SERUM CYTOKINE PROFILES AS BIOMARKER IN MULTI-DRUG RESISTANT TUBERCULOSIS (MDR-TB).

BY

BOAKYE ALAHAMAN NANA

(10551247)

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DECLARATION

This study was carried out at the Noguchi Memorial Institute for Medical Research, University of Ghana, and at the Deparment of Medical Laboratory Sciences, University of Ghana under the joint supervision of Dr Gloria Ivy Mensah, Dr Samuel Antwi-Baffour and Dr Enid Owusu.

"I hereby declare that, except for references to other people's work which have been duly acknowledged, this thesis is as a result of my own original investigation.

I further declare that the electronic version of the submitted thesis is congruent with the printed version both in content and format.

…… Date…….

Student

(ALAHAMAN NANA BOAKYE)

"We hereby declare that the preparation and presentation of this thesis were done in accordance with the guidelines on supervision of thesis laid down by the University of Ghana"

…… Date……..

Supervisor

(Dr Gloria Ivy Mensah)

…… Date …….

Supervisor

(Dr Samuel Antwi Baffour)

DEDICATION

This work is dedicated to my mentors,

Dr Gloria Ivy Mensah, Dr Samuel Antwi-Baffour and Dr Enid Owusu.

Your exceptional inspirations have been most helpful and dearly appreciated.

To God be the glory, great things He has done

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ABSTRACT

Background: Tuberculosis (TB) remains a major threat to public health despite several efforts to curb the disease. Contributions made by TB control programs have been tremendous; however, the emergence of multi-drug resistant (MDR) tuberculosis is a big threat to the control efforts and calls for innovative measures to mitigate the effect. New TB control programs with a comprehensive approach to dealing with MDR-TB is needed globally. In particular, new non-sputum based diagnostic tools capable of monitoring treatment response would be novel in this regard. The immune system with its associated cytokines plays a pivotal role in host response to *Mycobacterium tuberculosis* infection. The dynamics of cytokines at different stages of infection may therefore be useful as diagnostic biomarkers that could also be useful in monitoring response to TB treatment as well as in phase II clinical trials.

Aim: To evaluate the levels of eight cytokines in the serum of drug susceptible and MDR-TB patients and determine the utility of a single or multiple cytokine profile as a surrogate biomarker for MDR-TB.

Methodology: The study was cross-sectional using sixty-four archived serum samples of MDR (n=21), active TB patients (n=25) and healthy controls (n=18). Healthy controls were individuals who were not infected with TB (Quantiferon TB test negative). Serum levels of TNF-α, IFN-γ, IL-17A, IL-6, IL-10, Granzyme B, IL-4 & IL-12 p70 in each group was quantified by multiplexing using the Luminex™ 200 system. Data was entered into Microsoft Excel V10. Further analysis was performed using GraphPad Prism Version 7.03 and SPSS Version 23. Statistical significance underthe Graph Prism and Analysis of Variance (ANOVA) was used toassess the ability of each cytokine(s) to discriminate between different population groups. Mann-Whitney test was used to analyze concentration differences of each of the eight cytokines between the groups. The

relationship between two cytokines was assessed using Spearman correlation analysis (rho value). The sensitivity and specificity of the variables were analyzed using ROC curves. Differences between groups were considered significant at p–values less than 0.05.

Results: Serum levels of IFN-γ, IL-4 and TNF-α were significantly elevated in TB and MDR-TB cases compared to healthy controls (p=0.01, p=0.0001, p=0.0001 respectively). In addition to IFN-γ, IL-4 and TNF-α, serum levels of IL-6, IL-10 showed significantly higher levels in only TB cases compared to healthy controls (p=0.0001, p=0,01, p=0.00001, p=0.001, p=0.01 respectively). However, the serum cytokine concentrations of MDR-TB cases compared to their active TB counterparts showed no significant difference. Additionally, the diagnostic potentials of IL-4, TNF-α, Granz B, IL-10, IL-6and IFN-γ showed a trend of higher sensitivity between MDR-TB/TB and No TB cases. The sensitivity patterns IL-4, TNF-α, and Granzyme B were significant among all the population groups (p=0.009, p=0.003, p=0.009 respectively).

Conclusion: The results point to dysregulated serum cytokine profile in patients with MDR-TB similar to that observed in active TB cases. The study identified unique cytokine patterns characteristic of MDR-TB, TB and No TB subjects. Estimation of the serum levels of IFN-γ, IL-4, IL-6, IL-10 and TNF-α may be useful as biomarkers for distinguishing MDR-TB or TB cases from healthy individuals and thus may contribute to TB management and monitoring treatment responses.

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LIST OF ABBREVIATION

MDR-TB	Multidrug Resistant Tuberculosis
DOTS	Directly Observed Treatment-Short Course
MDR	Multidrug Resistant Tuberculosis
Mtb	*Mycobacterium tuberculosis*
TB	Tuberculosis
HIV/AIDS	Human Immune Virus/Acquired Immune Deficiency Syndrome
Granz B	Granzyme B
IL-6	Interleukin-6
IL-10	Interleukin-10
IFN-γ	Interferon Gamma
TNF-α	Tumor Necrosis Factor alpha
XDR	Extensively Drug-Resistant
IGRA	Interferon Gamma Release Assays
BCG	Bacille Calmette Guerin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
MHC	Major Histocompatibility Complex
Th-	T Helper cell

WHO	World Health Organization
IL-12p70	Interleukin-12p70
PBMC	Peripheral blood mononuclear cells
PPD	Purified Protein Derivative
IL-4	Interleukin-4
IL-17A	Interleukin-17A
PE	Phycoerythyrin
LJ	Lowenstein Jensen
LTBI	Latent tuberculosis infection
NTP	National Tuberculosis Control Programme-Ghana
INH	Isoniazid
ETH	Ethambutol
RIF	Rifampicin
PZA	Pyrazinamide
DNA	Deoxyribonucleic acid
ROS	Reactive Oxygen Species
TLR	Toll-like receptors
NADPH	Nicotinamide adenine dinucleotide phosphate

IL-2	Interleukin-2
IL-18	Interleukin-18
DC	Dendritic cell
Sp-A	Surfactant protein receptors
MyD88	Myeloid differentiation protein-88
PAMP	Pathogen associated membrane protein
µl	Microlitre
NMIMR	Noguchi Memorial Institute for Medical Research
Ml	Millilitre

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND

Tuberculosis (TB) remains one of the world's top ten leading infectious causes of death with about 1 million deaths in 2016 and 10.4 new (incident) cases worldwide (WHO annual TB report, 2017). Tuberculosis is typically an airborne disease mainly spread through aerosols from active pulmonary TB patients. However, it has been shown that approximately 10% of exposed individuals develop active clinical disease whiles the remaining 90% percentage succeeds in containment of the bacilli without any apparent clinical signs. The successful evasion mechanisms employed by the bacilli eventually results in dormant non-replicating bacilli located in lesions (Gengenbacher et al., 2012). This state of latency referred to as latent tuberculosis infection (LTBI) may further degenerate into active disease following resuscitation and disruption of host immune response (Walzl et al., 2015). This is due to the ability of Mtb to persist within granulomatous lesions engulfed within the macrophages in the lungs of individuals and the inability of the host immune response to completely eradicate mycobacteria from host tissues. This may be the principal reason for the higher susceptibility to TB infection in immunocompromised individuals (such as HIV/AIDS patients) compared to the immunocompetent community (Wells *et al.*,2007; Sissi *et al*., 2008).

The global fight against TB started decades ago and has seen a series of interventions. Amongst such interventions include the STOP TB strategy, Directly Observed Treatment Short course (DOTS) programme, and the current END TB programme aimed at eradicating TB by the year 2035. However, successes chalked by these interventions are under threat with the emergence of

MDR and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* (Matteelli et al., 2007). A *Mycobacterium tuberculosis* (Mtb) strain is said to be multi-drug resistant when it shows resistance to Isoniazid and Rifampicin: the two principal primary drugs for tuberculosis treatment (Ormerod *et al.,* 2005). On the other hand, extensively drug resistant strains are multidrug resistant strains with additional resistance to fluoroquinolone and an injectable agent such as amikacin, viomycin and kanamycin. In addition to high cost of management, MDR-TB is characterized by high morbidity and mortality rate. Hence, a major threat to public health. Mistreatment of tuberculosis, poor patient compliance, non-adherence among other factors selectively favor thriving of MDR-TB strains over drug susceptible species(Flora *et al.,* 2013). Reports from the National Tuberculosis Programme (NTP report, 2016), indicate an increase in MDR-TB prevalence in Ghana from 3.4% in 2015 to 4.0 in 2016. WHO guidelines recommend a standardized 9-12 months treatment regimen for MDR-TB cases established through phenotypic drug susceptibility testing by culture. However, efforts are underway to shorten the treatment course aimed at mitigating possible resistance.

The successive stages in TB pathogenesis are driven by host-pathogen interactions under the control of pro-inflammatory, anti-inflammatory and regulatory cytokines. Cytokines serve as indicators of treatment response, disease state as well as biomarkers for targeted interventions (Wallis et al., 2010). Pro-inflammatory cytokines such as TNF-a, IFN-g and IL-12p70 have been reported to vary with treatment in latent and active TB infections. On the other hand, anti-inflammatory and Treg associated cytokines such as IL-4, IL-2, and IL-10 also direct TB pathogenesis uniquely. However, there is paucity of data on the utility of these cytokines in the context of MDR-TB.

1.1 PROBLEM STATEMENT

Although progress has been made to reduce global incidence of drug-susceptible tuberculosis, the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis during the past decade threatens to undermine these advances(Gandhi *et al.*, 2010). Early detection and treatment is key in TB control, however detection of MDR-TB in Ghana like other developing countries may occur months after drug therapy failure. Despite substantial investments and progress made in expansion of the directly observed therapy, short course (DOTS) strategy, the STOP TB initiative and improved treatment completion rates, low case detection remains a major obstacle to global control of tuberculosis(Wallis *et al.*, 2014). Tuberculosis development is not spontaneous. It involves interplay of complex clinical stages and immunological processes from entry of bacilli, latent TB, persistence and reactivation of latent tuberculosis, active diseased state, and multi-drug resistance to extensively drug resistance.

The laboratory plays a crucial role in TB control programs. However diagnostic difficulties especially in developing countries have left majority of MDR-TB cases undetected. Currently, MDR-TB diagnosis is based on conventional methods for drug susceptibility testing (DST) on solid media. In addition, following recommendations from WHO, GeneXpert technology has also been adopted in some countries to address same. Limitations to DST diagnostic approach include long turnaround time and the inability to provide representative sputum for culture-based resistance testing after commencing treatment (Hartung *et al.*, 2002). Both techniques however, are not useful in resource–constrained settings. Also they are inefficient in monitoring treatment outcomes. Other limitation to the GeneXpert technique remains the inability to differentiate DNA from dead and live mycobacteria (Miotto et al., 2012). Furthermore, findings from several studies

have proved that DNA based techniques may not be the best solution to addressing this problem because there is a little or no change in levels following therapeutic success.

Despite new, sensitive, automated molecular platforms for detection of tuberculosis and drug resistance in advanced countries, a simple, inexpensive point-of-care test or diagnostic tool is still not available to monitor treatment efficacy and disease progression (Wallis *et al*.,2013). A more robust approach to MDR-TB control should focus on prevention of its emergence. A particular immune marker with the potential to serve as clinical and surrogate endpoint still remains a priority. Assessing the dynamics of different cytokine profiles in different populations groups will contribute greatly towards achieving this goal.

1.2 SIGNIFICANCE OF STUDY

Successful treatment outcomes among drug resistant (DR-TB) patients are of public health importance to prevent further spread of drug resistant strains to the general population. In addition, this will prevent the development of more resistance which would further pose more challenges in its management. Assessment of levels of expression of multiple cytokines may prove more effective than reliance on a single marker (Wassie *et al.*, 2008). Immunologically based diagnostic technique would be useful in addressing the challenges of the conventional sputum based approaches. It would also have an added advantage in extrapulmonary tuberculosis and where sputum production is difficult such as paediatric TB. Cytokine concentrations change rapidly during treatment (Hertog *et al*., 2013). The development of surrogate biomarkers for improving diagnosis and monitoring therapeutic effects are critical for effective TB control. The high morbidity and mortality rate associated with MDR-TB is threatening. Therefore a system to monitor and prevent the development of MDR-TB in the first place will be novel. In-depth analysis

of cytokines and its association with radiological recovery in TB patients may be useful in monitoring TB patients post chemotherapy for both clinicians and TB control program(Ansari*et al.*, 2016).

