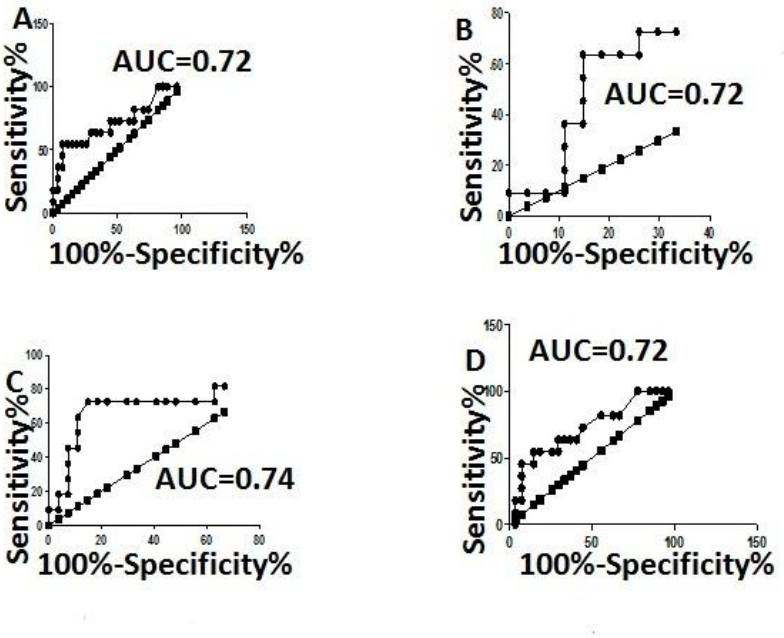

Figure 4.3: A= Fractalkine, B=IL-6, C=CRP, D=MIP-1β. Levels of markers detected in the saliva of pulmonary TB cases and individuals without TB disease and receiver operating characteristics (ROC) plots showing the accuracies of these markers in the diagnosis of TB disease. Error bars in the scatter-dot plots indicate the median analyte levels. Only markers for which the area under the ROC curve (AUC) was ≥ 0.70 are shown. CRP = C-reactive protein

4.3.6 Markers in serum

When the levels of markers detected in the serum samples of TB patients were compared to the levels obtained in the non TB cases significant differences were obtained in the levels of 4 markers (IL-6, IL-2, SAP and SAA). The levels of IL-6, IL-2 and SAP were significantly higher in the TB cases ($p \leq 0.03$) while those of SAA were higher in the non TB cases (Table 4.5).

When the diagnostic utility of the markers in serum was investigated by ROC analysis, the AUC for all four markers (IL-2, IL-6, SAP and SAP) was ≥ 0.70 (Figure 4.4). At the cut-off levels with the highest likelihood ratio, only SAP could ascertain TB disease with sensitivity up to 55% but all four markers diagnosed TB disease with specificity up to 96.3% (Table 4.5, Figure 4.4).

Table 4.5: Abilities of biomarkers detected in serum to discriminate between pulmonary TB cases (n=11) and individuals without active TB disease (n=27). Median levels (pg/ml) and Inter quartile range (25th to 75th percentile, in parenthesis), and accuracies of markers in discriminating between TB disease and no TB are shown. Only markers with significant Mann Whitney U p values or showing trends are shown. The cut-off values for the sensitivity and specificity for TB disease were selected based on the highest likelihood ratio. AUC = Area under the ROC curve. 95% CI = 95% Confidence interval.

Marker	TB cases	Non TB cases	P-value	AUC (95% CI)	Cut-off value	Sensitivity % (95% CI)	Specificity % (95% CI)(95% CI)
IL-6	11.49 (0.00-28.09)	0.00 (0.00-0.00)	0.01	0.73 (0.54-0.92)	>27.54	27.27 (6.02 to 60.97)	96.3 (81.03-99.91)
IL-2	0.56 (0.00-1.34)	0.00 (0.00-0.00)	0.01	0.73 (0.53-0.92)	>0.95	45.45 (16.75 to 76.62)	92.59 (75.71 to 99.09)
SAP	60894.78 (45137.41-65623.18)	42251.43 (36985.94-53804.84)	0.03	0.72 (0.54-0.90)	>58914	54.55 (23.38 to 83.25)	85.19 (66.27 to95.81)
SAA	239.06 (0.00-848.83)	6133.83 (2012.08-40070.18)	0.05	0.70 (0.52-0.88)	>941894	18.18 (2.28 to 51.78)	96.3 (81.03 to 99.91)

