In vitro profiling of *Mycobacterium tuberculosis* region of difference 1 antigen induced cytokine and chemokines production among patients with active pulmonary tuberculosis and latent tuberculosis

ZIPPORAH BOSIBORI MACHUKI

A DISSERTATION IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF A MASTERS OF SCIENCE DEGREE IN TROPICAL AND INFECTIOUS DISEASES AT THE UNIVERSITY OF NAIROBI, INSTITUTE OF TROPICAL AND INFECTIOUS DISEASES (UNITID)
DECLARATION

I declare that this is my original work and it has not been presented for a Degree or Diploma in any University or Institute.

Zipporah Bosibori Machuki

W64/60352/2010

Signature  

Date: 26/11/2012

Supervisors:

Dr Julius Oyugi
Lecturer, Dept of Medical Microbiology
University of Nairobi
Signature  

Date: 26/11/2012

Dr Florence Mutua
Lecturer Dept of Medical Microbiology
University of Nairobi
Signature  

Date: 26/11/2012

Prof. Aggrey Omu Anzala
Associate Professor, Dept of Medical Microbiology
University of Nairobi
Signature  

Date: 23/11/2012
DEDICATION

I dedicate this work to the Almighty God who has been so good to me. I cannot count his blessings in my life.

I also dedicate this to my parents Mr. and Mrs. Billy Mwencha for their tremendous support and encouragement.

I dedicate this to my daughter Esther Nyangara and my siblings.
AKNOWLEDGEMENT

I sincerely thank and acknowledge Dr. Julius Oyugi, Prof. Aggrey Omu Anzala and Dr. Florence Mutua, my supervisors for their guidance and supervision in writing this dissertation. I also thank Prof. T Blake Ball, Dr. Sandra Kiazyk for the support they gave me that enabled me to carry out this study. Finally I thank Dr. Joshua Kimani and Dr. Nyagol for reviewing this study and the KNH/UON Ethics and Research Committee for allowing this study to be conducted.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART</td>
<td>Anti-retroviral Treatment</td>
</tr>
<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrom</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmett-Guerin</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemotactic Chemokine Ligand</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>CD4+CD45RO</td>
<td>Memory helper cells</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of Differentiation 8</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and prevention</td>
</tr>
<tr>
<td>CFP-10</td>
<td>Culture Filtrate Protein 10</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC Chemokine Ligand</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DOT</td>
<td>Direct Observation Therapy</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>Early secretory Antigen Target-6</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Anti-retroviral Therapy</td>
</tr>
<tr>
<td>HHC</td>
<td>House Hold Contacts</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IFNa2</td>
<td>Interferon alpha 2</td>
</tr>
<tr>
<td>IGRA</td>
<td>Interferon Gamma Release Assays</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL1R</td>
<td>Interleukin 1 Receptor</td>
</tr>
<tr>
<td>IL1ra</td>
<td>Interleukin 1 Receptor Antagonist</td>
</tr>
<tr>
<td>IL1α</td>
<td>Interleukin 1 Alpha</td>
</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1 Beta</td>
</tr>
<tr>
<td>IP10</td>
<td>Inducible Protein 10</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MSMD</td>
<td>Mendelian Susceptibility to Mycobacterial Disease</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NTM</td>
<td>Non-Tuberculous Mycobacteria</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>QFT</td>
<td>Quantiferon® TB Gold</td>
</tr>
<tr>
<td>RD1</td>
<td>Region of Difference 1</td>
</tr>
</tbody>
</table>
RNI- Reactive Nitrogen Intermediates
TH- T Helper
TST- Tuberculin Skin Test
TB- Tuberculosis

TGF-β - Transforming Growth Factor Beta
TLRs - Toll like Receptors
TNF-α - Tumor Necrosis Factor Alpha
WHO- World Health Organisation
# TABLE OF CONTENTS

DECLARATION .............................................................................................................. ii  
DEDICATION .................................................................................................................. iii  
AKNOWLEDGEMENT .................................................................................................... iv  
ABBREVIATION ............................................................................................................ v  
ABSTRACT ...................................................................................................................... ix  
CHAPTER 1: INTRODUCTION ....................................................................................... 1  
CHAPTER 2: LITERATURE REVIEW ........................................................................... 4  
  2.1. Cytokines ............................................................................................................... 5  
  2.2. Chemokines .......................................................................................................... 8  
  2.3. RD1 Antigens ....................................................................................................... 9  
  2.4. Multiplex cytokine bead array ............................................................................. 10  
JUSTIFICATION ............................................................................................................ 11  
RESEARCH QUESTION ............................................................................................... 12  
Objectives ..................................................................................................................... 12  
CHAPTER 3: MATERIALS AND METHOD .................................................................. 13  
  3.1. Study Design ........................................................................................................ 13  
  3.2. Description of the larger study .......................................................................... 13  
  3.3. Study subjects ..................................................................................................... 13  
  3.4. Inclusion criteria ................................................................................................ 13  
  3.5. Exclusion criteria ................................................................................................ 14  
  3.6. Multiplex cytokine immunoassay (Luminex assay) ............................................ 14  
    3.6.1. Preparation of samples and reagents ............................................................ 14  
    3.6.2. Immunoassay procedure ............................................................................. 15  
  3.7. Data collection .................................................................................................... 16
3.8. Data analysis .......................................................................................................................... 16
3.9. Ethical consideration ............................................................................................................... 16

CHAPTER 4: RESULTS ................................................................................................................... 17

4.1 Study subjects .......................................................................................................................... 17

4.1.1 Demographic characteristics of the participants ............................................................... 17
4.1.2 Clinical characteristics of the participants ......................................................................... 17

4.2 Antigen-dependent cytokine and chemokine production ......................................................... 18

4.2.1 Cytokine and chemokine profile in active TB/HIV negative participants ............................ 18
4.2.2 Cytokine and chemokine profile in active TB/HIV co-infected patients ............................ 21
4.2.2.1 Effects of CD4+ T cell counts on antigen-dependent cytokine production ................. 23
4.2.3 Cytokine/chemokine profile in individuals with LTBI ...................................................... 25
4.2.4 Comparison of cytokine/chemokines production in active TB/HIV negative participants and individuals with LTBI .......................................................... 26

4.3 Discriminative ability of cytokine/chemokines in TB diagnosis ............................................. 29

4.3.1 Diagnosis of TB .................................................................................................................. 29
4.3.2 Comparison of active TB/HIV negative participants with active TB/HIV co-infected participants ......................................................................................... 30
4.3.3 Differentiating active TB from LTBI .................................................................................. 31

Chapter 5: DISCUSSION .............................................................................................................. 33

5.1 Limitations ............................................................................................................................. 36
5.2 Conclusion ............................................................................................................................. 36
5.3 Recommendations .................................................................................................................. 36

CHAPTER 6: REFERENCES ......................................................................................................... 37
ABSTRACT

Introduction: Tuberculosis (TB) is one of the leading causes of death worldwide and human immunodeficiency virus (HIV) co-infection poses a great challenge in its control. Furthermore, diagnosis of TB in HIV co-infected subjects is not easy. Early diagnosis of TB is important in the control of TB both for treatment of patients and curbing transmission to others in the community. In countries like Kenya where the prevalence of HIV is higher than 5% in the general population there is need to identify a biomarker that can be used to accurately diagnose Mycobacteria tuberculosis infection. A diagnostic technique that can differentiate between active TB and latent TB infection (LTBI) would also be a major breakthrough.

Objective: The aim of this study was to identify specific M. tuberculosis CFP-10 and ESAT-6 induced cytokine(s) and chemokine(s) that are consistently present amongst patients with latent and active tuberculosis regardless of their immune status and that can discriminate LTBI from active TB disease.

Design: This was a cross-sectional study.

Methodology: A total of 82 subjects were recruited into the study of which 62(75.6%) were pulmonary TB patients, 13 (15.86%) were household contacts (HHC) and 7(8.54%) were controls from another ongoing study. Luminex multiplex cytokine assay was performed to determine the levels of 17 cytokines/chemokines in QFT supernatants. The quantity of cytokine produced following stimulation with ESAT-6 and CFP-10 (antigen-dependent cytokine and chemokine) was determined by subtracting the concentration of cytokine in the nil tube from the antigen tube.