Analysis of the cytokines in this study will provide information about risk of a potential treatment failure, possibility of developing multi-drug resistance and or an eventual relapse. In addition, quantification of a panel of cytokines at different stages of TB infection will also allow immediate detection of multi-drug resistance, as well as prompt a timely change in therapy before MDR-TB ensues.

1.3 HYPOTHESIS

Ho: Serum cytokines can be used as biomarker in MDR-TB diagnosis.

1.4 AIM

To evaluate the levels of eight cytokines in the serum of drug susceptible and MDR-TB patients and determine the utility of a single or multiple cytokine profile as a surrogate biomarker for MDR-TB.

1.5 SPECIFIC OBJECTIVES

- To determine TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-17A, Granzyme B & IL-12p70 levels in serum of TB patients, MDR-TB patients and healthy controls.
- To determine the utility of a single or multiple combination of cytokines TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-17A, Granzyme B & IL-12p70 in discriminating between drug susceptible and MDR-TB.

CHAPTER TWO

LITERATURE REVIEW

2.0 BACKGROUND

This chapter reviews published articles related to *Mycobacterium tuberculosis* infection, multidrug resistance developments and discoveries in relation to biomarker development. The literature is discussed under the following:

- MDR-TB as a major public health concern, highlighting on global epidemiology and the risk factors for acquisition and spread of MDR-TB.
- An overview of the immunopathogenesis of tuberculosis in addition to both innate and adaptive immunity to tuberculosis.
- Trends and potential role of biomarkers in the quest to monitoring treatment response and predictors of relapse.

2.1 EPIDEMIOLOGY AND GLOBAL BURDEN OF MDR-TB

Globally in 2016, 6.6 million notified cases of tuberculosis were reported (WHO global report, 2017). Notified MDR-TB and rifampicin resistant cases were 153119 representing 2.1%. Of the nearly 1.3 million TB cases notified in Africa, 27828 were MDR-TB. Ghana is not rated as a high MDR-TB burden country but it is a high burden country for HIV/ TB coinfection. High HIV/TB burden correlates with the increase in MDR-TB cases and as such the country needs to do more to combat MDR-TB. Currently the prevalence of MDR-TB in Ghana stands at 4% of the total number of notified cases up from 3.4% in 2015. MDR-TB cases in Ghana are sparsely distributed across all the regions without restriction to a specific geographic area.

The emergence of MDR-TB has complicated global TB control programs. Amidst several risk factors for MDR-TB development(Ms *et al.,* 2013), the most predominant in Africa is treatment failure(Bpharm *et al,* 2008). Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases have MDR-TB (WHO Global Tuberculosis report 2015). Ghana is not exempted from the high prevalence of MDR-TB burden especially among previously treated patients (Otu*et al.,* 2014). In the early 1990s, Argentina experienced an outbreak of MDR-TB in hospitalized HIV/AIDS patients (Aita *et al.,* 1996; Ritaco *et al.,* 1997) which became an issue of much concern when it disseminated into the immunocompetent community (Palmero *et al*., 2003). A similar outbreak occurred at a hospital in Tugela Ferry, KwaZulu-Natal, South Africa, between January 2005 and March 2006 (Gandhi *et al.,* 2006). A stable anti-tuberculosis drug resistance was reported in Mali between 2006 and 2014 (Diarra *et al*., 2015). Between 2000 and 2004, 20% of 17,690 samples collected from both high and low HIV prevalence countries were MDR-TB (Centers for Disease Control and Prevention, 2000–2004). Global awareness of the persisting threat of MDR-TB became more evident following reports from South Africa on patients with AIDS rapidly dying of TB despite anti-retroviral treatment (NIAID Tuberculosis Working Group. NIAID research agenda: multidrug-resistant and extensively drug-resistant tuberculosis) as well as the United States (CDC investigation of U.S. traveler with extensively drug resistant tuberculosis (XDR TB)). In order not to reverse the gains made by several TB control strategies, an approach to MDR –TB requires urgent practical interventions. Understanding the biology, and immune-pathogenesis of host-pathogen interaction will accelerate biomarker development to rapidly diagnose and monitor MDR-TB(Fauci & Working, 2008).

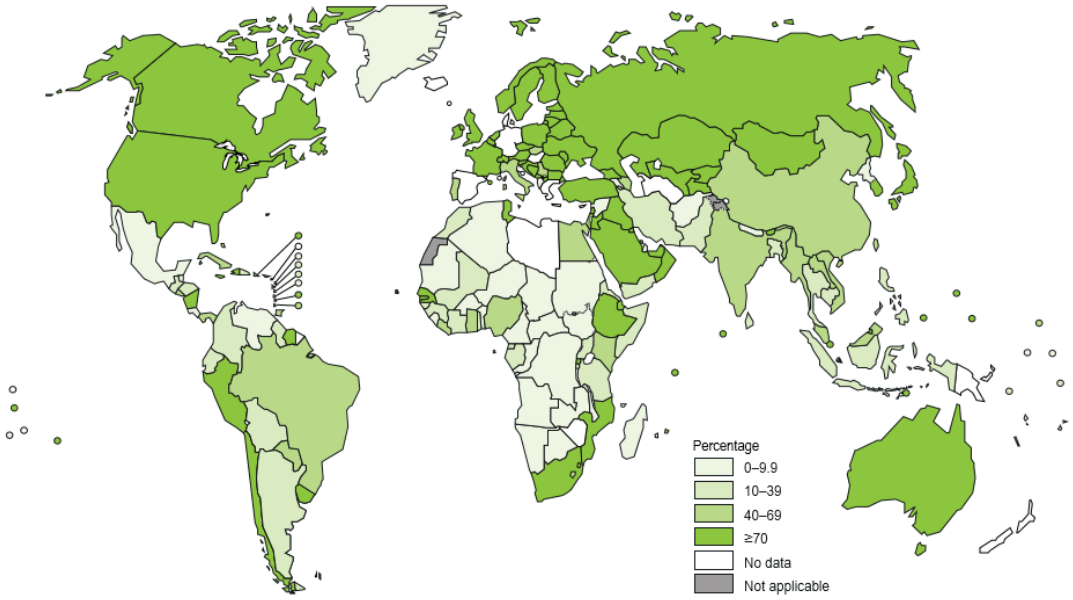

Figure 1.0 A map showing the Global percentage of bacteriologically confirmed MDR/RR-TB (Adapted from WHO global report on tuberculosis, 2017.)

2.2 TRANSMISSION AND RISK FACTORS FOR *Mycobacterium tuberculosis* INFECTION

Persons with active pulmonary tuberculosis (TB) serve as the main source of transmission. Infection is initiated by aerosol inhalation expectorated from active tuberculosis individuals. Clinically apparent TB infection is a function of several host and environmental factors which includes degree of infectiousness of droplet nuclei, proximity to contact, bacillary load and immune status of host (Young et al., 2008). The pivotal role of HIV as a risk factor in tuberculosis development has been extensively studied and well established. Corbett et al (2006) revealed HIV incidence rate as a primary contributing factor to the tuberculosis epidemic in Africa. Flora and colleagues also argued in favor of HIV/AIDS as a risk factor in addition to poverty. A random

effects model in a comprehensive meta-analysis study assigned the high incidence of tuberculosis in Africa to the rising HIV-infection rates, poverty and ineffective control programs (Viswanathan et al., 2008). Evidence of alcoholism, housing conditions, Regardless of diverse study populations, several risk factor assessment studies have revealed some similar generalizations in results.

Studies involving multidrug resistant tuberculosis have reported treatment failure, inadequate adherence to treatment, and treatment relapse (Wahab et al., 2009). Multidrug resistance may also arise from lack of effective treatment monitoring (Walzl et al., 2011). A poorly administered treatment regimen permits drug resistant mutant strains to dominate in active TB patients (Flora et al., 2013). However, these factors may be interrelated. As Karsel et al have reported failure in the implementation of the Directory Observed Treatment Short course (DOTS) programme by some countries as a contributing factor. Interestingly, similar risk factors for developing tuberculosis have also been implicated in multidrug resistant cases (Espinol et al., 2010). On the contrary, studies reviewed by Viswanathan et al (2008) among an African cohort showed a non-significant association between HIV status and MDR-TB. Nonetheless, drug resistant strains can be transmitted directly from an infected person to another individual

2.3 ENTRY MECHANISMS OF Mycobacterium tuberculosis

Invariably, airborne transmission of infectious droplet remains the successful route for *Mycobacterium tuberculosis* infections. However, there is paucity of information regarding characteristics of transmissible bacilli, survival time in the air as well as the exact travel distance limit the bacilli can travel to cause infection (Torrel et al., 2017). Following aerosol inhalation, *Mycobacterium tuberculosis* (Mtb) escapes bronchi ciliary action, overcome respiratory barriers, enters into host lungs and finally interacts with alveolar macrophages(Amett et al, 2016). Alveolar

macrophages, dendritic cell and other phagocytic immune cells engulf the bacilli prior to phagocytosis. In addition, Garcia-Perez (2003) indicated that non-phagocytic cell such as M cells, epithelial cells Type 1 and 2; endothelial cells could also be infected through micropinocytosis Internalization of Mtb by phagocytic antigen presenting cells is initiated by recognition of Mtb components by host receptors. Central to these interactions is recognition of Pathogen Associated Molecular Patterns (PAMP) by specific Pathogen Recognition Receptors (Trinchieri et al., 2007). Toll-like Receptors are significantly involved in this process (Harding et al., 2010), nonetheless mannose receptors, scavenger receptors dendritic cell-specific intercellular adhesion molecule grabing non-integrin (DC-SIGN), Nucleotide binding oligomerization domain (NOD) like receptors and C-type lectins have also been implicated in this phenomenon (Jo et al., 2008). Also documented are the potential role of complement receptors, surfactant protein receptors (Sp-A) and cholesterol receptors. These receptors associate with Mtb membrane components including lipoprotein, CpG- containing DNA phosphatidylinositol mannoside, and mannose-caped lipoarabinomannan. Interestingly, different receptor-ligand host-Mtb interactions culminate in different intracellular signaling thus resulting in divergent responses (Cao *et al.*, 2011; Mishra *et al.*, 2011). Binding of lung surfactant protein-D with Mtb surface lipoarabinomannan blocks the intracellular growth of Mtb by increasing the rate of phagolysosome formation (Fergusson et al., 2006). Mannose receptor stimulation results in anti-inflammatory cytokines, IL-4 and IL-13 production, antagonizing IL-12 production and deactivating oxidative responses (Nigou et al 2001). It is speculated that binding of MTB to MR induces phagocytosis, but phagosome-lysosome fusion is limited (Kang et al., 2005).

The TLR family is a transmembrane protein. TLR interaction with mycobacterial components such as lipopolysaccharides occurs in tandem with CD14 and myeloid differentiation factor 2 accessory

proteins located on the cell surface (Tartey et al., 2017). Its cytoplasmic domain TLR is homologous to the signaling domain of IL-1 receptor (IL-1R) and links to IRAK (IL-1R-associated kinase), a serine kinase that activates transcription factors like NF-kB to signal the production of pro-inflammatory cytokines and TNF, IL1β, IL-12, nitric oxide and chemokines (Jo et al., 2007). A study by Yamamoto and colleagues (2003) reported TLRs mediated production of nitric oxide and pro-inflammatory cytokines occurs through either myeloid differentiation primary response protein 88 (MyD88)- dependent or MyD88-independent pathway. In addition to mycobacterial cell surface, TLRs are also expressed on phagosomes and endosomal compartments (Kawai et al., 2010). Therefore, immune activation may occur with or without phagocytosis. On the other hand, immune activation does not solely depend on phagocytosis without the involvement of TLRs (van Crevel et al., 2002).