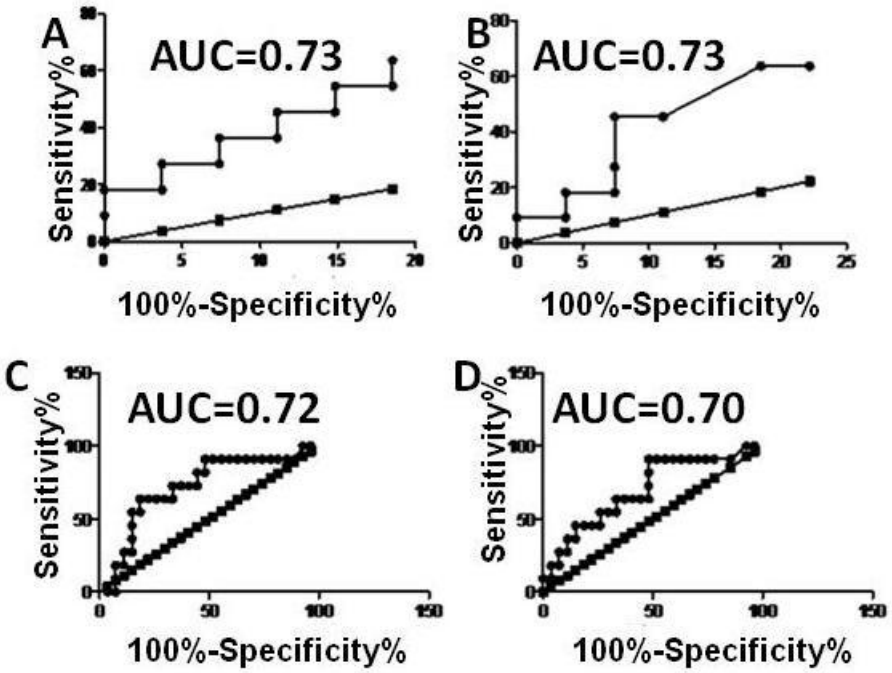

Figure 4.4: A=IL-6, B=IL-2, C=SAP and D=SAA. Levels of markers detected in the serum of pulmonary TB cases and individuals without TB disease and receiver operating characteristics (ROC) plots showing the accuracies of these markers in the diagnosis of TB disease. Error bars in the scatter-dot plots indicate the median analyte levels. Only markers for which the area under the ROC curve (AUC) was ≥ 0.70 are shown.

4.5 Discussion

The challenges currently faced by TB control programs, especially in the diagnosis of TB disease in patients with difficulty in providing good quality sputum samples, such as children and individuals with extrapulmonary disease, call for the development of new diagnostic approaches that would be suitable for such individuals. In this study, we investigated the levels of immunological host markers in saliva; a relatively easy-to-obtain sample type in individuals recruited as possible pulmonary TB patients, and compared maker levels in saliva to the levels obtained in serum. The main finding of our study was the dissimilar expression levels of different host markers in saliva and serum. While there were higher levels of CRP, G-CSF, GRO, IL-8, IP-10 and MIP-1β in serum of all study participants, the levels of EGF, fractalkine, GM-CSF, IFN-γ, IL-12(p70), IL-13, IL-15, IL-17, IL-1α were higher in saliva. Interestingly, some of the markers detected in saliva (IL-6, CRP, MIP-1β and fractalkine), and serum IL-2, IL-6, SAA and SAP, showed potential as diagnostic candidates for TB disease.

This study in relation to other studies

Most of the markers investigated in this study are commonly evaluated inflammatory markers, and have been shown to play different roles in the pathogenesis of several diseases including TB. Although many of the markers showed significant differences between TB disease and no active TB according to the Mann Whitney U test (p-values <0.05). IL-2, IL-6, MIP-1β, fractalkine, CRP, SAA and SAP were the only markers that showed potential as diagnostic candidates in either serum or saliva as ascertained by area under the ROC curve ≥0.70. IL-2 and IL-6 are Th1 related proinflammatory cytokines which have been shown to be involved in immunity to tuberculosis (129). IL-6 has been reported in a number of studies to be produced in greater amounts in TB patients than controls although its role is uncertain (146,147). It is interesting to note that IL-6 has shown to have diagnostic potential in both sample types (saliva and serum). MIP-1β and fractalkine are chemokines. Fractalkine is induced in endothelial cells and antigen presenting cells by various Th1 favourable signals including IFN-γ, CD40L and TNF-α, and inhibited in the presence of IL-4/IL-13 (148). Our findings are in agreement with this statement as no IL-4 was detected and an increased amount of Fractalkine was observed.

MIP-1β is mainly produced by macrophages, dendritic cells and lymphocytes (149) following exposure to bacterial endotoxins (150). It has been reported to be responsible for the activation of human granulocytes (neutrophils, eosinophils and basophils) which are essential for neutrophilic inflammation (151). CRP, SAA and SAP are acute phase proteins. They are mainly produced by the liver cells (hepatocytes). The liver is involved in scavenging bacteria and the clearance and production of inflammatory mediators (152), and thus all the three main cell types of the liver

(hepatocytes, Kupffer cells and sinusoidal endothelial cells) participate in host defense responses through an orchestrated and complex network to respond to various stress signals such as infection (153). SAA has been reported to increase in serum levels up to 1000-fold in response to infection (154). It has been implicated in various pathophysiologic processes of inflammation, lipid metabolism and tissue repair. It has been reported to function as a chemoattractant for immune cells such as monocytes, mast cells and T lymphocytes and its induction may lead to the recruitment of these immune cells and promotion of inflammation (155).

Mouse serum amyloid protein (SAP) is a physiological and functional counterpart of CRP in humans (156). SAP has been reported to function as an opsonin upon binding to polysaccharide and matrix components of bacterial surface and it subsequently mediates phagocytosis (157). On the contrary, another report suggested that binding of SAP to bacteria inhibits phagocytosis and augment their virulence (158). Other studies have reported a possible role of SAP in host defense. This is due to its ability to bind to some bacteria including the important human pathogen *Streptococcus pyogenes* (159). CRP levels rise in response to inflammation and physiologically CRP binds to phosphocholine which is expressed on the surface of dying cells and other bacteria to activate the complement system via the CQ3 complex to enhance phagocytosis by macrophages (160). It is also believed to play an important role in innate immunity as an early defense system against infections (161). SAP and CRP levels have been reported to rise drastically during the vast inflammatory processes in the body. This increment is due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages (161). The median levels of all of these markers (IL-2, IL-6, MIP-1β, Fractalkine, CRP, SAA and SAP) were higher in TB cases as compared to the non-TB cases in saliva. All the above mentioned markers seem to have a role in the control of *M.tb* and in host immune response to infection. Thus it is not surprising that they were found to be higher in TB cases in our study. Generally our findings show that chemokines have a potential as diagnostic markers in saliva whilst acute phase proteins have a diagnostic potential in serum. We show for the first time according to our knowledge that it is possible to detect markers that have a potential to discriminate between TB disease and no disease in saliva. However the lack of specificity should be addressed in future studies as other pulmonary diseases may present a similar result.