Results: Interleukin 2, IFNγ and IL1ra were produced in significantly high amounts in ESAT 6 and CFP 10 stimulated whole blood from M. tuberculosis infected compared to controls who were quantiFERON® TB Gold negative and HIV negative. Interleukin 17 was consistently produced in significantly lower amount in those participants with active TB regardless of the HIV status compared to controls. Interleukin 1α, IL2, MIP1α and TNFα were produce in significantly higher amounts in ESAT 6 and CFP 10 stimulated whole blood from participants with latent TB infection (LTBI) compared to active TB.
CHAPTER 1: INTRODUCTION

Tuberculosis (TB) is caused by Mycobacterium tuberculosis complex. The Mycobacterium tuberculosis complex consists of genetically closely related group of Mycobacterium species which includes: Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium bovis Bacille Calmette-Guerin (BCG), Mycobacterium microti, Mycobacterium canetti, Mycobacterium pinnipedii Mycobacterium mungi. Tuberculosis is spread from person to person by inhalation of droplet nuclei (1-5μm) containing M.tuberculosis, that are expectorated from a person with active pulmonary TB. After infection the bacteria can remain confined in the lungs or spread to other parts of the body. Tuberculosis can be latent or active. Active TB is symptomatic while LTBI is asymptomatic. Approximately 30% of individuals exposed to M. tuberculosis are thought to develop latent TB which is a reservoir of future TB epidemics. A third of the world’s exposed population is latently infected with M.tuberculosis [1].

In 2010 there were 8.8 million incident cases of TB globally [2]. Reports from World Health Organisation (WHO) show that, the top 22 High TB burden countries contribute 80% of all new TB cases. Kenya is among the top 22 High TB burden countries with an incident rate of 298 per 100 000 population. [2].

Persons who are latently infected with TB have a 5% to 10% chance of developing active TB in their lifetime [3]. However infection with HIV increases their risk of developing active TB with more than 10% annually [4,5]. There is evidence that treatment of LTBI in subgroups reduces the risk of developing active TB [5]. Therefore accurate diagnosis of LTBI is necessary for effective control of TB. The Centers for Disease Control and Prevention (CDC) recommends testing for LTBI especially among high risk group which includes HIV infected individuals [6]. Tuberculin skin test (TST) has been used for a century in diagnosis of LTBI. However TST requires that an individual returns for the results to be read by a trained professional and therefore its widespread use is affected by loss to follow up. Also, with TST there is the potential cross-reactivity with non-tuberculosis mycobacteria (NTM) and BCG vaccine. BCG vaccination affects the usefulness of TST in that, those who have been vaccinated test positive. TST also has poor sensitivity in immunocompromised patients and lacks standardizations and thus leads to variability in interpretation of results [7, 8].
Moreover TST cannot distinguish individuals who are latently infected with *M.tuberculosis* from those who have active disease.

There have been some improvements in the diagnosis of LTBI especially in low TB burden countries because of the discovery of interferon gamma release assays (IGRAs) [9]. Since IGRAs are performed by *in vitro* stimulation of whole blood collected with a single blood draw, a return visit for the result to be read is not needed. Moreover including standard operating procedures in the IGRAs reduces inter reader variability. In addition, the number of false positives in IGRAs is reduced because the test uses specific *M.tuberculosis* antigens encoded by region of difference (RD)-1 of *M.tuberculosis* gene which reduces cross-reactivity with NTM and in BCG-vaccine [10]. However IGRAs may not be applicable in countries like Kenya where the proportion of those infected with HIV is more than 5%. This is because like TST, IGRAs have low sensitivity in immunocompromised individuals and young children [8]. It is also not possible to differentiate between active TB disease and LTBI when using IGRAs. The WHO recommends against use of IGRAs in low and middle income countries for diagnosis of active TB and for identifying people at risk of active TB [11].

Most TB treatment programs at the country level focus on active TB case finding with diagnosis and treatment being of importance in public health [12]. Although TB is a public health challenge when the patient is infectious, control of TB is highly dependent on elimination of the reservoir which constitutes at least one third of the world’s population. Control of HIV associated TB epidemics and early diagnosis and treatment of active TB cases to prevent them from spreading to others is the world’s priority [13].

Tuberculosis is the most common opportunistic infection among immunocompromised individual. Nevertheless diagnosis of TB in immunocompromised individuals faces a lot of challenges because the point of care tests like sputum smear have low sensitivity and culture technique takes long for the results to be obtained [14,15]. The chest X-ray which has been used extensively is subject to inter-reader variability and has low sensitivity in severely immunocompromised individuals.
Problems encountered in the diagnosis of TB especially in immunocompromised individuals, emphasizes the need for identification of a biomarker for *M. tuberculosis* infection. An ideal biomarker should be one that is capable of differentiating between individuals who have active *M. tuberculosis* infection from those who have LTBI.
CHAPTER 2: LITERATURE REVIEW

The estimated global burden of TB in 2009 was 9.4 million out of which 1.1% were co-infected with HIV [1]. Eighty percent of these HIV/TB co-infected patients are from Africa [16]. In 2009 there were about 0.38 million deaths caused by TB among HIV infected people [16]. Kenya is currently ranked among the top 22 countries with the highest TB burden [17]. The incident rate of TB in Kenya is 298 per 100 000 population [2]. The case detection rate of TB in Kenya increased from 51 per 100 000 population in 1997 to 320 per 100 000 population in 2006. The average annual TB increase in Kenya is 7% and this has been the case for the past over 10 years. Tuberculosis resurgence in Kenya is attributed to human immunodeficiency virus (HIV), poverty and urbanization. About 40% of patients who have TB in Kenya are co-infected with HIV [18].

Development of IGRAs has improved diagnosis of \textit{M. tuberculosis} infection in low TB burden regions [19]. The IGRAs detect IFN-\(\gamma\) released by T lymphocytes that are specific to \textit{M. tuberculosis} RD1 antigens such as early secretory antigen target (ESAT) 6 and culture filtrate protein (CFP) 10 [8]. The IFN-\(\gamma\) released in response to stimulation by \textit{M. tuberculosis} specific antigens can be evaluated by enzyme linked immunosorbent assay (ELISA) or enzyme linked immunospot (ELISPOT).

There are three commercial kits based on the IGRA principle: T-SPOT.TB which is an ELISPOT, Quantiferon\textsuperscript{®}-TB Gold (QFT-G), assays which use only ESAT-6 and CFP-10, and Quantiferon\textsuperscript{®}-TB Gold in-tube with an additional antigen, TB 7.7 incorporated in the kit. These assays are better than TST in that they have higher specificity, better correlation with exposure to \textit{M. tuberculosis}, and less cross-reactivity due to BCG vaccination and NTM [20]. This is because ESAT-6 and CFP-10 are absent from BCG and from NTM except for \textit{M. kansasii}, \textit{M. szulgai}, \textit{M. marinum}, \textit{M. gastrii} and \textit{M. flavescens} [19,20]. However, the sensitivity of IGRAs in persons with HIV is low especially in those with low CD4\textsuperscript{+} T-cell counts [21, 22, 23]. This is because IFN\(\gamma\) is secreted by CD4\textsuperscript{+} T cells and therefore its production is influenced by HIV infection and low CD4 T cell counts [24].
2.1. Cytokines

The word cytokine is used for a diverse group of soluble proteins and peptides which act as regulators under both normal and pathological conditions to modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. All cells of the immune system are regulated by cytokines [25]. Some cytokines such as interferon alpha 2 (IFNα2), IFNγ, interleukin (IL) 10, IL12p40, IL12p70, IL15, IL17, IL6, IL7, IL2, IL1 receptor antagonist (IL1ra), IL1α, IL1β, interferon inducible protein (IP10), macrophage inflammatory protein (MIP)1α, MIP1β and tumor necrosis factor (TNF)α are directly related to *M. tuberculosis* infection and/or disease and therefore form a pool of potential biomarkers for detection of active TB disease and/or LTBI. Other infectious agents also induce the production of cytokines. For example infection with HIV leads to elevated IFNα and IL15 within 5 days after viremia is detected, followed by TNFα, IP10, and IFNγ, and then by IL12 [26].

2.1.1. Interferon gamma (IFNγ)

IFNγ is a key cytokine in the control of *M. tuberculosis* infection. IFNγ together with IL2 and IL6 induce cytotoxic T cells to produce granulysin which leads to death of *M. tuberculosis* in the macrophages. IFNγ is secreted by CD4+ T cells, CD8+ T cells and natural killer (NK) cells [27,28]. Currently there are *in vitro* T-cell based assays for diagnosis of LTBI which measure the IFNγ released by sensitized T cells after specific *M. tuberculosis* antigen stimulation. These assays are known as IGRAs. However, IGRAs have some limitation in that the release of IFNγ may vary among subjects and some studies have shown that IFNγ levels may be depressed in patients with active TB [29,30]. Other studies have also revealed that despite the highly active anti-retroviral therapy (HAART) there is impaired IFNγ production by mycobacteria-specific CD4+ T cells in HIV infected persons [31,32]. Also, IFNγ cannot be used to differentiate between active TB and LTBI. Therefore, there is need to determine another cytokine other that IFNγ that can be used as a biomarker for early detection of TB and that can distinguish between active disease and LTBI.
2.1.2. Interleukin 12 (IL12)

IL12 is a key cytokine of the innate immune response. Production of IL12 is induced by phagocytosis of *M. tuberculosis* by macrophages and dendritic cells (DCs) [33, 34]. IL 12 is produced by the macrophages and DCs. Some studies conducted among patients with mendelian susceptibility to mycobacterial disease (MSMD) revealed that deficiency of IL12 increases their susceptibility to TB [35,36]. Thus IL12 is important in preventing the development of active TB. Production of IL12 has been shown to be antigen dependent [37]. *M. tuberculosis*-driven IL12p40 and IL12p70 production is also important for the stimulation of CD8+ T cells to secrete IFNγ [34]. In HIV infected patients, low production of IL.12 has been shown to precede the development of TB [38].