The induction of pro-inflammatory cytokines and chemokines attract immune cells (neutrophils, NK cells, T cells, dendritic cells, macrophages) to the point of infection (Korbel et al., 2008). Intracellular killing is facilitated by increased vitamin D receptor (VDR) and vitamin D-1 hydrolase gene expression. Conversion of pro-vitamin D into active form activates antimicrobial peptides (cathelicidin and B defensing) to facilitate intracellular killing of *Mycobacteria* (Chocano-Bedoya et al., 2009). In another study Yang and Shin (2009) reported NADPH oxidase 2 interaction with TLR 2 upregulates VDR mediated cathelicidin expression from macrophages. This may directly restrict growth of Mtb directly and through the induction of autophagy. Furthermore, studies on VDR and TLR polymorphisms have ascertained their role in host innate immune response to tuberculosis infection (Raghavan et al., 2012). Indeed, TLR signaling may serve as a central drive of the innate immune response to tuberculosis infection, perhaps due to its

early expression and engagement with Mtb components particularly TLR 4 and TLR 2 (Harding et al., 2010).

Dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) interacts with Man-LAM and lipomannans (Geijtenbeek et al 2003). Lately, it was shown that α-glucan (a dominant capsular polysaccharide) is also a ligand for DCSIGN (Schluger et al., 2016). On the contrary, downstream signaling following engagement of DC-SIGN with mycobacterial components lead up to induction of anti-inflammatory cytokines; IL-10 and TGF-B. It has also been shown that DC-SIGN triggers acetylation of the NF-κB subunit p65 via Raf-1, NT-Kb which brings to bear immunosuppressive effects but only in the presence of simultaneous TLR stimulation (Gringhuis et al., 2007).

2.4 PATHOGENESIS OF *M. tuberculosis*

Mtb is an intracellular pathogen, mainly contained in granulomatous lesions, that survives successfully by subverting host immunity (Zumla et al., 2015). The overall immune response to Mtb is dependent on the host ability to amount a Th1 or Th2 response and the potential of each strain to induce the Th2 cytokine response in susceptible individuals. Patients with MDR-TB display Impaired Th1 responses and enhanced regulatory T-Cell response (Geffner *et al*., 2009). The interplay of cytokine effector mechanisms regulates the immune responses to Mtb(Chandrashekara *et al*., 2016). Cytokines play a critical role in immune response modulation, and screening of cytokines involved in different clinical stages would reflect the immune status of the host (BoseDasgupta *et al*., 2014).

Protective immunity against Mtb infection is principally executed via Th1 immune response with macrophages playing a major role in host response (Ehrt et al., 2009). Following inhalation of the

infectious respiratory droplets, Mtb evades the defensive mechanisms of the upper respiratory tract. Viable bacilli enter the lungs where they are phagocytosed by alveolar macrophages. Uptake by macrophages represents the first major host-pathogen interaction in tuberculosis infection(Schluger *et al.*, 2001) . However, the Mtb bacilli is able to resist bactericidal mechanisms and evade host immune response via mechanisms such as inhibition of phagosome acidification and maturation as well as subverting the activities of toxic reactive oxygen and nitrogen intermediates (Flannagan et al.,2009).

A major aspect of host response to TB is the secretion of IFN gamma by Th1 cells. IFN gamma is the key cytokine for a protective immune response against Mtb (Jacobs *et al*., 2016). It is a pro-inflammatory cytokine that has multiple beneficial roles. It tends to unblock the inhibition and thus facilitate the process of phagocytosis.

The exact correlates of protection in TB are not known, however, a Th1 cytokine profile is crucial for a protective immune response, although it may also cause immunopathologic damage if left uncontrolled. Th2 cytokines on the other hand are known to inhibit autophagic control of intracellular Mtb (Harris et al., 2007), although, an excessive down regulation might favour disease progression which may be eventually exploited by the bacilli to establish resistance (Larson *et al*., 2013). In addition to Interferon gamma, other cytokines that play a role in host immune response to tuberculosis include interleukin-12p70, interleukin-4 (IL-4), IL-10, Granzyme B, Tumor Necrosis Factor alpha (TNF alpha), IL-6, IL-17, is a pivotal cytokine in driving the immune system towards a Th1 type response and preventing a Th2 type immune profile. Interleukin 4 (IL-4) on the other hand induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. This is an approach to eventually abrogate and down regulate the expression of Th1 cytokines. Higher levels of Regulatory T cells (Tregs) and its associated cytokines (IL-10, TGF-beta, IL-4) have been

reported in patients with active TB and MDR-TB than in latent TB subjects (Hougardy et al., 2007; Wergeland *et al.*, 2011).

2.5 INNATE IMMUNITY TO Mycobacterium tuberculosis

Innate immunity is a crucial component of the immune response against Mtb. There is growing evidence indicating that innate immune cells provide a means of crosstalk with adaptive immunity and thus are uniquely positioned to determine that balance between protective and pathogenic immune responses in human TB (van Crevel et al., 2002).

Macrophages, dendritic cells, natural killer cells and neutrophils are the major innate cells implicated in tuberculosis infections (Jonathan et al., 2015) though other non- classically defined immune cells, such as airway epithelial cells, have been shown to contribute to the immune response against Mtb in animal models (Dorhoi et al., 2014).

2.5.1 MACROPHAGES

Alveolar macrophages are one of the first host cells to encounter Mtb after aerosol inhalation. While they function as the first line of defense against Mtb infection, macrophages have also been found to provide a cellular niche within which Mtb actively replicates thus serving as reservoirs for persistent bacteria within granulomas. Several aspects of macrophage functions have been investigated in human TB. These include phagocytosis of Mtb, induction of antimicrobial pathways and responsiveness to interferon gamma.

2.5.2 NATURAL KILLER (NK) CELLS

They are granular innate cells from the lymphoid lineage with cytolytic capacity. Unlike macrophages, and dendritic cells, NK cells are non MHC restricted thus activation is via receptor

ligand interactions. NK cells possess the capacity to lyse Mtb infected macrophages (Vankayalapati et al., 2002). They have also been shown to inhibit intracellular growth of Mtb by enhancing phagolysosomal fusion through IFN-gamma and IL-22 production (Dhiman et al. 2009). Vankayalapati et al. (2004) also reported that NK cells can promote the production of IFN-γ from CD8 T cells by stimulating IL-15 and IL-18 production from Mtb-infected monocytes.

2.5.3 DENDRITIC CELLS

Dendritic cells are crucial cells that are involved in bridging innate and adaptive immunity. Primarily, they are antigen presenting cells that initiate adaptive responses through their capacity to present antigen, their costimulatory capacity, and secretion of T-helper polarizing cytokines.

Secreted cytokines resulting from pathogen-host interactions are crucial mediators of innate immune response to tuberculosis. Interferon gamma secreted from activated T cells and NK cells plays a very crucial role in promoting bacterial killing, phagosomal maturation and production of reactive nitrogen and oxygen intermediates. Guteirrez and his colleagues observed that IFN-gamma induces autophagy in MTB infected macrophages. Several other studies have reported the monumental role of IFN gamma in unblocking and promoting phagosome and lysosome fusion. This stimulates release of ubiquitin conjugates unto lysosomes thereby increasing bactericidal activity of the lysosomal soluble fragment (Alonso et al., 2007). Consequently, powerful oxidants such as hydrogen peroxide and hydroxyl radicals are produced within the phagolysosome to execute Mycobacterial destruction (Vilteze et al., 2013). High levels of reactive oxygen species within the phagolysosomal milieu undergo spontaneous dismutation to produce hydrogen peroxide which stimulates lysosomal killing (Podinovskaia et al 2013).

In addition, TNF-alpha also plays significant role in innate immunity to tuberculosis. Studies in both mice and humans have elucidated the protective role of TNF alpha in Mtb infection (Keane

et al., 2005). TNF ALPHA acts in synergy with IFN gamma to inducible nitric oxide synthase thus enhancing mycobactericidal activity (Cooper et al., 2009). Roca and Ramakrishnan (2013) recently discovered TNF alpha induced reactive oxygen species (ROS) in Zebrafish models of tuberculosis. They reported mitochondria as the primary source of ROS. It would therefore be of great interest to have mitochondrial ROS play a substantial mycobactericidal role like its reported anti-Salmonella activities (West et al. 2011).

2.6 ADAPTIVE IMMUNE RESPONSE TO *Mycobacterium Tuberculosis*

The adaptive and innate immune responses to Mtb infection are interrelated. Infected antigen presenting cells, predominantly macrophages and dendritic cells engulf, process and present mycobacterial antigenic peptides to T cells. The release of apoptotic bodies following macrophage apoptosis has also been reported to elevate antigen presentation when assessed by bystander cells (Schaible et al., 2003, Winau et al., 2006). Antigen presentation is done by MHC class I and II, CD 1 molecules and gamma delta T cells (Ahmed 2010). MHC class I and II are respectively recognized by CD4 and CD8 T cells respectively. Antigenic peptides presented via CD1 molecules are recognized by MHC class CDI, restricted CD4+ and CD8+ T cells (Korb et al., 2016). Mtb lipid antigens can also be processed and presented to unconventional T cells such as γδ T cells and NKT cells, but their role in the immune response to Mtb is still unclear. CD8+ T cells destroy intracellular pathogens through granulysin and perforin- mediated pathways (Woodworth et al., 2006). Although largely associated with innate immune response, Ngai et al (2007) reported that CD8+ cytotoxic T lymphocytes are capable of effecting protective immunity against secondary mycobacterial exposure in the absence of CD4+ T cells. However, protective immunity by antigen specific CD8+ T cells, natural killer (NK) cells, γδ T cells, and CD1-restricted T cells cannot

compensate for that of CD4+ T cells (Flynn., 2001). Nevertheless, what remain unclear is whether the protective immunity elicited by CD8 + T cells is that offered by granulysin and perforin mediated cytolytic killing or they serve as a secondary source of Th1 cells (Lewinsohn et al.,2011).

Protective immunity to Mtb infection largely depends on CD4+ T cells (Ogara et al., 2015). A study on CD4+ deficient mice revealed inability to control mycobacterial growth. In addition, CD4+ T cell lymphopenic patients were susceptible to tuberculosis. Polarization into different T cell subsets from naïve T cell sets usually depends on distinct cytokine profile (Keir et al., 2008). Reports have shown that IL-12, IL-18 and IFN gamma promote Th1cell development while IL-4, IL-5, and IL-13 induce Th2 cell development (Kaufman et al., 2008). Synergistic effect of IL-6 and TGF beta also induce Th17 differentiation in mice (Betteli et al., 2006) while in humans IL-23 replaces IL-6 (Yang et al., 2008). Each pattern of immune response results in distinct effector mechanisms, however Th1 subsets is typically associated with sterility and impaired growth of the bacilli(Ogarra et al., 2015).

Differentiation of CD4+ T helper cells to Th1 cells induced by IL-12 culminates in TNF alpha and IFN gamma production. Eventually, Th1 cells express IL-2 in conjunction with IL-2 receptors. Therefore, IL-2 acts in autocrine fashion leading to active clonal expansion of Th1 cells. Induction of IFN gamma plays a monumental role in protective immunity against tuberculosis infection. Primed T cells guided by its associated chemokines and cytokines signal recruitment of more immune cells to the focus of infection. Recruited macrophages in an attempt to kill indigestible bacilli undergo conformational change to form epithelioid cells. In addition, T cells, fibroblasts, endothelial cells and other host cells accumulate leading to the formation of granuloma. Typical granuloma has been shown to consist of foamy macrophages and epithelioid cells, multinucleated giant cells, surrounded by a color of lymphocytes and fibroblasts (Torrelles et al., 2017). This

partitions the tubercle bacilli from the rest of the lung tissue. Several studies have reported different immunological responses leading to formation of physiologically distinct granulomatous lesions (Ahmed 2010). Some of these lesions suppress while others augment the thriving of active TB bacilli within the microenvironment (Young et al., 2009) by serving as a niche for multiplication and escape from antimycobacterial agents.