Clear differences were observed between markers expression in serum and saliva. Surprisingly a majority of the marker levels were higher in saliva which was unexpected considering the hostility of saliva as a biofluid. The distribution of markers was also different: the majority of the markers which were higher in serum where chemokines, acute phase proteins and a few proinflammatory markers. Saliva was dominated with growth factors, proinflammatory markers and markers which are involved in T cell regulation and activation (162). Our findings are in agreement with studies

that have identified the salivary gland as a reservoir of many growth factors in rodents. In humans, the epidermal growth factor, basic fibroblast growth factor, and insulin and insulin-like growth factor family have also been detected in this gland, but their physiological role remains unclear (163).

Although there have not been many investigations on saliva in the TB biomarker/diagnostic field, it is important to note that saliva has been widely investigated in other diseases. Saliva has been shown to have the potential to be used as a diagnostic fluid in leukoplakia, oral cancer, oral lichen planus (OLP) and periodontitis amongst others (164). A study conducted by Zhang and co-workers (165) compared serum and saliva of patients with oral lichen planus and identified salivary IL-8 as a promising biomarker for OLP over serum. Leigh and co-workers reported a dominant Th2-type cytokine (IL-4 and IL-10) profile in saliva of HIV –positive individuals which indicated susceptibility to mucosal candidiasis (166). Brailo and co-workers reported elevated levels of inflammatory cytokines (IL-1β and IL-6) in saliva of oral cancer patients compared to serum levels (167). In other reports, genes encoding for genes IL-8, Ornithine decarboxylase, spermidine acetyltransferase and IL-1β in saliva, were shown to potentially discriminate and predict whether a saliva sample was from a patient with cancer or a healthy individual, with both sensitivity and specificity of 91% (ROC 0.95).

The saliva results were compared to those of serum and it was reported that in oral cancer detection salivary transcriptome diagnostics have a slight edge over serum. Our results indicate that this may be true for tuberculosis. Saliva-based diagnostics have been reported to have applications in infectious diseases however very little has been reported to date. The markers IL-8 and IL-6 were among the markers that were present at higher levels in saliva of TB patients compared to controls in our study; IL-6 is the marker that showed the most promise as a diagnostic candidate for TB. This might imply that any possible diagnostic tests based entirely on saliva or serum markers might not be very specific for TB as these inflammatory markers are induced in different infectious diseases. However, the advantage of measuring such markers is the fact that they are highly abundant in this sample type which is relatively easy to obtain. Although diagnostic tests based on single markers may lack sensitivity and/ specificity, a biosignature comprising of a combination of markers detectable in saliva might be very useful, especially if combined with clinical signs or symptoms. Such an approach was not undertaken in this preliminary study, but could be done in future, larger studies.

Implications of this study

Although this study has many potential limitations, especially the fact that the sample size was small, it identified another diagnostic opportunity that could have important implications. The fact that rapid, simple, cheap point-of-care tests are needed in order to better control the TB epidemic has been widely publicised. Any such test that is developed, based on markers detected in saliva might be very useful, especially as saliva is a highly abundant and easy to collect sample type, and such tests might be useful even in individuals with difficulty in providing good quality sputum samples such as children.

Furthermore, saliva collection poses a reduced risk to healthcare workers and saliva itself as a sample type is easier to handle compared to other, relatively more easily obtainable samples such as blood (144) which may clot and require re-drawing. Oral based diagnostics offer the possibility of developing low-cost, hand-held devices which may be the ideal solution for developing countries that lack resources and infrastructure. Oral-based diagnostics could enable speedy, low-cost devices that do not depend on trained phlebotomists for sample collection. Saliva-based tests have the potential to provide rapid or even field-based results.

A few oral-based tests are already available commercially such as the OraQuick which was approved by the FDA in 2004 as an oral-based qualitative immunoassay for HIV-1 and HIV-2. Currently oral-based diagnostics are being investigated in a number of diseases including oral, breast, pancreatic, lung cancer, HIV (as a combined screening and confirmatory test) as well as tuberculosis, malaria and heart disease COPD among others. Some of the markers investigated in this study have been shown to have potential as predictors of treatment outcome when measured in sera from patients undergoing TB therapy (168). The fact that some of the markers are many fold higher in saliva than serum might show that it might be important to look at the levels of the markers in saliva of patients undergoing TB chemotherapy. In addition to the fact that saliva is more easily obtainable sample type than serum, the fact that most of the markers are expressed in very high levels means that they would be suitable for use in lateral-flow strip-based tests, which are very useful as point-of-care devices. Therefore salivary biosignatures of TB treatment response is another area that can be investigated, this study serving as proof of concept that it is possible to detect these non-specific inflammatory markers in saliva using the platform employed in this study.

The reasons why some markers were highly expressed in saliva compared to serum is unknown. However, we believe that inhibitors or enzymes in saliva may affect the detection of markers using this customized Milliplex kits (Merck Millipore, St. Charles, Missouri, USA), (Bio PlexTM, Bio Rad Laboratories) platform using Luminex technology. Also variations in saliva flow rate, oral hygiene status may complicate measurement of analytes in saliva (169). There have been no reports to

date of inhibitors in saliva however inhibitors of cysteine proteinases have been described (170). Saliva has been reported to contain mucin glycoproteins (171) and this may limit the detection of biomarkers but even so, large amounts of many biomarkers were detected in saliva, in comparison to serum. Saliva is also derived from serum thus as a result of the diffusion, absorption and secretion processes a large amount of markers may not be absorbed in the saliva. Serum may contain receptors that prevent certain markers to diffuse into saliva. This phenomenon explains why certain markers are lower in saliva compared to serum. There may be molecules that are responsible for the quicker clearance of markers in blood as opposed to saliva. On the other hand saliva represents a pooled sample with contributions from all periodontal sites, thus provides an overall assessment of disease status as opposed to site-specific serum analysis (172), and this may explain why certain markers are highly expressed in saliva.