2.1.3. Tumor necrosis factor alpha (TNF α)

Infection with *M. tuberculosis* also induces macrophages, DCs and T cells to produce TNFα [39]. Some studies have shown that TNFα is required for the control of acute *M. tuberculosis* infection. This is exemplified by studies where *M. tuberculosis* infection resulted in rapid death and higher bacterial burdens in mice deficient in TNFα or TNFα receptors compared to control mice [40]. Other studies have also revealed that TNFα acts synergistically with IFNγ to induce nitric oxide synthase (NOS2) expression from murine macrophages which then generate nitric oxide (NO) and reactive nitrogen intermediates (RNI) [41, 42, 43]. The NO and RNI have anti-mycobacterial activity. Also, TNFα is important in granuloma formation in TB and other mycobacterial diseases in that it is involved in setting chemokine circuitry in developing granuloma [44, 45, 46, 47]. In addition it is responsible for the host mediated tissue destruction. In an experiment where *M. tuberculosis* infected mice were treated with thalidomide which downgrades inflammatory cytokines including TNFα, IL6 and IL10, the size of the granuloma in the lungs was reduced without a change in bacterial numbers [48]. Production of TNFα has been shown to be antigen dependent [49].

2.1.4. Interleukin 10 (IL 10)

IL10 is produced by T helper (Th) cells, most CD4+ T cell subsets (including TH1 and TH17 cells), B cells, neutrophils, macrophages, and some DCs subsets [50]. It plays an important role in the
regulation of host immune responses against *M. tuberculosis* by down-regulating production of IL12. This leads to decrease in IFNγ production by T cells, deactivation of macrophage, modulation of pro-inflammatory cytokines and interference with antigen-presenting cell (APCs) function [51,52]. *M. tuberculosis* survives in APCs such as macrophages and DCs. The APCs present antigens in association with major histocompatibility complex (MHC) class II molecules and stimulate CD4+ T cells. This process is essential to contain *M. tuberculosis* infection. However IL10 has been shown to inhibit APCs function and therefore hindering CD4+ T cells responses [53]. There is a possibility that *M. tuberculosis* exploits IL10 regulatory mechanism in order to establish a chronic infection [54]. Production of IL10 is antigen dependent [50].

2.1.5. Interleukin 6 (IL6)

IL6 is a pro-inflammatory cytokine that has been shown to be important in acquired immune response to Mycobacterium infection [55]. IL6 works together with IL2 and IFNγ to induce cytotoxic T cells which secrete granulysin [56]. Granulysin acts by killing *M. tuberculosis* inside the macrophage.

2.1.6. Interleukin 15 (IL15)

IL15 is one of the cytokine released after stimulation with *M. tuberculosis*. There are reports that IL15 can be used to discriminate between active and latent TB [57]. IL15 is important for accumulation of CD8+ T cells in the lungs [58]. It also functions by up regulating cathelicidin, a protein with antimicrobial activity thus leading to reduced survival of *M. tuberculosis* [59].

2.1.7. Interleukin 17(IL17)

IL17 is important in the formation of granuloma in that it triggers recruitment of neutrophils to the lungs [60,61]. It is expressed by activated CD4+ CD45RO+ memory T cells [62]. The production of IL17 is antigen dependent [49].
2.1.8. Interleukin 2 (IL2)

IL2 is secreted by some subsets of T cells in response to *M.tuberculosis* infection [63]. IL2 promotes long term growth of activated CD4^+^ T cells and induce CD4 T cell to produce IFNγ [64]. Some studies have shown that IL2 can be used to differentiate between latent and active tuberculosis [65].

2.1.9. Interleukin 1(IL1)

IL1 is produced by macrophages infected with *M.tuberculosis* and consists of two subunits IL1α and IL1β. IL1 mediates innate immune response particularly to intra-cellular microorganisms like *M.tuberculosis* by acting on resting CD4^+^ T cells thus activating them and inducing expression of interleukin 1 receptor (IL1r) and secretion of IL2 [66]. IL1 has also been shown to help in B-cell activation and proliferation [67, 68]. It has been revealed that as part of their survival strategy in macrophages, mycobacteria regulate IL1β maturation through the induction of type I IFNs [69].

2.1.10. Interleukin 1 receptor antagonist (IL1ra)

IL1ra competitively inhibits binding of IL1α and IL1β to IL1r [70]. IL1ra has been found to be elevated in patients with active TB, and decreases during anti-tuberculosis treatment [71]. IL1ra has potential as an *in vitro* biomarker for *M.tuberculosis* infection [72].

2.2. Chemokines

2.2.1. Interferon inducible protein (IP)10

The production of IP10 is highly antigen dependent. It is produced by monocytes and T cells following *M.tuberculosis* infection [73]. The main function of IP10 is chemoattraction of activated TH1 cells to site of inflammation [74]. The levels of IP10 have been shown to be high in serum of TB patients [75]. In *in-vitro* stimulation of whole blood cells with RD1-specific antigens, HIV infection does not impair response detected by IP-10, while it significantly decreases IFNγ-mediated responses. [76].
2.2.2. Macrophage inflammatory protein 1 alpha (MIP1α)

MIP1α is also known as chemotactic chemokine ligand (CCL) 3 and it is a chemokine that function in recruitment and activation of polymophonuclear leukocytes during the acute inflammatory state. The other function of MIP1α is to induce activation and proliferation of T cells [77]. Secretion of MIP 1α in response to *M. tuberculosis* infection is promoted by TNFα [78].

It has been shown that MIP1α can be used as a biomarker of pleural TB [79]. In a study where murine macrophages were infected *in vitro* with *M. tuberculosis* there was a rapid induction of mRNAs for MIP1α [78].

2.2.3. Macrophage inflammatory protein 1 beta (MIP1β)

MIP1β is also known as CCL4. It is a chemotactic chemokine that acts as a chemoattractant for NK cells and monocytes. It also induces activation and proliferation of T cells [79]. Secretion of MIP1β in response to *M. tuberculosis* infection is promoted by TNFα [80].

2.3. RD1 Antigens

*Mycobacterium tuberculosis* genome consists of 16 regions of differences (RD). RD1 plays a major role in *M. tuberculosis* virulence and is present in *M. tuberculosis* and *M. bovis* but it is deleted from BCG vaccine strain of *M. bovis*. The RD1 gene region (Rv3871 to Rv3879c) has nine open reading frames (ORFs) [81] and can therefore code for nine proteins among them is early secretory antigen target 6 (ESAT 6) and culture filtrate protein 10 (CFP 10). It is absent from all BCG vaccine strains and from most environmental mycobacteria, but it is present in *M. tuberculosis* complex, including all clinical isolates of *M. tuberculosis* and *M. bovis* [82]. Anderson and colleagues identified two T-cell antigens encoded by RD1 gene, ESAT 6 and CFP 10 [82, 83]. The two antigens, ESAT 6 and CFP 10 are highly immunodominant and highly specific since they are not found in BCG and NTM strains of mycobacteria. Although the remaining seven RD1-encoded gene products are potentially highly specific, they do not encode strongly antigenic proteins and therefore lead to less immune response compared to ESAT 6 and CFP 10 [83].
2.4. Multiplex cytokine bead array