Within the granuloma, Mtb adapt to the hostile environment by transcriptionally inducing new genes such as efflux pumps (Cambier et al., 2014). Ramakrishman explained that macrophages at the centre of granuloma die by either apoptosis or necrosis. Apoptosis maintains membrane integrity of host cells. In contrast, lysis of macrophages via necrosis leads to the formation of hypoxic caseous necrotic core (Via et al., 2008). Histopathological studies have confirmed a large number of tubercle bacilli within this region. Incessant necrotic activities eventually rupture the granuloma thus resulting in resuscitation and dissemination of Mtb bacilli.

Mycobacterium tuberculosis internalized by phagocytic immune cells (alveolar macrophages) replicate intracellular, and the bacteria laden immune cells may cross the alveolar barrier to cause systemic dissemination (Bermudez *et al.,* 2002). This phenomenon may lead to the development of extra pulmonary tuberculosis. Alveolar macrophages and DC are then believed to transport Mtb to local lymph nodes where T cells are primed and clonally expanded (Dheda *et al.,* 2010).

Infection of human macrophages and dendritic cells with Mtb induces a differential cytokine gene expression that modulates T cell response (Giacomini *et al.,* 2001). This is characterized by an alveolitis of activated α/β T-cell receptor-positive lymphocytes, recently recruited immature macrophages (Schwander et al., 1996; Robinson *et al.,* 1994). Immune response during the active stage of the disease (TB patients) is characterized by both pro and anti-inflammatory cytokines irrespective of the nature of the antigen (Mensah *et al.,* 2014).

Protective immune response to Mtb is strongly enhanced by *M. tuberculosis* antigen-specific Th1 responses, with large amounts of locally secreted pro-inflammation

2.7 DRUG RESISTANCE MECHANISMS OF *M. tuberculosis*

Generally, bacterial drug resistance results due to mutations in the genotype resulting in phenotypic expression of resistance which can be transferred to subsequent generations.

Three drugs for tuberculosis, isoniazid (INH), ethambutol (ETH) and pyrazinamide (PZA) all require activation in order to function. Thus, resistance is mediated by mutations in prodrug activation enzymes which lead to termination of the activation step. Zdang and colleagues demonstrated such inactivation for katG (catalase-peroxidase), PncA (nicotinamidase/pyraminidase) in PZA resistance (Scopio et al., 1996) and EtaA/EthA (FAD-containing monooxygenase in ETH resistance (Johnson et al.,2002).

Rifampicin (RIF) on the other hand, acts by interfering in RNA biosynthesis through binding with bacterial RNA polymerase. Mycobacterial resistance to RIF is predominantly associated with mutations in the rpoB gene (Telenti et al., 1993).

Salicylate-induced resistant mechanisms have been shown to induce resistance to multiple antituberculosis drugs in vitro for both avirulent strain *M. tuberculosis* H37Ra and virulent strain *M. tuberculosis* H37Rv (Schaller et al., 2002).

2.8 TREATMENT AND DIAGNOSIS OF MDR-TB

Antimicrobial resistance has become a subject of immediate relevance and public health concern globally (General Assembly of United Nations, 2016). MDR-TB patients require treatment regimens which are longer than first-line regimens. The long duration in second-line MDR-TB

regimens poses a problem of strict adherence to therapy hence increasing the risk of drug resistance development.

The first line drugs recommended for the treatment of drug susceptible cases are rifampicin (INH), isoniazid, ethambuthol and pyrazinamide. The revised treatment guidelines include a strong recommendation to treat new pulmonary TB patients with drug-susceptible TB with a 6-month regimen comprising of 2 months of isoniazid, rifampicin, pyrazinamide and ethambuthol, followed by 4 months of isoniazid and rifampicin (WHO guidelines for treatment of drug susceptible tuberculosis, 2017). On the other hand, the WHO guidelines recommend a standardized 9-12 months' treatment regimen (fluoroquinolones or injectable agents) for MDR-TB cases.

Phenotypic drug susceptibility testing (DST) remains the gold standard for MDR-TB diagnosis (Falzon et al., 2016). However, rifampicin resistance is considered as a major surrogate marker for MDR-TB since greater than 90% of rifampicin resistant isolates are also resistant to isoniazid (Orenstein et al., 2009, Traore et al., 2000). Therefore, WHO recommends the use of Xpert MTB/RIF® assay for presumptive diagnosis of MDR-TB (WHO Global TB report, 2017). The Xpert MTB/RIF® assay is useful for the simultaneous detection of TB and rifampicin resistance.

2.9 BIOMARKERS OF *M. tuberculosis* CONTROL

A biomarker is defined as a parameter that can be objectively measured as an indicator of normal or pathogenic biological processes, or an indicator of pharmacological responses to therapeutic interventions (Biomarker working group, Wallis et al., 2010). It can be either host or pathogen-derived. In routine clinical care, biomarkers aid in the categorization of individual patients towards targeted specific interventions that might not otherwise produce overall benefits (McNervey et al., 2012). The roadmap to effective TB treatment monitoring, shortening treatment duration and

assessing therapeutic efficacy may fall within the domain of biomarker discoveries. A range of host biomarkers have been studied using various clinical samples such as saliva, sputum, urine, serum, cerebrospinal and pleural fluids (Khutso et al., 2013). These studies have been in the area of immunology, proteomics, metabolomics and transcriptomics (Jacobson et al., 2007, Esterhuyse et al., 2015). Currently, the only acceptable biomarker in TB is the "month two sputum culture status. This surrogate biomarker for treatment failure and relapse is based on sputum-culture status after two months of treatments (Kurbatova et al., 2015). Although this marker has been validated and supported by several studies (Wallis et al., 2013, Tang et al., 2015) other important studies such as the REMOX trial have found it unhelpful to predict treatment failure (Gillespie et al., 2014). In addition, difficulty in obtaining sputum specimen for culture after two months of therapy coupled with long duration of the culture process undermines the utility of this technique and also correlates crudely with disease relapse (Dedicoat et al., 2017).

Immunological biomarkers, of which cytokines form a major part, hold promise as surrogate markers to monitor treatment responses and predict eventual relapse (Doherty et al., 2009). Irrespective of the fact that several potential markers exist (Wallis et al., 2009), none has been validated in large prospective studies (Dheda et al., 2010). Cytokines such as TNF- alpha, IFN gamma, IL4/IL4&2 ratio have been found to vary with treatment in latent TB infection and active disease (Siawawa et al., 2008). Also, numbers and ratios of Treg cells and NK cells have been found to differ in TB and non-TB patients (Veenstra et al., 2006). However, these changes are not always consistent and thus may be confounded by the natural differences in stimulated cytokine responses over time (Pai et al., 2006; van Zyl –smit et al., 2009). Recent studies suggest that a profile comprising of several cytokines rather than a single biomarker may be much more useful (Talat et al., 2009; Djoba et al., 2009). Hence the bests approach might entail a comprehensive

evaluation of immunological variables in combination with other relapse prediction score parameters.

2.10 SPECIFIC ROLES OF CYTOKINES IN *Mycobacterium tuberculosis* INFECTION

Cytokines are short lived cell signaling protein molecules that aid cell to cell communication in immune responses and stimulate the movement of cells towards sites of inflammation, infection and trauma.

Interferon gamma (IFN GAMMA) and tumor necrosis factor alpha (TNF alpha) genes are activated from IL-12 mediated primed T cells.

2.10.1 The role of Interferon gamma (IFN-g) and TNF alpha.

The protective role of IFN gamma in tuberculosis has been extensively studied and well established both in human and animal models (Flynn *et al*., 2001). Interferon-γ secreted from activated T cells and NK cells have the capability to activate macrophages and promote bacterial killing by permitting phagosomal maturation and production of antimicrobial reactive nitrogen and oxygen intermediates (Kaufman et al, 2001). The block on phagolysosome formation (Malik *et al.,* 2000) is released by interferon gamma mediated delivery of ubiquitin conjugates to the lysosome, augmenting the bactericidal capacity of the lysosomal soluble fraction (Alonso *et al.,* 2007). In addition, IFN gamma induces autophagy in macrophages by activating INOS signaling pathway (Gutierrez et al., 2004). Nandi et al (2011) demonstrated using animal models, a positive correlation between low IFN gamma levels and severity of tissue damage. Individuals with IFN gamma mutations also developed disseminated TB infection following exposure to BCG and other non-tuberculosis species of mycobacteria (Casanova et al., 2002). Several studies have observed lower levels of IFN gamma in active TB patients from both stimulated and unstimulated PBMCs

(Marcos et al., 2015). Production of IFN gamma following specific antituberculosis therapy was lower as compared to patients with latent tuberculosis (Sahiratmadja et al., 2007). In addition, Mtb specific IFN-y producing T cells levels were reduced in TB patients after two weeks of efficient TB treatment (Feruglio et al., 2015).

However, Mtb specific IFN gamma producing T cells have been found in most people with active TB depicting the insufficiency of IFN- gamma alone in preventing active TB development (Nemeth et al., 2009). This suggests other pathways are involved in mounting protective immunity against tuberculosis (Nunes-Alves et al., 2014). Furthermore, production of higher amounts of IFN gamma is likely to aid progression into active disease than as observed in patients with weaker IFN-gamma response (Diel et al., 2011). According to Hirsch, these contradictory outcomes regarding the production of IFN gamma at the site of infection and in peripheral blood may be partly attributed to susceptibility of PBMC to apoptosis in active patients than in healthy controls. Co-production of IFN-gamma, TNF alpha and IL-2 have been reported to correlate with protective immunity in vaccination studies (Lindenstorm et al.,2009, Scriba et al., 2010) whiles other studies have shown correlation with active disease (Caccamo et al., 2010). Further studies would therefore be necessary to clarify these contrasting views.

Fluctuating levels of Mtb specific total IFN-y and single IFN-y producing T cells (Tonby *et al.*, 2014) makes Mycobacterial antigen-specific IFN-production in vitro useful as a surrogate marker of infection with Mtb (van Crevel *et al.*, 1999). Recently Hur and colleagues demonstrated a correlation between sputum clearance and IFN- gamma levels after two months of treatment.

TNF alpha acts in concert with IFN gamma in killing intracellular Mtb by activating reactive nitrogen intermediates. Both cytokines are involved in granuloma formation (Flynn *et al.*, 2001). They have been investigated as the two main cytokines of the Th1 lineage required to elicit

protective immunity against tuberculosis. Interestingly, Marcos et al (2015) established that increased production of TNF alpha and IFN gamma occurs later after treatment. TNF alpha is needed for controlling LTBI as anti-TNF antibody infliximab increases the risk of activating latent TB (Keane *et al*., 2001). Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mtb in humans (Bruns *et al*., 2009). Tuberculosis relapse has also been observed in patients under anti-TNF alpha therapy (Harris *et al*., 2010). The role of TNF alpha in tuberculosis has also been reported to correlate with protective immunity in vaccination studies (Lindenstrom *et al*., 2009).

2.10.2 Interplay of Cytokines in TB Pathogenesis

Various cytokines act in an interrelated fashion during TB pathogenesis. The distinct role of individual cytokines is still under study.

The synergistic activity of IL-6 and TGF-B directs differentiation of naïve T cells into Th17 cells which further results in the production of a proinflammatory cytokine IL-17A (Yang et al., 2011).

2.10.3 Interleukin-12p70(IL-12p70)

IL-12 is known to play an important role in anti-TB cell mediated immunity (Cooper *et al* 1995 and is the major cytokine for directing primary Th1 differentiation in CD4+ T cells.in-vitro and in-vivo (Pereira *et al*.,2004). Therefore, it plays a key role in host defense against intracellular pathogens such as Mtb. Interleukin-12 (IL-12) is a heterodimeric pro-inflammatory cytokine mainly produced by dendritic cells and macrophages in response to Mtb infection(Trinchieri *et al.*,2003). Dendritic cells (DCs) and macrophages produce IL-12 in response to pathogens during infection. Interleukin 12p40 homodimer induces pulmonary dendritic cell migration and T cell priming required for recruiting cytotoxic CD8+ cells after Mtb infection (Serbina *et al*., 2001).