The composition of saliva is influenced by numerous factors. The protein levels change due to medications, the time of day and whether the patient ingested food or drinks (Schmidt, University of California). Sensitivity is defined as the probability that a test result will be positive amongst patients that have the disease and specificity is the probability that the test will give a negative result in individuals that do not have the disease. Markers tested in saliva showed low sensitivity and high specificity. Thus a diagnostic test based on these makers in saliva will suggest that the patient is likely infected with TB and the test will serve as a rule in test for TB. One more limitation to this technique/assay was the lack of suitable controls (patients with other lung diseases) besides TB thus it is expected that other diseases such as pneumonia might give a similar biomarker signature, it is thus suggested that patients should be tested for other pulmonary diseases prior to enrolment into the study. Participants enrolled into this study were possible TB cases (so-called TB suspects) attending a primary health care facility, all with at least a cough and other symptoms. It is not known whether any factors such as food or drinks taken before coming to the clinic, the non fasting sample amongst others might have influenced the levels of cytokines detected in the serum or saliva of the study participants. Whether such factors have any influence on cytokine levels needs to be investigated in future studies. The influence of any of these factors on any potential diagnostic test based on these markers will need to be investigated once the diagnostic potential of the markers has been confirmed in other studies.

Future directions

This study highlights certain promising markers in saliva for TB research. Validation of these markers in a large set of individuals could identify saliva as an alternative biofluid for TB diagnosis.

4.6 Conclusion

In conclusion the data presented in this study indicates that there are many differences in the levels of host markers expressed in saliva in comparison to serum and some markers in both sample types have potential in the diagnosis of TB disease. Although it is premature to postulate about possibilities for actual clinical use, these preliminary findings warrant further investigation in larger studies.

Acknowledgements

Shirley and nursing assistants/ SUN-IRG lab staff

Funding

This work was funded by the EDCTP through the African European Tuberculosis Consortium (AE-TBC, grant number IP_2009_32040) and the Trials of Excellence in Southern Africa (TESA, project code CG_cb_07_41700).

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 Introduction

A complex interaction occurs between *M.tb* and its host thus it is important to understand pathogenesis and protective immune responses during infection. The global control of tuberculosis which is responsible for the deaths of nearly 2 million people every year can be achieved through the improvement of diagnosis and the development of effective vaccines. The identification of correlates of protection/protective immunity to *M.tb* in children has been challenging. Literature has identified certain markers such as IFN-γ that are essential but not sufficient for protective immunity against *M.tb*. The complexity of the immune system makes it apparent that other biomarkers are required. This thesis has identified other markers that could have the potential to be markers associated with protective immunity (chapter 3).

A lot of research has been done towards the development of better diagnostic tools for both latent and active tuberculosis. However despite the numerous attempts there is still a need for a rapid point-of-care test which is suitable for resource limited and high TB burden settings. A test which is cost effective and utilises a sample type which is easily obtainable is required. This thesis evaluated the utility of saliva for biomarker evaluation and the possibility of its usage in TB diagnosis (chapter 4).

5.2 Summary of findings

CYTOKINE PROFILES IN CHILDREN WITH DOCUMENTED *MYCOBACTERIUM TUBERCULOSIS* EXPOSURE AND INFECTION

In this investigation (Chapter 3) we compared the cytokine profiles in *M.tb* exposed and infected children with those who are exposed but uninfected as well as an unexposed and uninfected control group. Cytokine concentrations that were higher in the infected (QFT and TST positive group) compared to the uninfected (QFT and TST negative) were IL-6, IP-10, IL-1α, IL-2 and IL-1β. Cytokine concentrations that were higher in the exposed uninfected group compared to the control group (unexposed uninfected) were IL-23 and IL-33. In the combined IL-1β data from the previous Loebenberg study and the current validation study a difference in the exposed uninfected and the unexposed uninfected group was observed however this difference was not statistically significant, although this may be due to the small number of participants. Future work should include IL-23 and IL-33 in addition with IFN-γ and IL-1 β in research that focuses on mechanisms of protection

against infection in children. These findings, which differentiate the exposed uninfected from unexposed children, could suggest that the exposed uninfected children did actually inhale the bacteria but that cytokines like IL-1β may be associated with a rapid clearance of *M.tb* before the adaptive immune system is engaged.

Our study also found higher levels of IL-23 and IL-33 in the exposed uninfected groups compared to the exposed infected and unexposed groups. This indicates that higher IL-23 and IL-33 production may play a very important role during early exposure and that these cytokines are likely to be associated with protection from infection. Future work should involve the investigation of these cytokines and the involvement of the IL-23/IL-17 as well as the pathways that are involved in the production of IL-33 during *M.tb* infection in a larger population to further define potential protective mechanisms. Such investigation would have to be conducted in conjunction with a careful measurement of the degree of TB exposure through validated exposure gradients. Future work should also be done to determine the role of these cytokines in HIV infected children, who represent a very susceptible phenotype.

As previously discussed in this thesis one of the strategies that could be employed towards the development of rapid immunodiagnostic tests for the diagnosis of *M.tb* infection is the identification of novel host markers that are differentially expressed between patients with active TB and healthy patients. The identification of an easily obtainable sample type for biomarker evaluation will also aid towards the development of rapid point-of-care tests for the diagnosis of *M.tb* infection. We have assessed (chapter 4) the levels of cytokines detected in saliva of TB cases and controls in comparison to the levels detected in serum and evaluated if any of the markers detected in serum and saliva had the ability to discriminate between the TB cases and controls and so could possibly be investigated further as a serum or saliva diagnostic marker for TB.