Multiplex cytokine immunoassays allow for the detection of multiple cytokines in a small volume of clinical samples [84, 85, 86]. This is important because many cytokines have closely related biological effects and therefore quantification of a single cytokine will not be of much value. Antibodies are used in a sandwich immunoassay fashion and are immobilized and conjugated to micro beads as “suspension arrays”. The immune-complex is then excited by the laser to emit colour. The bead specific emission is quantified and identified by the luminex. Examples of luminex software are luminex IS 2.3 (Luminex Corporation), Luminex 100/200™ (Luminex Corporation), Luminex™ 100 (MirabiovUSA) and Bio-plex Manager 4.1, 5.0, and 6.0 (Bio-Rad).
Diagnosis of pulmonary TB among adults in developing countries is based on techniques such as clinical assessment, sputum smear microscopy, chest x-ray and cultures. Although sputum smear examination for acid fast bacilli (AFB) is the most commonly used technique for routine diagnosis of TB, it has a low sensitivity and there is a high rate of false negatives especially among the immunocompromised patients owing to the paucibacillary nature of pulmonary TB in patients with HIV infection. Mycobacteria culture technique is the gold standard. However, it takes a long time (6-8 weeks) for sputum culture results to be obtained and most primary care centers in resource-limited countries lack TB culture facilities. Chest X-ray has a high inter-observer variability making this method subjective and less reliable. Despite this, diagnosis of tuberculosis in developing countries relies heavily on it. There is need to improve diagnosis of TB especially in immunocompromised individuals, by identifying biomarkers for *M. tuberculosis* infection. Diagnosis of LTBI is also important especially in immunocompromised individuals. This is because the annual risk of an HIV infected patient developing active TB is 10% and it increases with time. Treatment of latent TB has been shown to reduce the risk of developing active TB by 60%. TST has been used for a century for diagnosis of LTBI but it has several limitations including a high false positive rate due to NTM or previous BCG vaccination and false negative results due to cutaneous anergy due to underlying immunosuppression. Development of IGRAs has improved the diagnosis of LTBI. IGRAs are better than TST in that they have higher specificity, better correlation with exposure to *M. tuberculosis*, and less cross-reactivity due to BCG vaccination and NTM infection. However, some studies have shown that IGRAs have impaired sensitivity in the diagnosis of TB in immunocompromised patients. IGRAs cannot distinguish between active disease and latent TB. A biomarker that can distinguish active disease and LTBI would be useful for timely diagnosis and treatment of active TB and in the detection of those with LTBI.
RESEARCH QUESTION

Can *Mycobacteria tuberculosis* specific antigens early secretory antigen target 6 and culture filtrate protein 10 induced cytokines and/or chemokines be used as biomarkers to distinguish active from latent tuberculosis?

Objectives

Broad objective

To investigate using *in vitro* assay, the profile of cytokine and chemokine production induced by *Mycobacterium tuberculosis* ESAT 6 and CFP 10 specific antigens among patients with active and latent tuberculosis with a secondary aim of identifying some of the cytokines and chemokines as potential diagnostic biomarkers.

Specific objectives

To determine the *M.tuberculosis* specific antigens (ESAT 6 and CFP 10) induced cytokine and chemokine profile in HIV uninfected/tuberculosis subjects.

To determine the *M.tuberculosis* specific antigens (ESAT 6 and CFP 10) induced cytokine and chemokine production in TB/HIV co infected subjects.

To determine the *M.tuberculosis* specific antigens (ESAT 6 and CFP 10) induced cytokine and chemokine production in persons with latent *M. tuberculosis* infection.
CHAPTER 3: MATERIALS AND METHOD

3.1. Study Design
This was a cross sectional study nested within an ongoing study titled: “Use of immune assays to improve the diagnosis of active/latent TB”.

3.2. Description of the larger study
The larger study had been approved by Kenyatta National Hospital Ethics and Research Committee and approval was also obtained for this substudy to allow use of leftover samples. The larger study had recruited patients with active TB who visited Mbagathi District Hospital for medical services and their household contacts. The study subjects were adults aged 18 years and above.

3.2.1 Sample collection
After consenting, a total of 3ml of blood had been collected by venipuncture and blood collected in specialized blood collection tubes for quantiFERON® TB Gold (QFT) assay. This included the nil control (grey cap), TB antigen (red cap) and mitogen control (purple cap). The blood had been transported to University of Nairobi, Department of Medical Microbiology where it was processed and QFT ELISA performed to determine the concentration of IFN-γ.

3.3. Study subjects
There were 62 pulmonary TB patients and 20 household contacts (HHC). Active pulmonary TB subjects had clinical signs of TB, positive AFB sputum smear test, chest X-ray suggestive of TB and or positive QFT test. Latent TB infection was defined as HHC who did not have clinical signs of TB and had a positive QFT test. Controls (TB naïve group) did not have clinical signs of TB, were HIV negative and had a negative QFT test.

3.4. Inclusion criteria
- Cases were AFB sputum smear positive and/or had X-ray suggestive of TB.
- Individuals who had HIV test results.
• House hold contacts (HHC) of individuals with active TB were living in the same house with active TB cases.

• TB naïve control group were QFT negative and HIV negative

3.5. Exclusion criteria

• Individuals who did not have HIV test results.

• Children under the age of 18 years.

• Those who refused to consent.

3.6. Multiplex cytokine immunoassay (Luminex assay)

Cytokine/chemokine levels were evaluated using LINCO-plex® kits (Millipore, St. Charles, Missouri, USA) on the Bio Plex platform (Bio Plex™, Bio Rad Laboratories) according to the Linco instructions [87]. Briefly luminex involved preparation of reagents and immunoassay procedure outlined below.

3.6.1. Preparation of samples and reagents

The samples and reagents were prepared according to manufacturer’s instructions [87]. Briefly the supernatants that remained in the nil and antigen tube after QFT assay had been performed were centrifuged to remove particles before being used in the immunoassay. The antibody-immobilized beads were sonicated for 30 seconds and then vortexed for 1 minute. This was followed by addition of the antibody-beads into a mixing bottle. The quality control 1 and 2, serum matrix and human cytokine standard were reconstituted with deionised water and mixed well. The reconstituted human cytokine standard was used to prepare working standards by serial dilution with assay buffer. The wash buffer was first brought to room temperature and mixed to bring all salts into solution. It was then diluted with deionised water. All reagents were allowed to warm to room temperature before use in the assay. The room temperature in the laboratory was always measured and recorded and it ranged between 20° C and 25° C.
3.6.2 Immunoassay procedure

Luminex assay was performed according to Linco instructions [87]. In brief, a filter plate was pre-wet by instilling 200μL of assay buffer into each well of the microtiter filter plate and sealed before mixing on a plate shaker for 10 minutes at room temperature. This was followed by removal of the assay buffer by vacuum filtration. The excess assay buffer was removed from the bottom of the plate with absorbent pad or paper towels.

Twenty five micro litres of each standard and control were put in duplicate into appropriate wells. This was followed by addition of 25μL of matrix solution to the standards and control wells. Twenty five micro litres of assay buffer was then put into each sample well before adding 25μL of sample in duplicate. The mixing bottle containing antibody beads were vortexed and then 25μL of premixed beads were added to each well. The plate was then sealed with a plate sealer and incubated with agitation in the dark overnight at 4°C. The following day the fluid was removed gently by vacuum filtration and then the plate was washed 2 times with 200μL/well of wash buffer, removing the wash buffer by vacuum filtration between each wash. The excess wash buffer was blotted from the bottom of the plate by an absorbent pad or paper towels. Twenty five microliters of detection antibodies were then added into each well. This was followed by sealing of the plate and incubating it with agitation in the dark for 1 hour at room temperature. After that 25μL of Streptavidin-Phycoerythrin was added to each well and the plate was sealed and incubated with agitation in the dark for 30 minutes at room temperature. Finally, all the contents were removed gently by vacuum filtration and the plate was washed 2 times with 200μL/well wash buffer, removing the wash buffer by vacuum filtration between each wash. The excess wash buffer was wiped from the bottom of the plate with a tissue. Finally, 150μL of sheath fluid was added to all the wells. The beads were re-suspended on a plate shaker for 5 minutes and the plate was run on luminex 100TM IS, 200TM, or HTS. The median fluorescent intensity (MFI) data was saved and analysed using weighted 5-parameter logistic for calculating cytokines/chemokines concentration in samples.
3.7. Data collection

Information on age, AFB sputum smear test results, QFT test results, chest X-ray results, HIV status and CD4\(^+\) T cell count of the patients was obtained from the questionnaire of the larger study in which this sub study was nested in.

3.8. Data analysis

The biomarker response was defined as the concentration of the cytokine in the TB antigen (Ag) tube minus the concentration of cytokine in the nil tube (Ag-Nil) as detected by multiplex bead assay. The median of each type of cytokine produced in participants was determined. The concentration of each biomarker was compared using Mann Whitney U test in different groups. Mann Whitney U test was also used to compare participants who had CD4\(^+\) T cell count less than 250 cells/mL and more than 250 cells/mL with controls. Receiver operator characteristic (ROC) curve analysis was used to determine cut off point for differentiating between the two groups in the diagnosis of \textit{M. tuberculosis} infection and discrimination LTBI from TB disease. Statistical analysis was conducted using SPSS v17.0. [88] and statistica 8 [89]. Statistical significance was accepted if the \(P\) values \(\leq 0.05\).