2.10.4 IL-10 and Tuberculosis: A careful balance between Bacillary Persistence and Reducing Immunopathology

Several studies have reported relapse from previous infection as the major cause of recurrence of tuberculosis coupled with eventual drug resistance development. Thus, immunity to tuberculosis after treatment may result in clinical recovery without necessarily offering a sterilizing cure. The incessant actions of several proinflammatory cytokines such as TNF-alpha and IFN-gamma can be detrimental to the host when left uncontrolled (Silva., 2015).

Interleukin-10 was originally referred to as cytokine synthesis inhibitory factor (CSIF) due to its ability to inhibit T. lymphocyte mediated cytokine production (Fiorentino et al., 1989). It is an anti-inflammatory cytokine that has been shown to mediate the prevention of immunopathology in tuberculosis infections (Jamil et al, 2007, Handzel et al., 2007). Contrary to previous reports, IL-10 production has been shown not only inTh2 and T regulatory cells but also in CD8+ T cells, Th17 and Th1 cells (Hill et al., 2006, Gerosa et al., 2012). Results from Silva et al., (2013), demonstrated that mounting protective immunity to Mtb is a time dependent effect. He further argued that Th1 response characterized by increased levels IFN-gamma and TNF-alpha occurred later accompanied by increased production of IL-10. Their results suggest that the clinical cure process progresses with potentiation of Th1 cytokine production as well as higher IL-10 levels to regulate the production and deleterious effect of these proinflammatory cytokines (O'Gara et al., 2007).

In patients with tuberculosis, expression of IL-10 mRNA has been demonstrated in circulating mononuclear cells at the site of disease in pleural fluid, and in alveolar lavage fluid (Abdalla *et al*., 2016). IL-10 antagonizes proinflammatory cytokine response by down regulating IFN-y, TNF-alpha, and IL-12 production (Tracey *et al*., 2008). However, there is a growing body of evidence

suggesting that the relationship between Th1 cytokines and IL-10 is not as antagonistic as originally believed but rather appears to act in complementary form (Jankovic et al., 2007). There are contrasting views regarding the dynamics of IL-10 in various categories of tuberculosis infection. While some studies show that there are higher levels of IL-10 in active tuberculosis (Winkler et al., 2005) others argue that such an increase only happens after clinical cure (Sahiratmadja et al., 2007). It has also been demonstrated from animal models that repeated antigenic stimulation may direct Th1 cells to express IL-10 in order to extend cell survival (Saraira et al., 2009).

2.10.5 Interleukin-6 (IL-6) and Tuberculosis

Interleukin-6 is a pleiotropic cytokine expressed by several cell lineages (Bettelli et al., 2006). Increased production of IL-6 has been implicated in many human chronic inflammatory diseases. In association with chronic inflammatory reactions such as in tuberculosis, IL-6 contributes to host resistance by its proinflammatory activity as well as the ability to influence other cytokine secretion (Periasamy*et al.,* 2011; Lyadova *et al.,* 2010). IL-6 has an innate protective role against tuberculosis infection and has been implicated in initiation and maintenance of acquired immunity (Chandrashekara et al., 2016). Genetic variants in IL-6 promoter gene have been associated with susceptibility to tuberculosis (Gualiang *et al*., 2012). A deficient protective immune response coupled with susceptibility to mycobacterial infection was observed in IL-6 deficient mice as compared to their wild type counterparts (Saunders *et al*., 2000; Lamas *et al*., 1997). Ladel also reported IL-6 as a crucial cytokine to resistance against tuberculosis after intravenous delivery of high doses of Mtb. Similarly, Leal et al (1999) reported IL-6 mediated protective immunity during a vaccination with a tuberculosis subunit vaccine. It has been demonstrated that IL-6 is required for the rapid expression of an initial protective IFN-g response during M. tuberculosis infection

(Tsao et al., 1999) which conflicts with other study reports that IL-6 has an inhibitory effect on interferon signaling (Nagabhushaman et al., 2003). To buttress this finding, Martinez et al (2015) also reported that IL-6 production inhibits interferon signaling and eventually facilitate disease progression, possibly because interferon gamma is a major activator of macrophages towards intracellular destruction of Mtb.

2.10.6 GRANZYME B

Cytolytic activity against Mtb within the infected macrophages is an important step in the immunity against TB infection, as Mtb is an intracellular bacterium. Granzymes are a family of serine proteases found in granules of cytotoxic lymphocytes. It is also expressed constitutively in natural killer cells (Susanto et al., 2012).A coordinated expression of effector functions such as cytolytic perforin and antimicrobial granulysin seems to be required for the control of human TB (Kumar et al., 2010). Genetics studies in knockout mice have revealed that perforin and granzyme B are essential components of granules important in the GE mechanism of target cell death Granzyme B enters cell through a perforin-dependent mechanism and acts in intracellular substrates including pro-caspase 3 (Trapani et al., 2002). Although commonly associated with T cells and natural killer cells, it has also been found to be expressed by macrophages (Veernoy et al., 2007). However excessive Granzyme B expression may result in pathologic conditions (Boivin et al., 2009). The role of Granzyme B in TB has been difficult to ascertain. A murine model using the vaccine strain BCG indicates that Granzyme B is unregulated in lung cells by 14 days post infection (Arandey et al., 2012). Other studies using knockout mice have demonstrated that stopping perforin expression limits CD8+ T cell-mediated cytotoxicity and thus impairs bacterial control (Woodworth et al., 2008) but eliminating Granzyme B expression has little effect on

bacterial control. Suggesting alternative mechanisms in addition to perforin mediated granzyme B cell cytotoxicity.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Study design:

The study was Cross-sectional.

3.1 Sample collection

Archived serum samples were used for the study

3.1.1 Description of Archival samples

Archived serum samples used for the study were obtained from blood samples of TB, MDR-TB patients and healthy controls (No TB). These study participants were recruited from the chest clinic of Korle-Bu teaching hospital and other regional TB referral centres across Ghana between January and May 2015 by convenient sampling for an MDR study. Active TB cases were confirmed by sputum microscopy using the auramine stained fluorescence microscopy technique. Multi drug resistant species were confirmed by culture on Lowenstein Jensen media, followed by drug sensitivity testing using the proportion method. Only participants who showed resistance to both isoniazid and rifampicin were considered as MDR-TB patients. The serum was separated and stored in triplicates at -80 at the Noguchi Memorial Institute of Medical Research (NMIMR). The active TB cases and MDR-TB cases were known cases on treatment at their respective healthcare facilities. The healthy controls were obtained from QuantiFeron negative samples used for a related study at the Noguchi Memorial Institute for Medical Research, Ghana.

3.1.2 Exclusion Criteria

Sampling excluded TB patients co-infected with HIV/AIDS, cancer and patients on steroid therapy.

3.1. 3 Selection of archived serum sample for this study

The criteria for selection of archived serum samples to be used for this study were availability of detailed demographic and personal data of the participant from whom the sera was obtained. Sixty-four (64) archival serum samples met this criterion and were processed for this study. The serum samples were obtained from three different population pools comprising twenty one (21) multi-drug resistant tuberculosis patients, twenty five (25) active TB patients and twenty (25) healthy controls.

3.2 Sample processing and cytokine quantification

Cytokine quantification was performed by multiplexing using the Luminex™ 200 system (Luminex, Austin, TX, USA) following manufacturer's instructions. The Luminex-200 system uses uniformly sized microspheres internally labeled with graded proportions of a red and a near infrared fluorophore, 658 and 712nm, providing the capacity to interrogate and classify 100 discrete beads (Fulton et al., 1997; Oliver et al., 1998; Swartzman et al., 1999; Martins, 2002). This technology facilitates the simultaneous evaluation of multiple cytokines with advantages of higher throughput, smaller sample volume, and lower cost.

3.3 Principle of Luminex Assay

The LUMINEX assay kits were procured from R&D systems, USA. This system employs color-coded magnetic beads pre-coated with analyte-specific capture antibodies which binds to the analyte of interest. Secondary antibodies (Biotinylated detection specific antibodies) specific to the analyte of interest are added thus forming an antibody-antigen sandwich. A fluorochrome, phycoerythrin conjugated streptavidin is added and binds to the biotinylated specific antibodies. The magnetic beads are read on the Luminex 200 MAGPIX system. A magnet in the Luminex 200 MAGPIX analyzer captures and holds the magnetic beads in a monolayer, while two spectrally distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and, the second LED determines the magnitude of the PE-derived signal which directly corresponds to the concentration of analyte in the sample.

3.4 Reagent Preparation

Preparation of standards

Three lyophilized standard cocktails namely L, A, and B were brought to room temperature before analysis. The standards were reconstituted using the Calibrator Diluent RD6-52. A volume of 225µl, 250µl, and 275µl each of the Calibrator Diluent were respectively pipetted into the standard cocktails L, A and B. They were left on the bench at room temperature for fifteen (15) minutes with gentle agitation prior to making dilutions.

A total volume of 1000µl was prepared from the three standards by pipetting 300µl that is 100µl each from standards L, A and B into an Eppendorf tube already containing 700µl of Calibrator Diluent. Prior to dilution, each standard is vortexed for 60 seconds. The tube was labeled as Standard 1 from which three fold dilutions of the Standards were prepared.

Preparation of wash buffer

The Wash buffer concentrate was warmed to room temperature and mixed gently until the complete dissolution of the crystals. A total volume of 500ml working solution was prepared by adding 20ml of the Wash Buffer Concentrate was to 480ml of deionized water.

Preparations of three-fold dilutions from standard 1

Five (5) other Eppendorf tubes were labeled 2,3,4,5, and 6. A volume of 200µl of Calibrator Diluent RD6-52 was pipetted into each of the tubes. Standard 1 was used to produce a threefold dilutions series by pipetting 100µl from Standard 1 into the tube labeled Standard 2. The solution was therefore mixed thoroughly before the next transfer of 100µl into Standard 3. The process was repeated up to Standard 6.

Preparation of diluted microparticle cocktail

The diluted Microparticle Cocktail was prepared based on the number of wells in the microtitre place to be used, that is 96 wells. The Microparticle cocktail vial was resuspended by centrifuging for 30 seconds at 1000 x g prior to removing the cap.

It must be noted that lights in the room were switched off to protect microparticles from light and avoid photo bleaching. The Microparticle Cocktail was diluted by pipetting 5000µl of Diluent RD2-1 into 500µl of Microparticle Cocktail in a mixing bottle

3.5 Loading of the wells

Samples were loaded in two fold dilutions by pipetting into each well by adding 75µl of sample to 75µl of Calibrator Diluent RD6-52and mixed thoroughly. This was done in a plain microtitre plate. In another microtitre plate (opaque), 50µl of the Microparticle Cocktail was pipetted into each well away from light. From the plain microtitre plate, 50µl of the two fold diluted samples were added unto the Microparticle Cocktail. The plate was covered with foil and incubated at 2-8 Degrees Celsius overnight.

After overnight incubation, the microplate was placed on a horizontal orbital microplate shaker for 60 seconds at 800+/- 50 rpm. The microplate was then removed and attached to a strong magnetic field designed to accommodate the microplate. The magnetic device was applied at the bottom of the microplate with the aid of a cello tape to facilitate the attachment (the magnetic beads are strongly attracted by the magnet device in order to avoid falling off during the washing process).

3.6 The washing process

The content of the microplate plate was poured away whiles still attached to the magnetic device. The first washing was done by pipetting 100µl of the wash buffer into each Well with the microplate detached from the magnetic device. It was then placed on the microplate shaker for 60 seconds. After 60 seconds of shaking, the plate was re-attached to the magnetic device for 60 seconds before pouring off the liquid. The wash procedure was performed three times.

3.7 Diluted biotin antibody cocktail preparation

Preparation of diluted Biotin Antibody Cocktail was also based on the number of well: 96 microtitre well plate. Prior to removing the cap, the Biotin Antibody Cocktail vial was centrifuged

for 30 seconds at 1000 x g. The Biotin Cocktail was diluted by pipetting 500µl of the Biotin Cocktail into 5000µl of Diluent RD2-1.