We show vast differences in the levels of markers expressed in saliva and serum and that some of the salivary markers have potential in the diagnosis of TB disease and warrant further investigation. The most important findings in this work was the ability to detect markers that discriminate TB cases from controls in saliva (IL-6, CRP, IL-9, IL-5, MIP-1beta, Fractalkine,IL-17 and VEGF) and markers that discriminated TB cases from controls in serum (IL-6, IL-2, SAP and SAA). Although proteins are present in minute amounts in saliva the ability to detect markers in this biofluid is advantageous in the field of diagnostics as saliva is easily obtainable, provides a reduced risk to healthcare workers as no needles are required to collect the samples. It is also easier to obtain as compared to the traditionally used sputum which is difficult to obtain from patients that cannot expectorate (children). We show for the first time, according to our knowledge, that host markers are expressed at different levels in individuals with active TB and in healthy.

Future work may have to investigate combinations of markers in saliva as the discriminatory ability of single markers was not sufficient for a diagnostic test.

Our study has also shown that there are clear differences in markers detected in saliva compared to serum. Although serum constituents affect the composition of saliva the mechanisms that lead to the differences in marker expression in these bodily fluids are not known and it is also not known if these differences are particularly prominent in infectious diseases like TB. Our results show that more markers are present at higher levels in saliva compared to serum. However, we are aware that the findings established in the present study are of limited value due to the small study cohort. We also report that the evaluation of markers in saliva is straightforward and hence it will facilitate the screening in large populations.

5.3 Implications of main study findings

A review by Barry et al 2009 suggested that a continuous response spectrum to *M.tb* infection exists (49). Our study is in agreement with these findings. In the first phase following *M.tb* exposure the innate immune system may successfully control the infection without the involvement of adaptive immune cells (exposed uninfected group). Macrophages and dendritic cells secrete cytokines and chemokines which play a role in host defence against *M.tb*. We have identified IL-23 as one such cytokine. We showed that IL-23 levels are increased in exposed uninfected children and significantly higher than in unexposed children, serving as an indication of an effective innate immune response against *M.tb.* The lower levels of this cytokine in the exposed infected group could be due to failure in the bacterial clearance (depletion of cytokine) and the progression of the infection spectrum to the recruitment of adaptive T cells to the site of infection in order to assist in fighting the infection. Similar observations were found with IL-33.

A number of studies have reported the importance of the activation of the IL-1β inflammasome for first line defence against *M.tb* (36). Elevated levels of IL-1β have been found in the lungs of patients with active tuberculosis. In the present study higher levels of IL-1β were observed in the exposed infected and exposed uninfected groups than in unexposed children, even if the differences were not statistically significant. This again suggests that exposed uninfected children display an immunological phenotype that is different from unexposed children and that they may have dealt with the pathogen on an innate immune level rather than having not been infected at all.

In the study (Chapter 4) evaluating the use of saliva for TB biomarker discovery we have identified markers that have the potential to discriminate between active TB and non disease measuring a limited number of analytes on a small amount of sample. This could have significant implications for the diagnostic utility of saliva as a preferred sample type as compared to serum. This may imply

that in our quest to develop better immunodiagnostic tools to discriminate between the two groups we should be focused on the use of a combination rather than single analytes as no single host marker can perform with the required optimal sensitivity and specificity. The microbiological methods used to diagnose active TB have been reported to perform with high specificity but lack sensitivity similar findings have been reported in our study using saliva this could be because a high bacillary load is required in biological specimens including sputum. Future studies should investigate the combination of these promising markers with the attempt to maximize sensitivity.

5.4 Future directions

Children living in the same household with adult tuberculosis cases are a vulnerable subpopulation as they are more likely to be highly exposed to *M.tb* due to the closeness of contact and the duration of contact with the index case, who is often the primary caregiver. Children in household contact with TB provide an opportunity to study recent infection, as they are less likely to have been exposed previously, which hampers the interpretation of household contact studies in adults in high transmission settings. Children in household contact with TB may also revert from positive IGRA tests to negative, providing another important susceptibility/protection phenotype for which the mechanism of protection is not clear but which may hold important information for post-exposure TB vaccine design.

The identification of the markers and the mechanisms involved in this reversion could lead to the development of a shorter prophylactic treatment regimen. Longer follow-up periods would provide opportunities to identify factors that may contribute to differential IL-23 and IL-33 levels. Future work should also focus on the role of IL-1β and the inflammasome in TB infection and disease. Proteomic studies should be done to allow the delineation of protective and non-protective anti-mycobacterial responses. Studies of multiple immunological markers and correlates of protection should focus on biosignatures and pathways rather than single markers that would give us a clearer understanding of the mechanisms of different outcomes. Functional studies could be performed to assess the role of the different host markers on innate cell function in tuberculosis.

Our study (chapter 4) only evaluated 33 analytes for the abilities to discriminate active TB from non-disease in both saliva and serum. Most of these analytes where chemokines and cytokines (102). Future studies should consider validating the analytes identified (Fractalkine, IL-17, IL-6, IL-9, MIP-1β, CRP, VEGF and IL-5 in saliva, and those of IL-6, IL-2, SAP and SAA in serum) in this study and other biomarkers including angiogenesis mediators and other immune modulators that have been previously implicated in the pathogenesis of TB using saliva. The use of saliva in the identification of candidate biomarkers at the gene expression level should be considered.

In addition to multiplex assays non-biased, discovery techniques for proteomic studies, including mass spectrometry should be considered for the identification of novel biomarkers which may be useful for diagnostic purposes. Despite the recent introduction of molecular beacon assays such as the GeneXpert into clinical practice, the diagnosis of TB disease remains challenging in individuals with difficulty in providing good quality sputum samples such as children. Host biosignatures of inflammatory markers could be valuable in such cases, especially if they are based on more easily obtainable samples such as saliva, and are developed into rapid, point-of-care tests. Our study has shown that it is possible to use saliva for biomarker discovery and there are potential markers that have the ability to distinguish between TB disease and non-disease this findings warrant further investigation. Similarly, the rapidly expanding technologies used for metabolomics, transcriptomics and lipidomics in biomarker discovery need to be employed in infectious diseases like TB. Studies like the present one help to increase the confidence with which certain body fluids and marker classes (i.e. cytokines and chemokines) will be targeted in future discovery efforts.