3.9. Ethical consideration

This study was approved by Kenyatta National Hospital/University of Nairobi Ethics and Research Committee.
CHAPTER 4: RESULTS

4.1 Study subjects

4.1.1 Demographic characteristics of the participants

A total of 82 participants were recruited into the study out of which 62 (75.6%) had active pulmonary tuberculosis while 13 (15.9%) were household contacts (HHCs) and 7 (8.5%) were controls. The mean age of the participants was 36 years with an age range of 18 years to 82 years. Out of the 82 participants 41 (48.8%) were male. The gender of one person was not indicated. The median age of pulmonary TB patients was 30.5 years, with the inter-quartile range (IQR) of 18 years to 82 years. The median (IQR) age of HHC was 39.5 (18-63) years respectively while the median (IQR) age of controls was 34 (27-58) years respectively. Table 1 shows the gender distribution of the participants.

Table 1: Gender distribution of the participants

<table>
<thead>
<tr>
<th>Gender</th>
<th>Pulmonary TB patients</th>
<th>HHCs</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male n (%)</td>
<td>34(54.8%)</td>
<td>5(38.5%)</td>
<td>1(14.3%)</td>
</tr>
<tr>
<td>Female n (%)</td>
<td>27(43.6%)</td>
<td>8(61.5%)</td>
<td>6(85.7%)</td>
</tr>
<tr>
<td>Unknown n (%)</td>
<td>1 (1.6%)</td>
<td>0(0.0%)</td>
<td>0(0.0%)</td>
</tr>
</tbody>
</table>

4.1.2 Clinical characteristics of the participants

Out of the 62 pulmonary TB patients who participated in the study, 26 (41.9%) were HIV positive. Of the 36 active pulmonary TB and HIV negative patients, 17 (47.2%) had positive AFB sputum smear test results; 19 (52.8%) were clinically and radiologically diagnosed but did not have sputum smear test results. The QFT test was positive in all pulmonary TB/HIV negative patients except one. The QFT negative patient among the active pulmonary TB/HIV negative group had a positive sputum smear result.

Out of the 26 active pulmonary TB/HIV co-infected patients, 7 (26.9%) were positive by AFB sputum smear test while 19 (73.1%) were clinically and radiologically diagnosed. Of the 26 HIV positive participants 14 (53.9%) had $CD_4^+$ T cell count results. The median (IQR) of $CD_4^+$ T cell count was 242 cells/mL (7-695) cells/mL respectively.
A total of 13 house hold contacts (HHCs) were recruited into the study. One HHC was HIV positive while the rest were HIV negative. One HHC had indeterminate QFT result and was not included in the analysis. The HIV positive HHC was QFT negative for latent TB and was also not included in the analysis. All the 7 controls were HIV negative and QFT negative.

4.2 Antigen-dependent cytokine and chemokine production

Antigen-dependent cytokine/chemokine production was evaluated by subtracting the amount of cytokine/chemokine in the nil (unstimulated) tube from the antigen (stimulated) tube i.e. (Ag-nil). The cytokines and chemokines analysed for were IFNα2, IFNγ, IL10, IP 10, IL12p40, IL12p70, IL15, IL17, IL6, IL2, IL1ra, IL7, IL1α, IL1β, MIP1α, MIP1β and TNFa.

4.2.1 Cytokine and chemokine profile in active TB/HIV negative participants.

Comparison of cytokine/chemokine levels was carried out between TB+/HIV negative individuals and controls using Mann Whitney U test. The levels of antigen-dependent IL1ra (p=0.032), IFNγ and IL2 (p=<0.0001) were significantly higher among active TB/HIV negative participants compared to the controls as shown in table 2 and figure 1. The level of IL17 (Ag-nil) (p=0.004) was significantly lower among active TB/HIV negative participants compared to the controls as shown in table 2 and figure 1. There was no significant difference between active TB/HIV negative participants and controls in antigen-dependent levels of the other analysed cytokines/chemokines. However, the median (1427.4pg/mL) of IP10 in active TB/HIV negative participants was higher than the median (94.2pg/mL) of IP10 in the controls, although the difference was not statistically significant. See table 2. The antigen dependent IFNγ and IL12p40 among the controls was negative because more cytokine was produced in the unstimulated compared to M.tuberculosis specific antigen stimulated tube.
Table 2: Comparison of antigen-dependent cytokine/chemokine production in active TB/HIV negative participants and controls.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Median pg/ml in TB+/HIV- (n=36)</th>
<th>Median pg/ml in controls (n=7)</th>
<th>P-Value (TB+/HIV- vs controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.468</td>
</tr>
<tr>
<td>IFNγ</td>
<td>297</td>
<td>-7.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL10</td>
<td>0.8</td>
<td>8.5</td>
<td>0.210</td>
</tr>
<tr>
<td>IL12p40</td>
<td>0.0</td>
<td>0.0</td>
<td>0.220</td>
</tr>
<tr>
<td>IL12p70</td>
<td>0.0</td>
<td>-0.9</td>
<td>0.784</td>
</tr>
<tr>
<td>IL15</td>
<td>0.0</td>
<td>0.0</td>
<td>0.292</td>
</tr>
<tr>
<td>IL17</td>
<td>0.8</td>
<td>6.3</td>
<td>0.004</td>
</tr>
<tr>
<td>IL1ra</td>
<td>433.5</td>
<td>17.4</td>
<td>0.032</td>
</tr>
<tr>
<td>IL1α</td>
<td>6.2</td>
<td>62.6</td>
<td>0.410</td>
</tr>
<tr>
<td>IL1β</td>
<td>103.8</td>
<td>28.9</td>
<td>0.687</td>
</tr>
<tr>
<td>IL2</td>
<td>122.6</td>
<td>0.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL6</td>
<td>135.5</td>
<td>494</td>
<td>0.735</td>
</tr>
<tr>
<td>IL7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.987</td>
</tr>
<tr>
<td>IP10</td>
<td>1427.4</td>
<td>94.2</td>
<td>0.114</td>
</tr>
<tr>
<td>MIP1α</td>
<td>101</td>
<td>773.8</td>
<td>0.711</td>
</tr>
<tr>
<td>MIP1β</td>
<td>1071.5</td>
<td>1022.5</td>
<td>0.595</td>
</tr>
<tr>
<td>TNFα</td>
<td>45.6</td>
<td>17.1</td>
<td>0.860</td>
</tr>
</tbody>
</table>

Table 2 illustrates the median antigen-dependent cytokine/chemokine production among active TB/HIV negative participants (TB+/HIV-) and Controls in pg/mL. The P-value for TB+/HIV- vs controls were calculated using Mann Whitney U test.
Figure 1: Comparison of antigen dependent cytokine production between active TB/HIV negative participants, Active TB/HIV positive participants and individuals with LTBI vs controls.

Figure 1 illustrates the antigen-dependent cytokine/chemokine production among active TB/HIV negative patients (TB/HIV−), active TB/HIV co-infected patients (TB/HIV+), individuals with LTBI and Controls. Only those biomarkers that were significantly different were plotted.
4.2.2 Cytokine and chemokine profile in active TB/HIV co-infected patients