3.8 Streptavidin-PE preparation

A polypropylene amber bottle was wrapped with aluminium foil. Preparation was done away from light. The Streptavidin-PE vial was centrifuged at 1000x g for 30 seconds prior to removing the cap without inversion. To make enough Streptavidin-PE for a 96 Well plate, 220µl of the Streptavidin-PE concentrate was diluted with 5350µl of Wash Buffer in the polypropylene amber bottle wrapped with foil.

3.9 Addition of biotinylated antibody and streptavidin-PE

Fifty microliters (50µl) of the diluted Biotin Antibody Cocktail was added to each well. It was securely covered with a foil plate sealer and incubated for 1 hour at room temperature on a plate shaker. After one hour of incubation, the microplate was placed on the magnetic device for 60 seconds after which the liquid was poured out. Wash buffer (100µl) was pipetted into each well again and left for 60 seconds on a plate shaker. After 60 seconds of shaking, the liquid was removed after attaching it to the magnetic device. The wash procedure was repeated three times.

After the washing process, 50µl of diluted Streptavidin-PE was added to each well and securely covered with a foil plate sealer. The plate was incubated again for 30 minutes at room temperature on the shaker.

After 30 minutes of incubation, the microplate was placed on the magnetic device for 60 seconds after which the liquid was poured out. Wash buffer (100µl) was pipetted into each well again and

left for 60 seconds on a plate shaker. After 60 seconds of shaking, the liquid was removed after attaching it to the magnetic device. The wash procedure was repeated three times.

Finally, the microparticles were resuspended by adding 100µl of Wash Buffer to each well and incubated for two minutes. The cytokines were quantified by reading on Luminex® 200™system. Final read out of results were displayed in concentrations.

3.10 Data management and analysis

All data were entered into Microsoft Excel 2010 and GraphPad Prism V 7.03 for analysis and graphs. Statistical analysis was performed using SPSS (V 23). Demographic details (age profile, geographical distributions, educational status and other risk factors) was assessed using descriptive statistics. Pairwise analysis was performed by Chi square. Numeric variables were expressed as median (interquartile range) for all cytokines in the different population groups. Analysis of Variance (ANOVA) was used to analyze differences among group (MDR-TB, active TB, Negative controls) means. Mann-Whitney test was used to analyze concentration differences of each of the eight cytokines between the groups. Spearman analysis was employed to assess correlations among continuous variables. Receiver operating characteristic (ROC) curves was constructed and the area under the ROC curve (AUROC) was used to evaluate the sensitivity of each cytokine level to discriminate between different population groups.

All data about participants were kept private and confidential. Names were coded and used for analysis of results. Results were stored in secured password-protected archival systems.

3.11 Dissemination of results

The findings of this study were disseminated through oral presentations at the Department of Medical Laboratory Sciences, University of Ghana.

3.12 Ethical issues

Ethical guidelines and protocols were duly followed in all procedures undertaken in this study. Retrieved archival sample and data of all study subjects were treated with high level of confidentiality. Ethical clearance and administrative approval were sought from the Ethical and Protocol Review of the School of Biomedical and Allied Health Sciences, University of Ghana and Noguchi Memorial Institute for Medical Research respectively with Ethics Identification Number: SBAHS – MD. /10551247/AA/5A/2016-2017.

CHAPTER FOUR

RESULTS

4.0 Demographic details of study cohorts

A total of sixty four (64) archived serum samples were retrieved for the study. Of the 64 samples, 21 (32.8%) were multidrug resistant tuberculosis (MDR-TB) cases, 25 (39.1%) were active tuberculosis patients and 18 (28.1%) were QuantiFeron TB test negative individuals. The average ages were similar for the population groups and the proportion of males and females were equally distributed (Table 4.0).

TABLE 4.0 AGE AND SEX PROFILEOF STUDY GROUPS.

Group	Age range (years)	Mean age	Male n (%)	Female n (%)
MDR-TB	15-65	46	12 (57.1)	9 (42.9)
TB	20-58	36	10 (40)	15(60)
No TB	25-52	40	8 (44.4)	10(55.6)

Multidrug Resistant Tuberculosis (MDR-TB), Active TB (TB), Negative controls (No TB)

None of the patients with MDR-TB had an educational background beyond tertiary level. Only one (1) out of twenty-five (25) active TB cases had tertiary education. No difference in educational attainment and occupation were observed in MDR-TB and TB cases though in both cases ‘no educational background’ recorded the highest figure. Alcohol intake, smoking and marital status showed no significant association with MDR-TB and TB cases (Table 4.1).

TABLE 4.1 Socio-demographic characteristics of MDR-TB and TB cases

Variables	MDR-TB n (%)	TB n (%)	Chi-square	p
Educational level				
Primary	5 (23.8)	4 (16)		
JHS	6 (28.6)	8 (32)		
Secondary	2 (9.5)	1 (4)	2.4	0.085
Tertiary	0 (0)	1 (4)		
Nil	8 (38.1)	11 (44)		
Occupation				
Skilled	2 (9.5)	4 (16)		
Unskilled	9 (42.9)	9 (36)	5.8	0.463
Retired	1 (4.8)	2(8)		
Unable to work	9 (42.9)	10 (40)		
Marital Status				
Married	11(52.4)	6 (24)		
Separated	2 (9.5)	3 (12)	7.0	0.221
Divorced	5 (23.8)	14 (56)		
Never married	3 (14.3)	2 (8)		
Smokes?				
Yes	3 (14.3)	9 (36)	12.4	0.002
No	18(85.7)	16 (64)		
Drinks Alcohol?				
Yes	10 (47.6)	15 (60)	4.5	0.301
No	11 (52.4)	10 (40)		

Under DOTS?				
Yes	18 (85.7)	8 (32)	13.6	0.001
No	3 (14.3)	17 (68)		
Previous TB History?				
Yes	21 (100)	2 (8)	16.8	0.0001
No	0 (0)	23(92)		
Previous TB treatment				
Yes	21 (100)	1 (4)	16.4	0.0002
No 0 (0)	24 (96)			

4.1 Comparison of the cytokine expression profile among the three groups

To determine whether any single cytokine profile has the potential to discriminate between any two of the three groups, the mean concentration of individual cytokines was compared between the groups.

Differences between group means were estimated using one-way ANOVA. The results (Table 4.2) show varried cytokine profiles in relation to the three population groups. The mean TNF-α concentration was significantly higher in MDR-TB and TB cases compared to the controls (p=0.001, p=0.0004 respectively). An equally significantly higher mean concentration of IL-4 was observed between MDR-TB cases and controls (p=0.002) but such was not shown between TB cases and negative controls (p=0.113).IL-10, IFN-γ, and IL-6 cytokine concentrations were significantly higher in TB caese cpmpared to controls (p=0.003, p=0.002, p= 0.010 respectively) but not between MDR-TB and controls (p=0.074, p=0.496, p=0.519 respectively). Generally the

analysis showed no significant variation between MDR-TB and TB cytokine concentrations for any single one of the eight cytokines.

Table 4.2 Comparison of single cytokine concentrations between groups (only p-values are shown in the table, p> 0.05 was considered statistically significant).

ANOVA

Cytokines	IL-10	IFN-g	IL-17A	IL-12p70	IL-4	Granz B	TNF-a	IL-6
MDR-TB/ TB	0.624	0.071	0.997	0.845	0.208	0.706	0.968	0.093
MDR-TB/ No TB	0.074	0.496	0.587	0.269	**0.002**	0.956	**0.001**	0.519
TB/No TB	**0.003**	**0.002**	0.436	0.112	0.113	0.582	**0.004**	**0.010**

A scatter plot (GraphPad Prism) comparing single serum cytokine levels in the different population groups (Figures 4.0-4.7) indicated similar patternsas in ANOVA. The p- values from the Mann-Whitney U-test (Table 4.3) depicted rather more significant variations (Fig 4.0). In contrast to the one-way ANOVA results, IFN-g and IL-4 were significantly higher for both groups MDR-TB/No TB and TB/No TB.

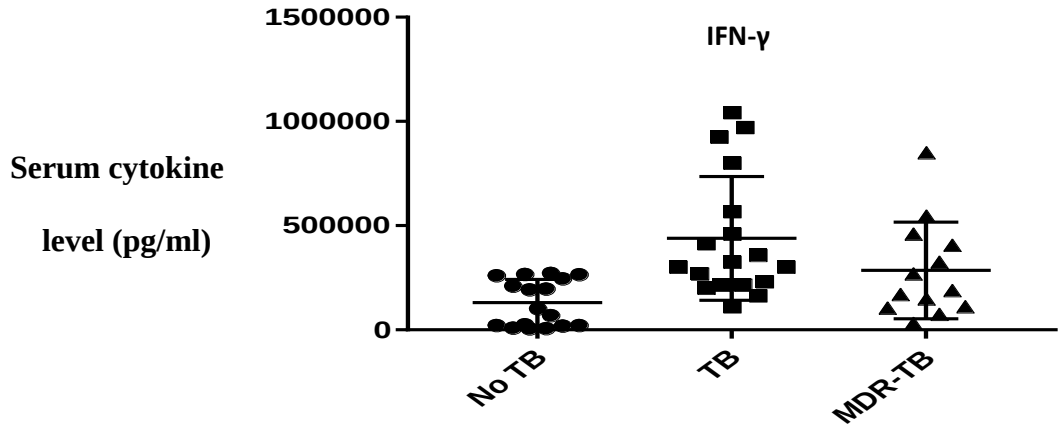

Figure 4.0 Serum cytokine concentration of IFN-γ in the three groups MDR-TB, TB and No TB. Statistical significance of concentration difference between groups were evaluated (MDR-TB/TB: p=0.121, MDR-TB/No TB: **p=0.01**, TB/No TB: **p=0.0001**)

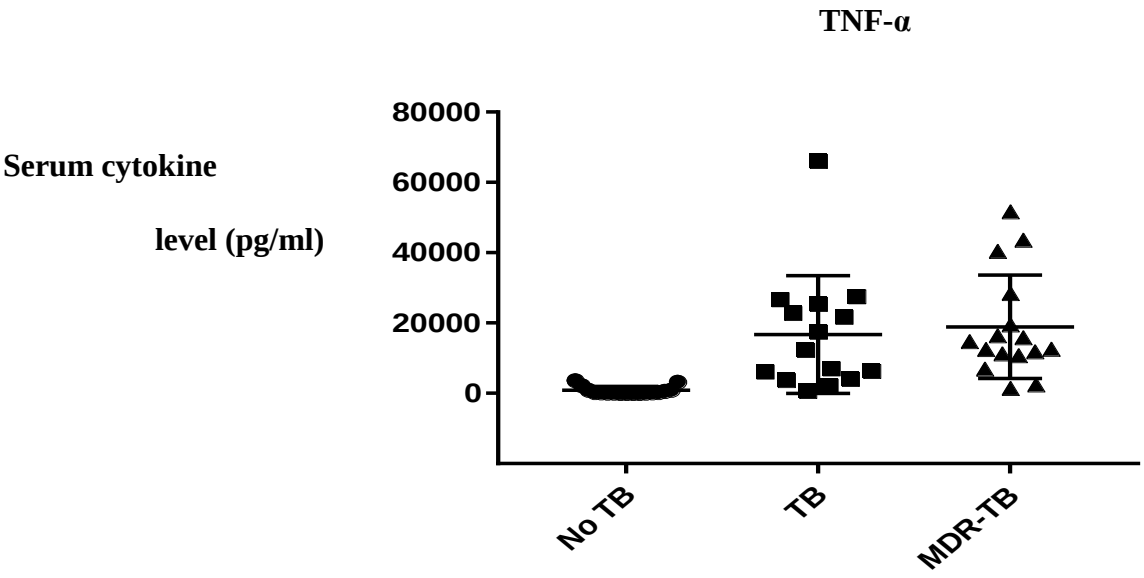

Figure 4.1 Serum cytokine concentration of TNF-α in the three groups MDR-TB, TB and No TB. Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.096, MDR-TB/No TB: **p=0.0001**, TB/No TB: **p=0.00001**).