5.5 Conclusion

In conclusion, the preliminary results presented in this thesis show that the identification of host markers in saliva is possible and the utility of saliva for the development of rapid immune-based tests for active TB is promising. We can putatively conclude that saliva has potential to be used in TB biomarker discovery. However the validations of these findings are needed before diagnostic tests are developed. This thesis has also identified markers that may grant an understanding on the mechanisms that are associated with protection against *M.tb* in children. The combination of multiple biomarkers is required for the characterisation of biosignatures which are necessary for the diagnosis of TB infection and disease. These findings if further investigated would assist in the development of TB diagnosis and vaccine development.

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Appendix A

Table A.1. Median levels of biomarkers detected in saliva and serum of TB cases and proportion above the minimum detectable concentration. IQR = inter quartile range

Marker	MDC (pg/ml)	Saliva		Serum		P value
		%> MDC	Median (IQR)	% > MDC	Median (IQR)	
IL-1α	1.5	100.00	5069.08 (1840.83-9441.46)	18.00	0.00 (0.00-0.00)	0.0000
IL-1β	0.7	91.00	25.11 (9-234.64)	45.00	0.00 (0.00-3.16)	*0.0033*
IL-2	0.4	91.00	14.35 (1.59-34.46)	64.00	0.56 (0.00-1.34)	0.0245
IL-4	0.6	0.00	0.00 (0.00-0.00)	0.00	0.00 (0.00-0.00)	
IL-5	0.1	55.00	0.92(0.00-9.17)	0.00	0.00 (0.00-0.00)	*0.0065*
IL-6	0.4	73.00	37.34 (0.00-52.18)	64.00	11.49 (0.00-28.09)	0.2566
IL-7	1.0	64.00	10.27 (0.00-29.31)	27.00	0.00 (0.00-8.81)	*0.0446*
IL-8	0.3	100.00	143.49 (80.85-237.31)	100.00	27.68 (7.63-46.32)	*0.0006*
IL-9	1.1	36.00	0.00 (0.00-11.02)	9.00	0.00 (0.00-0.00)	0.2106
IL-10	0.3	55.00	11.63 (0.00-24.10)	0.00	0.00 (0.00-0.00)	*0.0065*
IL-12p70	0.9	82.00	12.56 (3.71-22.34)	18.00	0.00 (0.00-0.25)	*0.0057*
IL-13	0.3	73.00	24.16 (0.00-30.28)	0.00	0.00 (0.00-0.00)	*0.0009*
IL-15	0.6	55.00	5.73 (0.00-14.27)	9.00	0.00 (0.00-0.00)	0.0235
IL-17	0.4	100.00	18.87 (7.58-36.97)	18.00	0.00 (0.00-0.00)	*0.0001*
sIL-2Rα	7.5	9.00	0.00 (0.00-0.00)	36.00	0.00 (0.00-88.02)	0.0543
TNF-α	0.2	82.00	9.36 (0.00-28.68)	99.00	11.35 (7.56-14.34)	0.6686
IFN-γ	0.4	93.00	7.14 (0.56-16.71)	73.00	3.60 (0.00-6.28)	0.4454
sCD40L	5.2	100.00	779.20 (104.64-1315.56)	100.00	734674.60 (278617-1179730)	*0.0001*
GRO	11.4	91.00	132.84 (48.3-223.04)	100.00	1357.19 (935.89-2132.75)	0.0001
G-CSF	3.9	100.00	1423.19 (525.37-2391.56)	99.00	90.72 (38.10-148.0)	*0.0002*
GM-CSF	2.3	100.00	137.91 (81.92-219.33)	18.00	0.00 (0.00-0.00)	*0.0011*
TGF-α	1.4	100.00	10.60 (7.18-20.19)	100.00	12.82(4.59-29.19)	1.0000
EGF	5.3	100.00	6187.53 (3331.7-7964.38)	100.00	98.08(60.17-186.97)	*0.0001*
VEGF	10.1	100.00	457.33(307.73-754.92)	100.00	374.23(163.41-493.18)	0.2776
IP-10	1.3	100.00	103.26 (72.85-138.93)	100.00	476.76 (326.53-1026.17)	0.0001
MIP-1β	3.2	100.00	17.02 (11.32-22.18)	100.00	43.63(24.98-148.40)	*0.0010*
MCP-1	1.2	100.00	89.14(10.67-193.6)	100.00	451.66 (288.55-736.23)	*0.0058*
Fractalkine	7.6	100.00	772.85 (225.79-1148.32)	18.00	0.00 (0.00-0.00)	*0.0001*
CRP	0.0012	82.00	246.50 (21.99-353.91)	100.00	38409.83(13125.58-362069.20)	*0.0001*
SAA	0.21	55.00	239.06 (0.00-848.83)	100.00	38539.80(7529.76-481018.4)	*0.0001*
SAP	0.055	36.00	0.00 (0-126.39)	100.00	60894.78(45137.41-65623.18)	*0.0001*
MMP-2	48.0	9.00	0.00(0-0)	100.00	1126.10 (1026.52-1295.13)	*0.0007*
MMP-9	1.0	100.00	167196.80(105484.3-479530.9)	100.00	2598.94 (2107.89-4271.68)	*0.0020*

Table A.2. Median levels of biomarkers detected in saliva and serum of non TB cases and proportion above the minimum detectable concentration