Comparison of cytokine/chemokine production in active TB/HIV co-infected participants and controls who were QFT negative and HIV negative was done using Mann Whitney U test. The levels of IFN\(\gamma\) (Ag-Nil) \((p=0.003)\) and IL2 (Ag-Nil) \((p=0.030)\) were significantly higher among active TB/HIV co-infected participants compared to the controls as shown in table 3 and figure 1. The level of IL17 (Ag-Nil) \((p=0.009)\) was significantly lower among active TB/HIV co-infected participants compared to the controls as shown in table 3 and figure 1. There was no significant difference in the antigen-dependent levels of the rest of the cytokine/chemokines between active TB/HIV co-infected participants and the controls. However, the antigen-dependent median (838.6pg/mL) levels of IP10 in active TB/HIV co-infected were higher than the median (94.2pg/mL) levels of IP 10 in the controls although not statistically significant as shown in table 3.
Table 3: Comparison of antigen-dependent cytokine/chemokine production in active TB/HIV co infected participants and controls.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Median in pg/mL among TB+/HIV+ (n=26)</th>
<th>Median in pg/ml among controls (n=7)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα2</td>
<td>0.6</td>
<td>0.0</td>
<td>0.813</td>
</tr>
<tr>
<td>IFNγ</td>
<td>26.4</td>
<td>-7.1</td>
<td>0.003</td>
</tr>
<tr>
<td>IL10</td>
<td>0.0</td>
<td>8.5</td>
<td>0.054</td>
</tr>
<tr>
<td>IL12p40</td>
<td>1.2</td>
<td>0.0</td>
<td>0.651</td>
</tr>
<tr>
<td>IL12p70</td>
<td>0.2</td>
<td>-0.9</td>
<td>0.199</td>
</tr>
<tr>
<td>IL15</td>
<td>0.2</td>
<td>0.0</td>
<td>0.949</td>
</tr>
<tr>
<td>IL17</td>
<td>1.01</td>
<td>6.3</td>
<td>0.009</td>
</tr>
<tr>
<td>IL1ra</td>
<td>112.2</td>
<td>17.4</td>
<td>0.330</td>
</tr>
<tr>
<td>IL1α</td>
<td>1.3</td>
<td>62.6</td>
<td>0.143</td>
</tr>
<tr>
<td>IL1β</td>
<td>4.4</td>
<td>28.9</td>
<td>0.215</td>
</tr>
<tr>
<td>IL2</td>
<td>21.4</td>
<td>0.0</td>
<td>0.03</td>
</tr>
<tr>
<td>IL6</td>
<td>10.1</td>
<td>494.1</td>
<td>0.143</td>
</tr>
<tr>
<td>IL7</td>
<td>-0.4</td>
<td>0.0</td>
<td>0.399</td>
</tr>
<tr>
<td>IP10</td>
<td>838.6</td>
<td>94.2</td>
<td>0.268</td>
</tr>
<tr>
<td>MIP1α</td>
<td>35.5</td>
<td>773.8</td>
<td>0.352</td>
</tr>
<tr>
<td>MIP1β</td>
<td>415.7</td>
<td>1022.5</td>
<td>0.424</td>
</tr>
<tr>
<td>TNFα</td>
<td>12.5</td>
<td>17.1</td>
<td>0.232</td>
</tr>
</tbody>
</table>

Table 3 illustrates the median antigen-dependent cytokine/chemokine production among active TB+/HIV co infected patients (TB+/HIV) and Controls. The P-value for TB+/HIV+ vs controls were calculated using Mann Whitney U test.
4.2.2.1 Effects of CD4+ T cell counts on antigen-dependent cytokine production.

The active TB/HIV co infected participants were grouped into two depending on their CD4+ T cell count i.e. those with less than 250 cells/mL and those who had more than 250 cells/mL. There were 14 HIV positive participants with CD4+ T cell count, out of which 7(50%) had <250 cells/mL. The active TB/HIV positive patients who had CD4+ T cell count less than 250 cells/mL and more than 250 cells/mL were compared with controls using Mann Whitney U test as shown in figure 2. There was a significant difference in the amount of IFNγ (p=0.008) and IL2 (p=0.019) between active TB/HIV co infected participants with CD4+ T cell count >250 cells/mL compared to controls. However, there was no significant difference in the amount of IFNγ and IL2 between active TB/HIV co infected participants with CD4+ T cell count <250 cells/mL compared to controls. There was a significant difference in amount of IL17 between TB/HIV co infected participants with CD4+ T cell count <250 cells/mL compared to controls, but there was no significant difference in amount of IL17 between TB/HIV co infected participants with CD4+ T cell count >250 cells/mL compared to controls. More IL1ra was produced in active TB/HIV positive participant with CD4+ T cells >250 compared to controls even though the difference was not significant as shown in figure 2.
Figure 2: comparison of active TB/HIV positive participants with CD4 T cells <250 cells/mL and more than 250 cells/mL with controls who were QFT negative and HIV negative.

Figure 2 illustrates the antigen-dependent IL2, IL1ra, IL17 and IFNγ production among active TB/HIV co-infected participants with CD4 T cell count <250 cells/mL, >250 cells/mL and controls.
4.2.3 Cytokine/chemokine profile in individuals with LTBI

Comparison of cytokine/chemokine levels was carried out between individuals with LTBI and controls, who were QFT and HIV negative, using Mann Whitney U test. The levels of IFNγ (Ag-nil) (p=0.007) and IL2 (Ag-nil) (p=0.004) were significantly higher among individuals with LTBI compared to controls. The level of IL17 (Ag-nil) (p=0.020) was significantly lower among individuals with LTBI compared to controls. See table 4 and figure 1. There was no significant difference in antigen-dependent levels of other analysed cytokines/chemokines between individuals who had LTBI and controls. However, the antigen-dependent IL1ra median (783.4pg/mL) levels were higher in individual with LTBI than the mean median (17.4pg/mL) in the controls. Also the antigen-dependent IP10 median (5066.6pg/mL) levels were higher in individuals with LTBI compared to the median (94.2pg/mL) in the controls. The median (2525.8pg/mL) levels of MIP1α were higher in the individuals with LTBI than the median (773.8pg/mL) in the controls. The median (276.9pg/mL) of TNF α were higher in individuals with LTBI than the median (17.1pg/mL) in the controls. See table 4.
Table 4: Comparison of antigen-dependent cytokine/chemokine production in whole blood from individuals with LTBI and controls.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Median pg/mL in Latent TB (n=11)</th>
<th>Median pg/ml in controls (n=7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.930</td>
</tr>
<tr>
<td>IFNγ</td>
<td>404.1</td>
<td>-7.1</td>
<td>0.007</td>
</tr>
<tr>
<td>IL10</td>
<td>1.5</td>
<td>8.5</td>
<td>0.328</td>
</tr>
<tr>
<td>IL12p40</td>
<td>0.0</td>
<td>0.0</td>
<td>0.659</td>
</tr>
<tr>
<td>IL12p70</td>
<td>1.0</td>
<td>-0.9</td>
<td>0.328</td>
</tr>
<tr>
<td>IL15</td>
<td>0.0</td>
<td>0.0</td>
<td>0.479</td>
</tr>
<tr>
<td>IL17</td>
<td>1.3</td>
<td>6.3</td>
<td>0.015</td>
</tr>
<tr>
<td>IL1ra</td>
<td>783.4</td>
<td>17.4</td>
<td>0.126</td>
</tr>
<tr>
<td>IL1α</td>
<td>72.4</td>
<td>62.6</td>
<td>0.860</td>
</tr>
<tr>
<td>IL1β</td>
<td>100.7</td>
<td>28.9</td>
<td>0.930</td>
</tr>
<tr>
<td>IL2</td>
<td>645.2</td>
<td>0.0</td>
<td>0.004</td>
</tr>
<tr>
<td>IL6</td>
<td>166.1</td>
<td>494.1</td>
<td>0.659</td>
</tr>
<tr>
<td>IL7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.860</td>
</tr>
<tr>
<td>IP10</td>
<td>5066.6</td>
<td>94.2</td>
<td>0.179</td>
</tr>
<tr>
<td>MIP1α</td>
<td>2525.8</td>
<td>773.8</td>
<td>0.285</td>
</tr>
<tr>
<td>MIP1β</td>
<td>3086.7</td>
<td>1022.5</td>
<td>0.285</td>
</tr>
<tr>
<td>TNFα</td>
<td>276.9</td>
<td>17.1</td>
<td>0.479</td>
</tr>
</tbody>
</table>

Table 4 illustrates the median antigen-dependent cytokine/chemokine production among individuals with LTBI (latent TB) and Controls.

The P-values for LTBI vs controls were calculated using Mann Whitney U test.

4.2.4 Comparison of cytokine/chemokines production in active TB/HIV negative participants and individuals with LTBI

Comparison of cytokine/chemokine level was carried out between LTBI and active TB/HIV negative by Mann Whitney U test. The antigen-dependent IL1α (p=0.04), MIP1α (p=0.049) and IL2 (p=0.049) was significantly higher in individuals with LTBI than in active TB/HIV negative participants as shown in table 5 and figure 3. There was no significant difference in antigen-dependent the other analysed cytokines/chemokines between active TB/HIV negative patients and individuals with LTBI. However the antigen dependent MIP1β median (3086.7pg/mL) in
participants with LTBI was higher than the median (1071.5 pg/ml) of MIP1β in active TB/HIV negative participants even though the difference was not significant. See table 5. Also the antigen dependent TNFα median (276.9 pg/mL) in participants with LTBI was higher than the median (12.5 pg/ml) of TNFα in active TB/HIV negative participants even though the difference was not significant as shown in table 5.