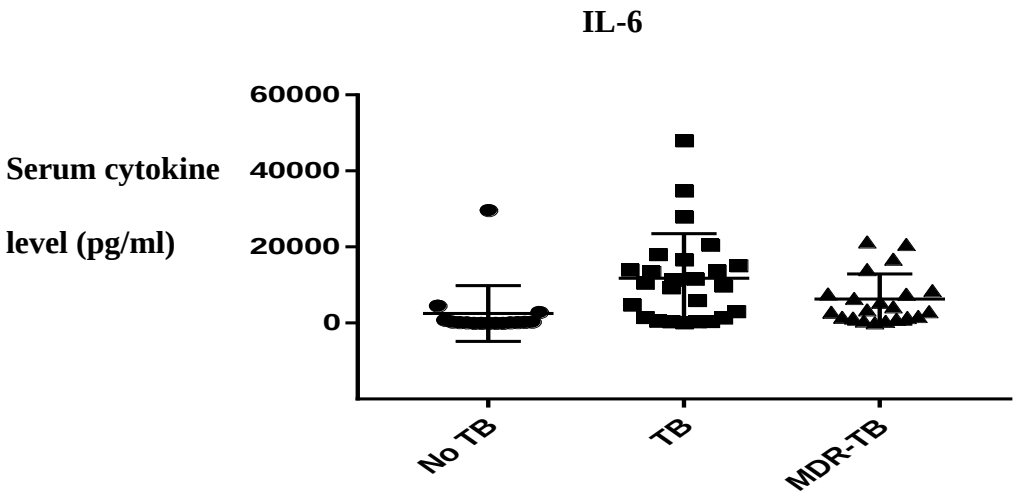

Figure 4.2 Serum cytokine concentration of IL-6 in the three groups MDR-TB, TB and No TB. Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.124, MDR-TB/No TB: p=0.055, TB/No TB: **p=0.001**)

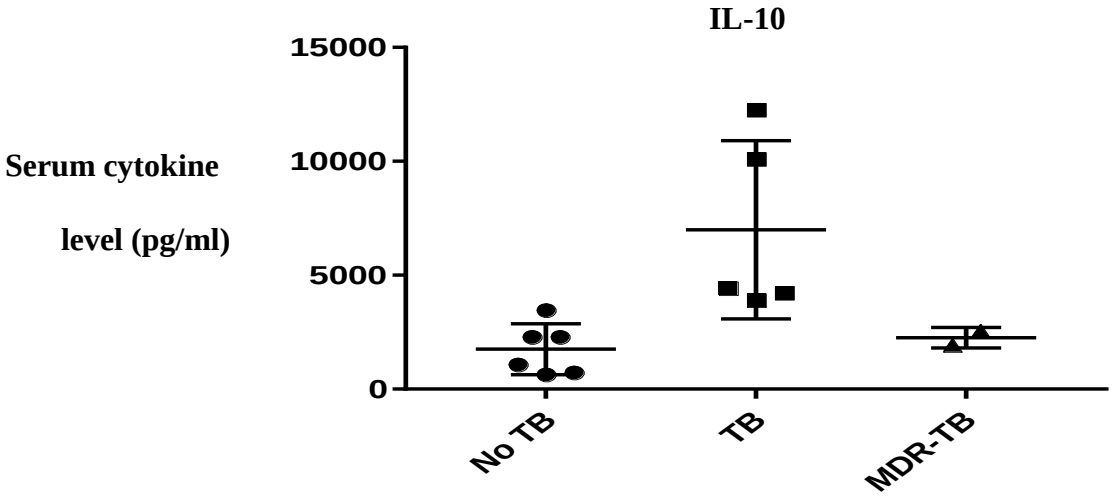

Figure 4.3 **Serum cytokine concentration of IL-6 in the three groups MDR-TB, TB and No TB.** Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.134, MDR-TB/No TB: p=0.082, TB/No TB: **p=0.01**)

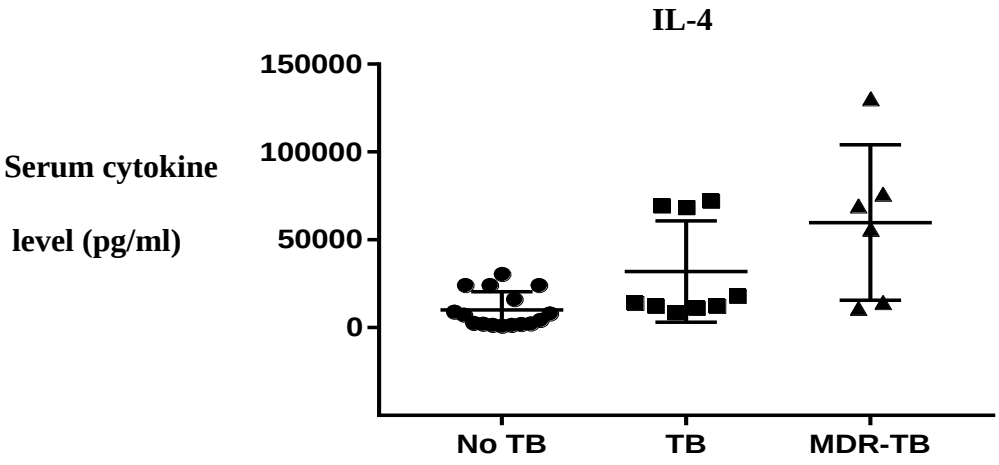

Figure 4.4 Serum cytokine concentration of IL-4 in the three groups MDR-TB, TB and No TB. Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.084, MDR-TB/No TB: **p=0.0001**, TB/No TB: **p=0.01**)

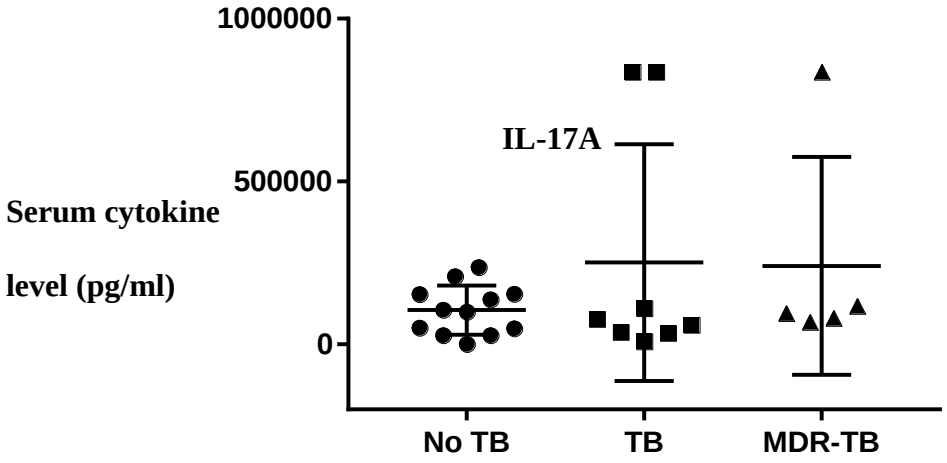

Figure 4.5 Serum cytokine concentration of IL-17A in the three groups MDR-TB, TB and No TB. Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.084, MDR-TB/No TB: p=0.374, TB/No TB: p=0.436).

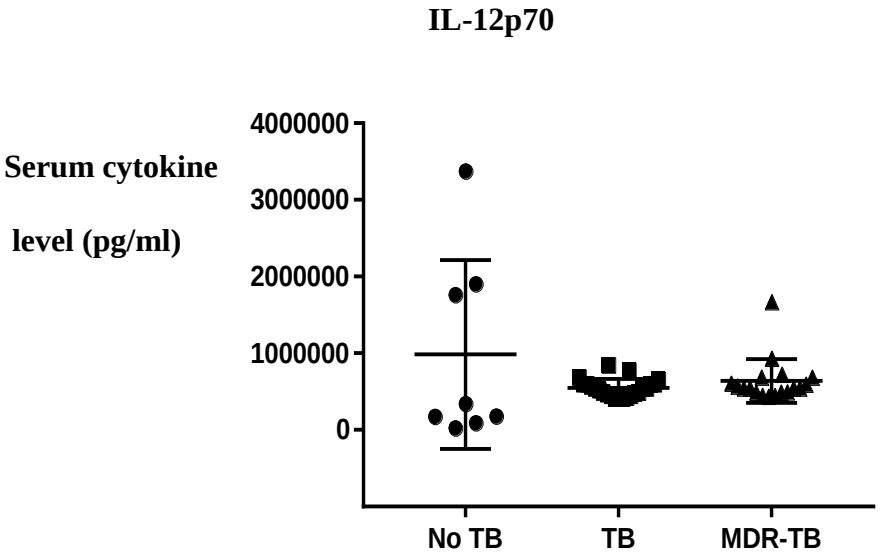

Figure 4.6 Serum cytokine concentration of IL-12p70 in the three groups MDR-TB, TB and No TB. Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.505, MDR-TB/No TB: p=0.072, TB/No TB: p=0.112)

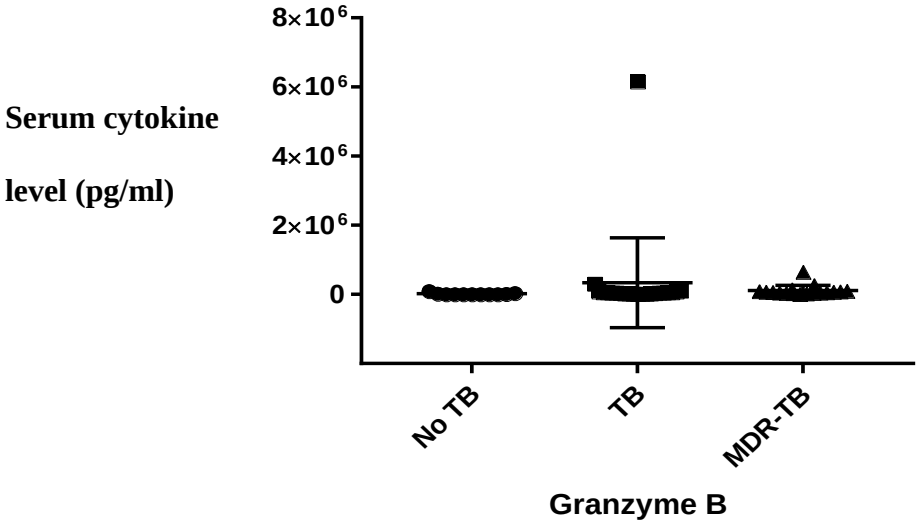

Figure 4.7 Serum cytokine concentration of Granzyme B in the three groups MDR-TB, TB, and No TB. Statistical significance of concentration difference between groups were analyzed (MDR-TB/TB: p=0.616, MDR-TB/No TB: p=0.748, TB/No TB: p=0.994).

Table 4.3 Comparison of single cytokine concentrations between groups (only p values are shown in the table, p> 0.05 was considered statistically significant).

Mann-Whitney U test

Cytokines	IL-10	IFN-g	IL-17A	IL-12p70	IL-4	Granz B	TNF-a	IL-6
MDR-TB/ TB	0.134	0.121	0.845	0.505	0.084	0.616	0.096	0.124
MDR-TB/ No TB	0.082	**0.010**	0.374	0.072	**0.0001**	0.748	**0.0001**	0.055
TB/No TB	**0.01**	**0.0001**	0.436	0.112	**0.01**	0.994	**0.00001**	**0.001**

4.2 Evaluation of the diagnostic performance of cytokines as biomarker for MDR TB

Receiver operator curves were generated to determine the cut-off values for optimal sensitivity and specificity for all the cytokine for each population group. The area under the curve <0.55 (55%) indicates poor performance in predicting substantial difference between the groups and vice versa. Figures 4.3-4.14 and Tables 4.4-4.6 presents details of the various dynamics in the AUROC in addition to their corresponding p-values.