Marker	MDC (pg/ml)	Saliva		Serum		P value
		%> MDC	Median (IQR)	% > MDC	Median (IQR)	
IL-1α	1.5	100.00	4504.78 (2185.56-10000)	19.00	0.00 (0.00-0.00)	*0.0000*
IL-1β	0.7	96.00	24.05 (14.76-54.73)	45.00	0.00 (0.00-0.00)	*0.0000*
IL-2	0.4	100.00	6.45 (2.34-11.36)	19.00	0.00 (0.00-0.00)	*0.0000*
IL-4	0.6	0.00	0.00 (0.00-0.00)	0.00	0.00 (0.00-0.00)	
IL-5	0.1	19.00	0.00 (0.00-0.00)	0.00	0.00 (0.00-0.00)	*0.0212*
IL-6	0.4	67.00	0.00 (0.00-13.16)	19.00	0.00 (0.00-0.00)	0.1708
IL-7	1.0	37.00	0.00 (0.00-19.01)	11.00	0.00 (0.00-0.00	*0.0159*
IL-8	0.3	100.00	146.81 (72.81-257.08)	96.00	13.02 (5.13-19.16)	*0.0001*
IL-9	1.1	18.00	0.00 (0.00-0.00)	4.00	0.00 (0.00-0.00)	0.5418
IL-10	0.3	33.00	0.00 (0.00-8.43)	4.00	0.00 (0.00-0.00)	*0.0042*
IL-12p70	0.9	96.00	9.27 (3.71-16.92)	15.00	0.00 (0.00-0.00)	*0.0001*
IL-13	0.3	100.00	19.41 (11.41-40.99)	0.00	0.00 (0.00-0.00)	*0.0001*
IL-15	0.6	19.00	0.00 (0.00-6.63)	41.00	0.00 (0.00-0.00)	*0.0013*
IL-17	0.4	96.00	12.56 (8.6-16.62)	15.00	0.00 (0.00-0.00)	*0.0001*
sIL-2Rα	7.5	7.40	0.00 (3.71-16.92)	26.00	0.00 (0.00-1.66)	0.0620
TNF-α	0.2	93.00	12.52 (9.05-16.56)	96.00	9.52 (6.13-12.95)	0.0692
IFN-γ	0.4	100.00	101.84 (0.56-8.81)	33.00	0.00 (0.00-4.97)	*0.0029*
sCD40L	5.2	100.00	332.99 (183.5-602.74)	100.00	620087.80 (317610.40-1037918	*0.0001*
GRO	11.4	91.00	132.14 (74.08-181.19)	100.00	1014.31 (760.68-1917.1)	*0.0001*
G-CSF	3.9	100.00	1339.54 (841.98-2263.20)	100.00	78.38 (52.25-114.37)	*0.0001*
GM-CSF	2.3	100.00	88.50 (53.19-121.73)	4.00	0.00 (0.00-0.00)	*0.0001*
TGF-α	1.4	100.00	9.40 (6.42-16.56)	89.00	6.81 (2.93-12.47)	0.0547
EGF	5.3	100.00	5427.41 (3991.86-79990.62)	96.00	99.44 (35.59-185.14)	*0.0001*
VEGF	10.1	100.00	680.00 (512.44-802.58)	93.00	242.12 (125.16-467.88)	*0.0002*
IP-10	1.3	100.00	101.84 (64.34-229.88)	100.00	380.29 (302.53-675.09)	*0.0001*
MIP-1β	3.2	100.00	11.32 (8.44-15.60)	100.00	47.96 (20.78-73.23)	*0.0001*
MCP-1	1.2	100.00	150.45 (63.74-211)	100.00	495.08 (314.28-636.96)	*0.0001*
Fractalkine	7.6	96.00	338.23 (104.29-565.54)	7.40	0.00 (0.00-0.00)	*0.0001*
CRP	0.0012	67.00	45.92 (0.00-122.04)	100.00	20186.95 (5136.52-69082.49)	*0.0001*
SAA	0.21	48.00	0.00 (0.00-848.83)	96.00	6133.83 (2012.08-40070.18)	*0.0001*
SAP	0.055	15.00	0.00 (0.00-0.00)	100.00	42251.43 (36985.94-53804.84)	*0.0001*
MMP-2	48	15.00	0.00 (0.00-0.00)	100.00	1171.53 (910.55-1369.43)	*0.0001*
MMP-9	1.0	100.00	162066(94288.88-348292.9)	100.00	2722.63 (1723.36-3883.14)	*0.0001*