Table 5: Comparison of antigen-dependent cytokine /chemokine production in active TB/HIV negative participants and individuals with LTBI.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Median pg/ml TB+/HIV- (n=36)</th>
<th>Median pg/mL in Latent TB (n=11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNa2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.776</td>
</tr>
<tr>
<td>IFNγ</td>
<td>297.1</td>
<td>404.1</td>
<td>0.833</td>
</tr>
<tr>
<td>IL10</td>
<td>0.8</td>
<td>1.5</td>
<td>0.683</td>
</tr>
<tr>
<td>IL12p40</td>
<td>0.0</td>
<td>0.0</td>
<td>0.095</td>
</tr>
<tr>
<td>IL12p70</td>
<td>0.0</td>
<td>1.0</td>
<td>0.527</td>
</tr>
<tr>
<td>IL15</td>
<td>0.0</td>
<td>0.0</td>
<td>0.629</td>
</tr>
<tr>
<td>IL17</td>
<td>0.8</td>
<td>1.3</td>
<td>0.611</td>
</tr>
<tr>
<td>IL1ra</td>
<td>433.5</td>
<td>783.4</td>
<td>0.872</td>
</tr>
<tr>
<td>IL1α</td>
<td>6.2</td>
<td>72.4</td>
<td>0.039</td>
</tr>
<tr>
<td>IL1β</td>
<td>103.9</td>
<td>100.7</td>
<td>0.378</td>
</tr>
<tr>
<td>IL2</td>
<td>122.6</td>
<td>645.2</td>
<td>0.048</td>
</tr>
<tr>
<td>IL6</td>
<td>135.5</td>
<td>166.1</td>
<td>0.931</td>
</tr>
<tr>
<td>IL7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.990</td>
</tr>
<tr>
<td>IP10</td>
<td>1427.4</td>
<td>5066.6</td>
<td>0.576</td>
</tr>
<tr>
<td>MIP1α</td>
<td>101.1</td>
<td>2525.8</td>
<td>0.048</td>
</tr>
<tr>
<td>MIP1β</td>
<td>1071.5</td>
<td>3086.7</td>
<td>0.290</td>
</tr>
<tr>
<td>TNFα</td>
<td>45.6</td>
<td>276.9</td>
<td>0.051</td>
</tr>
</tbody>
</table>

Table 5 illustrates the median antigen-dependent cytokine/chemokine production among active TB/HIV negative participants (TB+/HIV-) and individuals with LTBI (latent TB). The P-values for TB+/HIV- vs LTBI were calculated using Mann Whitney U test.
Figure 3: cytokines/chemokines that can discriminate LTBI from TB Disease

Figure 3 illustrates the antigen-dependent IL1α, IL2, MIP1α and MIP1β production among active TB but HIV negative participants and participants with LTBI. Only those cytokines/chemokines that were significantly different between participants with LTBI and controls were plotted.
4.3 Discriminative ability of cytokines/chemokines in TB diagnosis

The receiver operator characteristic (ROC) curve analysis was done for cytokines/chemokines that demonstrated significant difference in the various group comparisons.

4.3.1 Diagnosis of TB

The ability of IFNγ, IL1ra, IL2 and IL17 to diagnose TB irrespective of HIV status and the stage of *M.tuberculosis* infection was determined using ROC curve analysis.

Figure 4: Ability of IFNγ, IL1ra, IL2 and IL17 to discriminate *M.tuberculosis* infected from un-infected individuals

![ROC curves for IFNγ, IL1ra, IL2, and IL17](image)

Figure 4: Receiver operator characteristic curve analysis were performed by plotting sensitivity vs (100%-specificity) to determine the amount of measured IFNγ, IL17, IL1ra and IL2 that would discriminate *M.tuberculosis* infected from controls who were QFT negative and HIV negative. The area under the curve (AUC) was above 0.80 except for IL1ra which had an AUC of 0.73.
The cut-off value was determined by taking the highest point on the vertical axis and the furthest to the left on the horizontal axis (upper left corner) as shown in figure 4. This cut-off value was used to determine the sensitivity and specificity which were reported in percentage form. See table 6.

Table 6: Cut-off points, sensitivity and specificity values of IFNγ, IL1ra, IL2 and IL17 in diagnosis of M.tuberculosis infection.

<table>
<thead>
<tr>
<th>Cytokine/ Chemokine</th>
<th>Cut-off pg/mL</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All</td>
<td>TB+/HIV-</td>
<td>TB+/HIV+</td>
</tr>
<tr>
<td>IFNγ</td>
<td>14.5</td>
<td>80.8%</td>
<td>100%</td>
<td>53%</td>
</tr>
<tr>
<td>IL1ra</td>
<td>28.0</td>
<td>83.6%</td>
<td>88.9%</td>
<td>76.9%</td>
</tr>
<tr>
<td>IL2</td>
<td>1.4</td>
<td>84.9%</td>
<td>94.4%</td>
<td>88.5%</td>
</tr>
<tr>
<td>IL17</td>
<td>3.7</td>
<td>75%</td>
<td>75%</td>
<td>73.1%</td>
</tr>
</tbody>
</table>

Table 6 illustrates the cut-off point, percentage sensitivity and specificity of IFNγ, IL1ra, IL2 and IL17 in diagnosis of M.tuberculosis infection among active TB and HIV negative (TB+/HIV-) participants, active TB and HIV co infected (TB+/HIV+) participants and individuals with latent TB infection (LTBI).

4.3.2 Comparison of active TB/HIV negative participants with active TB/HIV co infected participants

The cut-off point of IFNγ was 14.5pg/mL for all stages of TB irrespective of the HIV status of the participants. Using a cut-off point of 14.5pg/mL for IFNγ in participants with active TB, the specificity was 100% for both HIV positive and HIV negative participants, while the sensitivity for HIV positive and HIV negative participants was 53% and 100%, respectively.

For IL2 the cut-off point, sensitivity and specificity in diagnosis of M. tuberculosis infection among active TB/HIV negative participants was 13.4pg/mL, 94.4% and 82.3%, respectively. Application of this cut-off point on the HIV infected participants gave a sensitivity and specificity of 50% and 83.3%, respectively. The cut-off point, sensitivity and specificity of IL17 in diagnosis of M. tuberculosis infection among HIV un-infected individuals was 2.9pg/mL, 83.3% and 71.4%, respectively. When this cut-off point was applied to HIV infected individuals, the sensitivity and specificity of IL17 in diagnosis of M. tuberculosis infection was 73.1% and 83.3%, respectively.
4.3.3 Differentiating active TB from LTBI

The ability of IL1α, IL2, MIP1α and TNFα to discriminate LTBI from active TB disease was determined by ROC curve analysis as shown in figure 6. The AUC was less than 75% for all the 4 cytokines. Since ROC curves with an AUC= 0.75 are not clinically useful [86] these 4 cytokines did not have clinical value in discriminating between LTBI and TB disease individually.

Figure 6: Ability of IL1α, IL2, MIP1α and TNFα to discriminate between active TB from LTBI

Figure 6 ROC curve analysis were performed by plotting sensitivity vs (100%-specificity) to determine the amount of measured IL1α, IL2, MIP1α and TNFα that would discriminate TB disease from LTBI.
The cut-off value was determined by taking the highest point on the vertical axis and the furthest to the left on the horizontal axis (upper left corner) as shown in figure 6. This cut-off value was used to determine the sensitivity and specificity which were reported in percentage form as shown in table 7. With a cut-off value of 53pg/mL the sensitivity and specificity of IL1α in differentiating active and latent TB was 63.6% and 65.5%, respectively. See table 7. The cut-off value of IL2 in discriminating active and latent TB was 172.1pg/mL. Using this cut-off value the sensitivity and specificity of IL2 in differentiating active and latent TB was 72.7% and 65.8%, respectively. See table 7. For MIP 1α the cut-off value, sensitivity and specificity were found to be 717.1pg/mL, 72.7% and 62.9%, respectively. While for TNFα the cut-off value, sensitivity and specificity was 119.4pg/mL, 63.6% and 68.6%, respectively. See table 7.

Table 7: Median, IQR of antigen depended IL1α, IL2, MIP1α and TNFα, cut-off points, sensitivity and specificity in discriminating LTBI and TB disease.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>cytokine/chemokine in LTBI median(IQR)</th>
<th>cytokine/chemokine in Active TB median(IQR)</th>
<th>Cut off point</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1α(Ag-nil)</td>
<td>72.35 (3.9-925.2)</td>
<td>6.22 (-216.9-667.2)</td>
<td>53.0</td>
<td>63.6%</td>
<td>65.5%</td>
<td>0.046</td>
</tr>
<tr>
<td>IL2(Ag-nil)</td>
<td>645.22 (-254-4685.2)</td>
<td>122.61 (0-685.1)</td>
<td>172.1</td>
<td>72.7%</td>
<td>65.8%</td>
<td>0.055</td>
</tr>
<tr>
<td>MIP1α(Ag-nil)</td>
<td>2525.78 (-178.2-9550.7)</td>
<td>101.05 (-2555.0-9934.7)</td>
<td>717.1</td>
<td>72.7%</td>
<td>62.9%</td>
<td>0.055</td>
</tr>
<tr>
<td>TNFα(Ag-nil)</td>
<td>276.9 (-30.2-989.3)</td>
<td>45.57 (-537.7-644.8)</td>
<td>119.4</td>
<td>63.6%</td>
<td>68.6%</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Table 7 illustrates the cut-off point, percentage sensitivity and specificity of IFNγ, IL1ra, IL2 and IL17 in discriminating active TB and HIV negative (TB+/HIV-) participants and individuals with latent TB infection(LTBI)
CHAPTER 5: DISCUSSION

The problems encountered in diagnosis of TB especially in immunocompromised individuals, emphasizes the need for a highly conserved biomarker for *M.tuberculosis* infection. A biomarker that can differentiate individuals who have active *M. tuberculosis* infection from those who have LTBI will also be a major breakthrough.