Additional graphs

General discriminant analysis (GDA) for markers in saliva

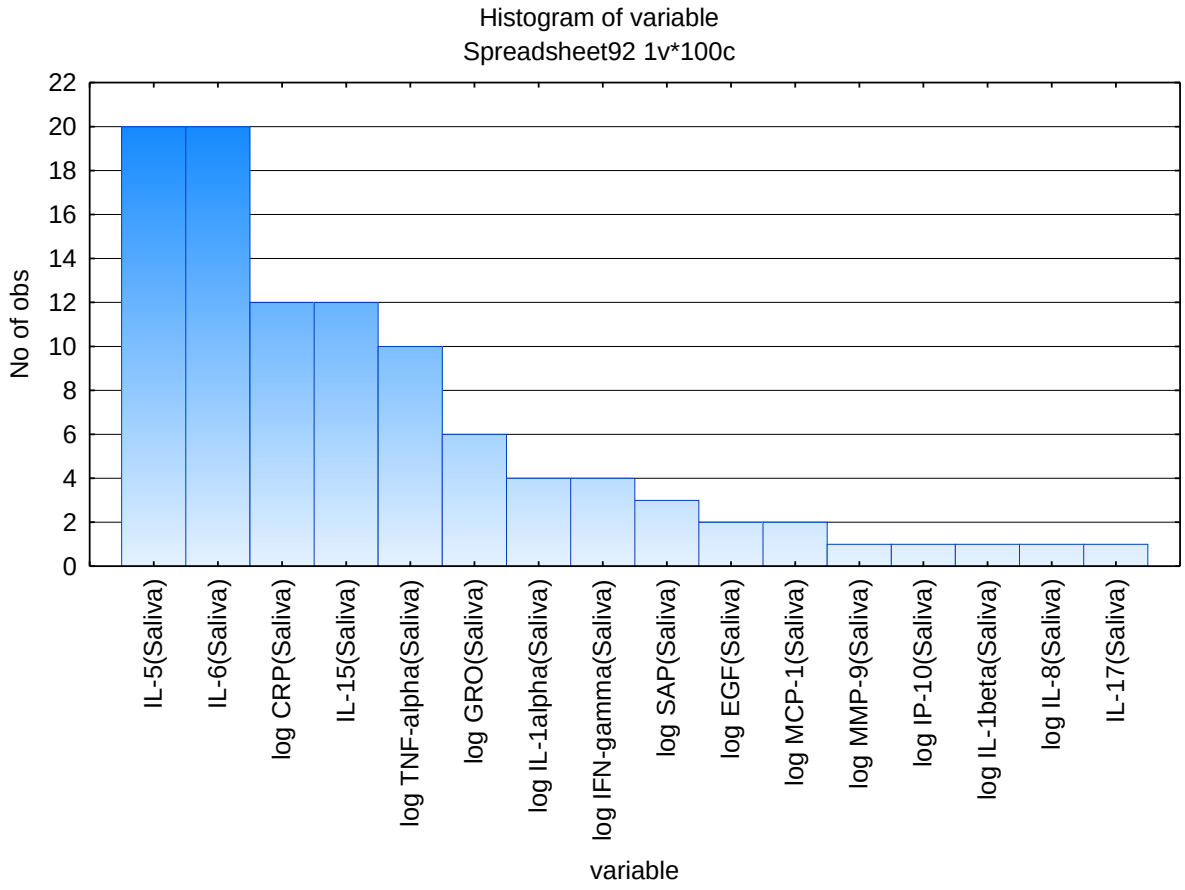

Figure A.1.General discriminant analysis (GDA) for markers in saliva that best discriminate between TB cases and non-TB cases. IL-5 and IL-6 are the best predictors of disease in saliva.

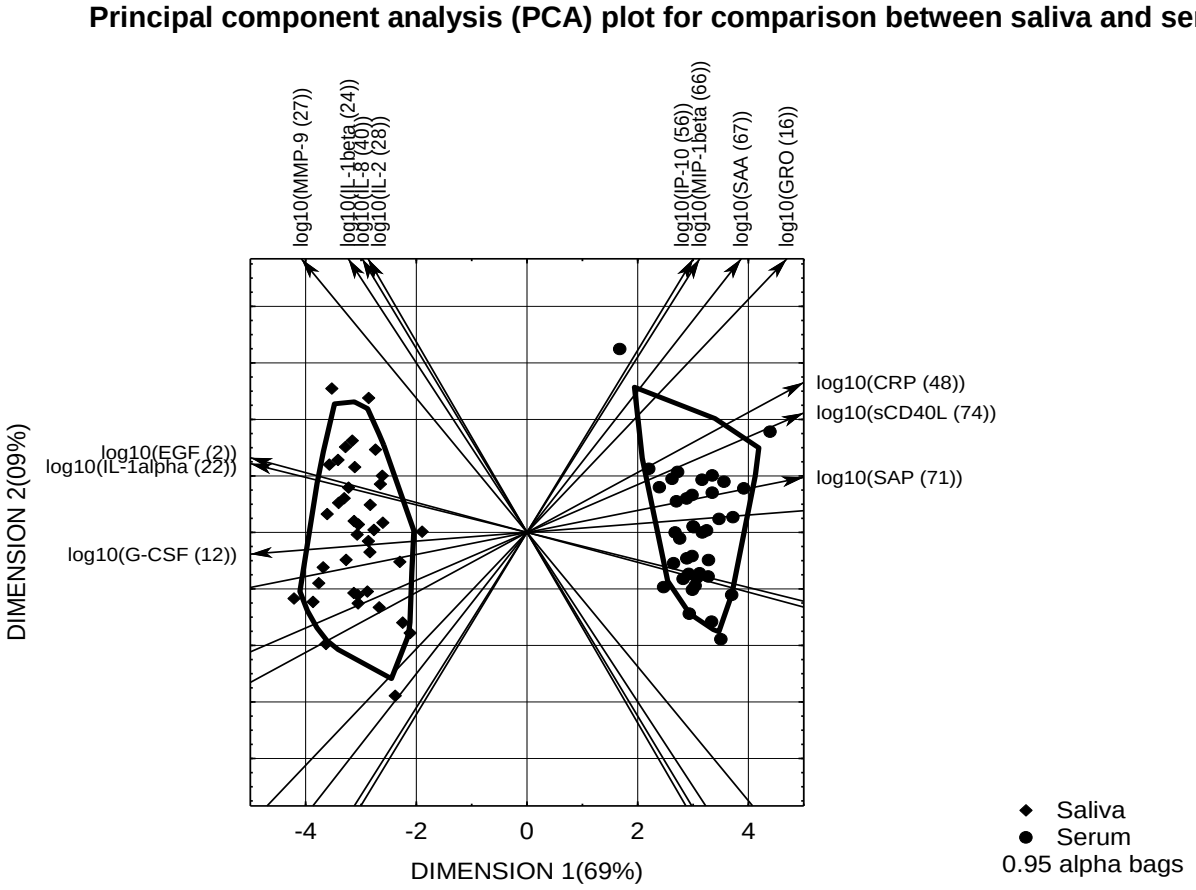

Figure A.2. Principal component analysis (PCA) plot for comparison between saliva and serum.