Interferon gamma is a key cytokine in control of *M.tuberculosis* infection. In this study, IFNγ was induced *in vitro* in response to *M.tuberculosis* ESAT-6 and CFP 10 antigens in significantly higher amounts among *M.tuberculosis* infected/HIV un-infected individuals compared to controls. Although IFNγ was produced in significantly high amount in active TB/HIV co infected patients than in controls, futher analysis in participants with CD4+ T cell less than <250 cells/mL showed that there was no significant difference in the levels of *in-vitro* induced IFNγ between *M.tuberculosis* infected and controls. A previous study has shared that despite HAART, there is impaired IFNγ production by mycobacteria-specific CD4+ T cells in HIV infected persons [28]. The sensitivity of IFNγ in diagnosis of active TB/HIV negative patients was 100%. However in active TB/HIV co-infected patients, the sensitivity reduced to 53%. This findings show that IFNγ is a poor biomarker in diagnosis of TB among HIV infected patients. The IGRAs which measure the level of IFNγ released after *M.tuberculosis* RD1 antigens have been reported to have low sensitivity in diagnosis of *M.tuberculosis* infection among HIV infected individuals [90, 91, 92].

Interleukin 2 is secreted by some subsets of T cells in response to *M.tuberculosis* infection [57]. Interleukin-2 promotes long term growth of activated CD4+ T cells and induce CD4+ T cell to produce IFN γ [58]. In this study, IL2 was induced *in vitro* in response to *M.tuberculosis* ESAT-6 and CFP 10 antigens in significantly higher amounts among individuals with active TB but HIV un-infected compared to controls. A study by Biselli et al had previously reported high amount of IL2 in quantIFERON-TB Gold in tube supernatants of blood from individuals with TB than in controls [65]. The sensitivity of IL2 in active TB/HIV negative patients was 94.4%. However, in active TB/HIV co-infected patients, the sensitivity reduced to 88.9%. Further analysis in active TB/HIV co infected patients with CD4+ T cell less than <250 cells/mL showed that there was no significant difference in the levels of *in-vitro* induced IL2 between *M.tuberculosis* infected and controls. This
findings show that IL2 is a poor biomarker in diagnosis of *M. tuberculosis* infection among HIV infected patients with low CD4⁺ T cell count. The impaired IL2 response at low CD4⁺ T cell count could be attributed to less Th1/Th17 subset of CD4 T cells in HIV infection [69]. Interleukin 2 also showed ability to discriminate individuals with LTBI from patients with active TB. Some studies have also shown that IL-2 can be used to differentiate between latent and active tuberculosis [59, 61]. Another study showed that IL2 is produced in larger amounts in LTBI individuals if incubation is prolonged to more than 72 hours [61]. Studies that evaluated IL2 as a biomarker for TB reported that IL2 cannot be used alone as a biomarker of TB [93, 94, 95].

Interleukin 1 receptor antagonist competitively inhibits binding of IL1α and IL1β to IL1r [64]. This study reported high levels of IL1ra production following *in vitro* stimulation with *M. tuberculosis* ESAT-6 and CFP 10 active TB/ HIV negative individuals compared to controls. Another study had also reported that IL1ra was induced in vitro in response to antigen stimulation in significantly higher amounts in those with tuberculosis than in controls [90]. The sensitivity IL1ra in active TB/HIV negative patients was 88.9%. However, in active TB/HIV co-infected patients, the sensitivity reduced to 76.9% re. This findings show that IL1ra is also a poor biomarker in diagnosis of *M. tuberculosis* infection among HIV infected patients.

Interleukin-17 is important in the formation of granuloma in that it triggers recruitment of neutrophils to the lungs [54, 56]. It’s expressed by activated CD4⁺ CD45RO⁺ memory T cells [59]. In this study IL17 showed ability to diagnose *M. tuberculosis* infection both in HIV infected and HIV uninfected individuals in that more IL17 was produced following ESAT 6 and CFP 10 antigen stimulation in controls compared to individuals with active TB and LTBI irrespective of the HIV status. The results of this study demonstrate that IL17 can be used as a biomarker of *M. tuberculosis* infection both in HIV infected and HIV uninfected individuals. However in active TB/HIV co-infected participants with CD4⁺ T cell count >250 cell/mL, the antigen-dependent IL17 was not significantly different compared to controls.
In this study, MIP1α showed ability to discriminate individuals with LTBI from participants with active TB. Macrophage inflammatory protein (MIP) 1 alpha is a chemokine that function in recruitment and activation of polymophonuclear leukocytes during the acute inflammatory state. The other function of MIP 1α is to induce activation and proliferation of T cells [71].

In this study IL1α showed ability to discriminate individuals with LTBI from patients with active TB. Interleukin 1 is produced by macrophages infected with *M. tuberculosis* and consists of two subunits IL1α and IL1β. Interleukin 1 mediates innate immune response particularly to intra-cellular microorganisms like *M. tuberculosis* by acting on resting CD4+ T cells thus activating them and inducing expression of interleukin 1 receptor (IL1R) and secretion of IL2 [60]. Interleukin 1 has also been shown to help in B-cell activation and proliferation [61, 62].

Interferon inducible protein 10 was produced in higher mounts among participants with TB irrespective of the HIV status compared to controls. However this difference was not significant. A study by Goletti et al had also demonstrated that IP10 response to RD1 selected peptides is associated with active TB in HIV-infected subjects and there were significantly higher proportion of responders to IP-10 than to IFN-γ [73]. The levels of IP10 have also been found to be high in unstimulated blood from active TB patients [69, 70].

The levels of IFNα2, IL10, IFNγ and IL7 were lower in *M. tuberculosis* ESAT 6 and CFP 10 stimulated whole blood cultures compared to unstimulated whole blood. This might be because of difference in the expression kinetics of the different cytokines/chemokines after stimulation with the *M. tuberculosis* ESAT 6 and CFP 10 antigens [95]. Also, it could be that some soluble or membrane bound receptors are co-expressed after stimulation and they consume the cytokines/chemokines [95]. Future studies are required to investigate this observation.

The concentration of each biomarker was compared using Mann Whitney U test in different groups. Mann Whitney U test is used to compare two independent continuous variables to test for statistical significance, when the sample size is small and normal distribution cannot be assumed [96].
Receiver operator characteristic (ROC) curve analysis was used to determine cut off point for differentiating between the two groups in the diagnosis of *M.tuberculosis* infection and discrimination LTBI from TB disease. The analysis is used to determine the cut-off points, sensitivity and specificity [97]. The cut-off value determines sensitivity and specificity of a diagnostic test [97]. Sensitivity and specificity is always a trade-off in that the decrease in one leads to increase in the other and ROC curves offer a graphical illustration of this trade-off [97]. Thus an ideal cut-off gives the highest sensitivity and specificity [97].

5.1 Limitations

Since this was a cross sectional survey some participants might have been wrongly stratified to the various groups. The sample size was also small.

5.2 Conclusion

Interferon gamma, IL2, IL1ra and IL17 can be useful biomarkers of TB. However IFNγ, IL2 and IL1ra cannot be relied on in the diagnosis of TB among HIV patients especially those who are severely immunosuppressed. Interleukin 2, MIP1α, and IL1α have poor sensitivity in discriminating active TB and LTBI and therefore they cannot be used individually.

5.3 Recommendations

In future longitudinal studies may be required to evaluate this cytokines performance. Since IL17 showed ability to diagnose TB both in HIV infected and uninfected individuals a longitudinal study is required to evaluate its performance individually and in combination with other cytokines/chemokines. Further studies are also needed to evaluate the usefulness of IL2, MIP1α, and IL1α in discriminating active TB and LTBI both individually and in combination with other cytokines/chemokine.
CHAPTER 6: REFERENCES


88. SPSS v17. IBM USA. SPSS predictive software and solutions.

89. Statistica 8. Ohio, USA. [www.statsoft.com](http://www.statsoft.com)