IL-4

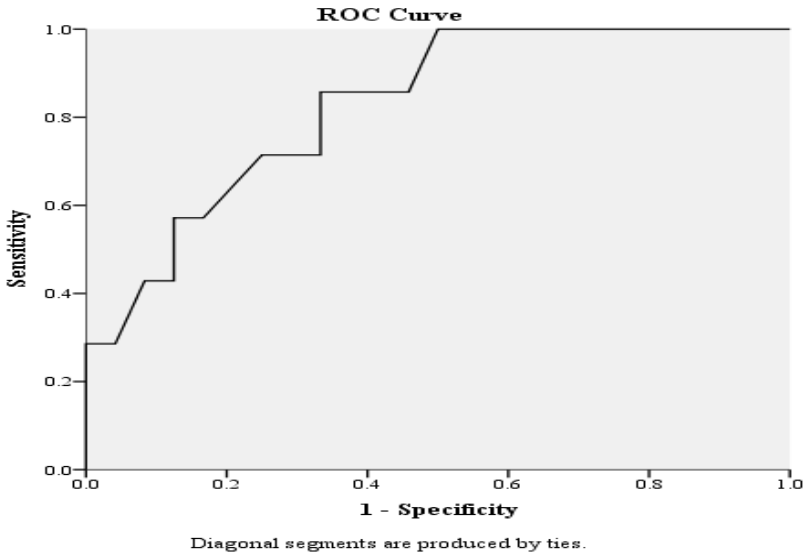

Figure 4.8 Area under the ROC curve of IL-4 in the three groups MDR-TB, TB and NO TB (AUC=0.759**, p=0.003**)

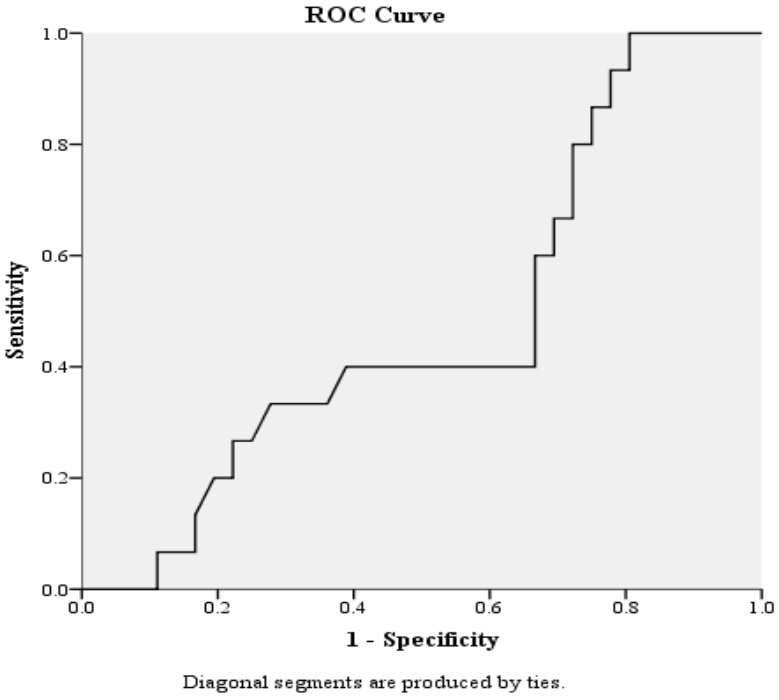

Figure 4.9 Area under the ROC curve of IFN-γ in the three groups MDR-TB, TB and NO TB (AUC=0.481, p=0.828)

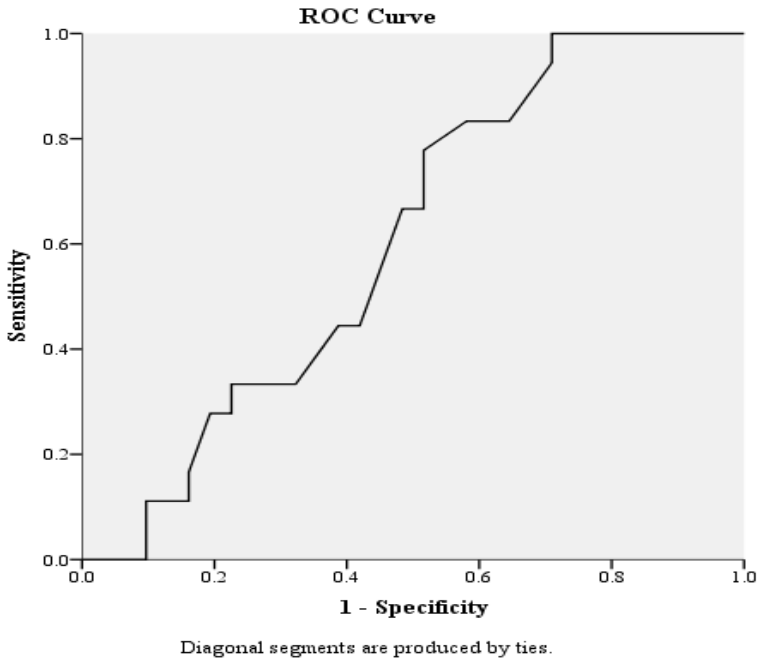

Figure 4.10 Area under the ROC curve of IL-12p70 in the three groups MDR-TB, TB, and No TB (AUC= 0.606, p= 0.221)

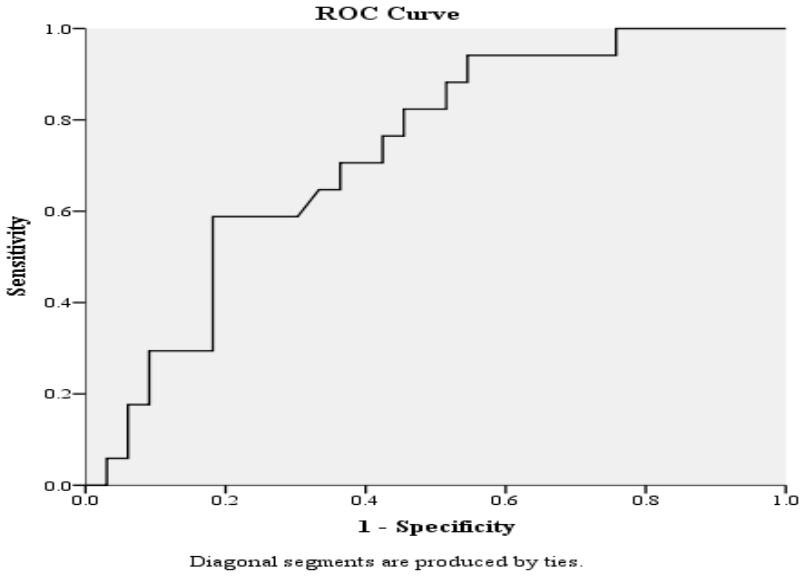

Figure 4.11 Area under the ROC curve of Granzyme B in the three groups MDR-TB, TB, No TB (AUC= 0.728, p=0.009)

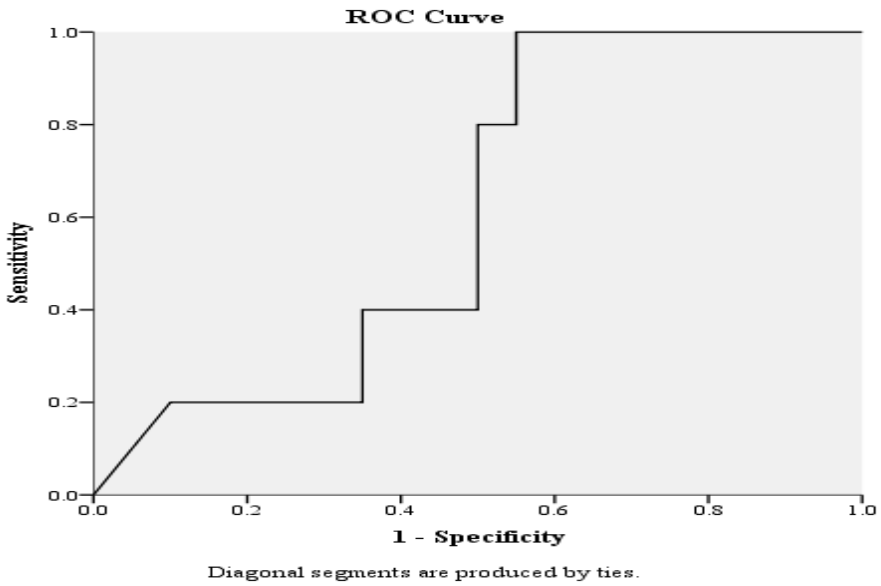

Figure 4.12 Area under the ROC curve of IL-17A in the three groups MDR-TB, TB, No TB (AUC= 0.610, p=0.455)

IL-10

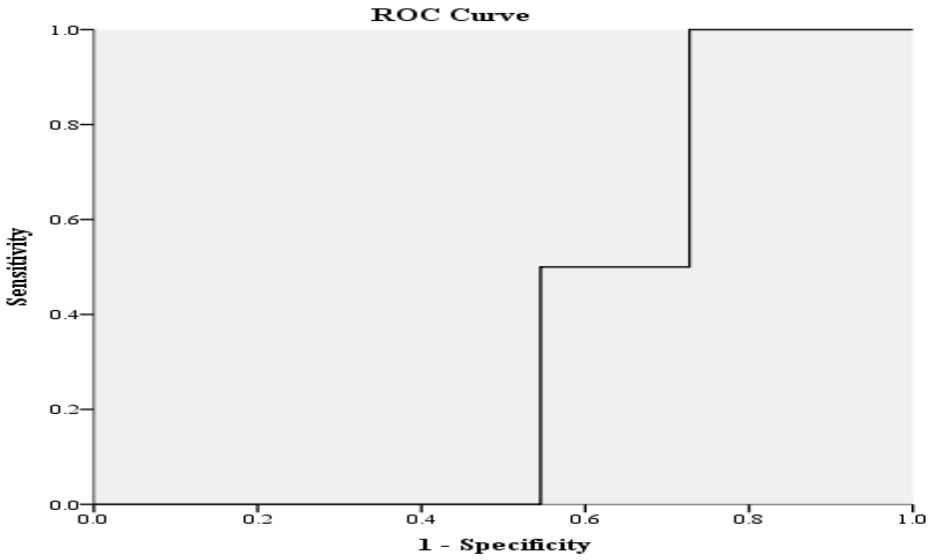

Figure 4.13 Area under the ROC curve of IL-10 in the three groups MDR-TB, TB, No TB (AUC= 0.364, p=0.554)

IL-6

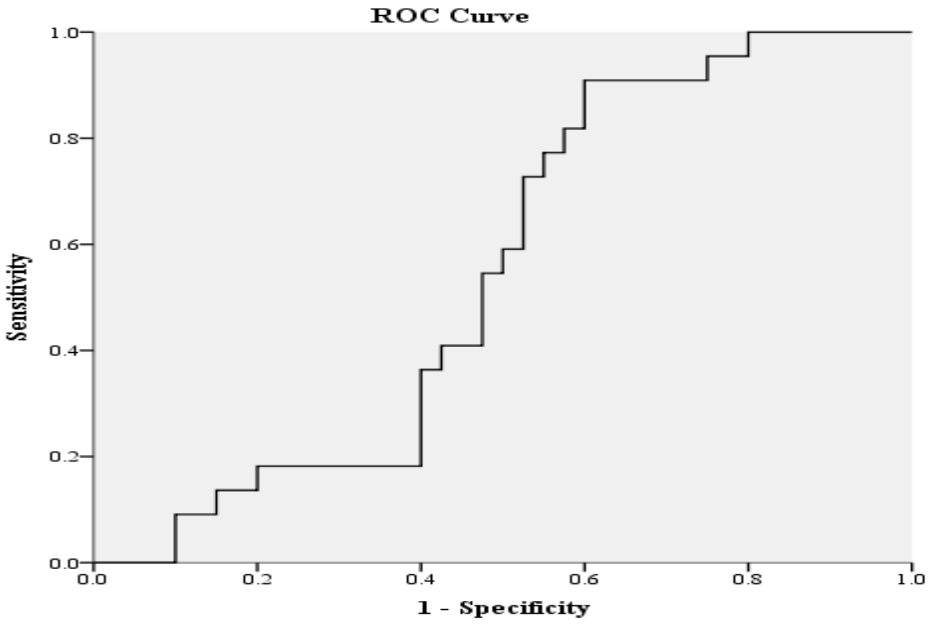

Figure 4.14 Area under the ROC curve of IL-6 in the three groups MDR-TB, TB, No TB
(AUC= 0.548, p=0.537)

TNF-α

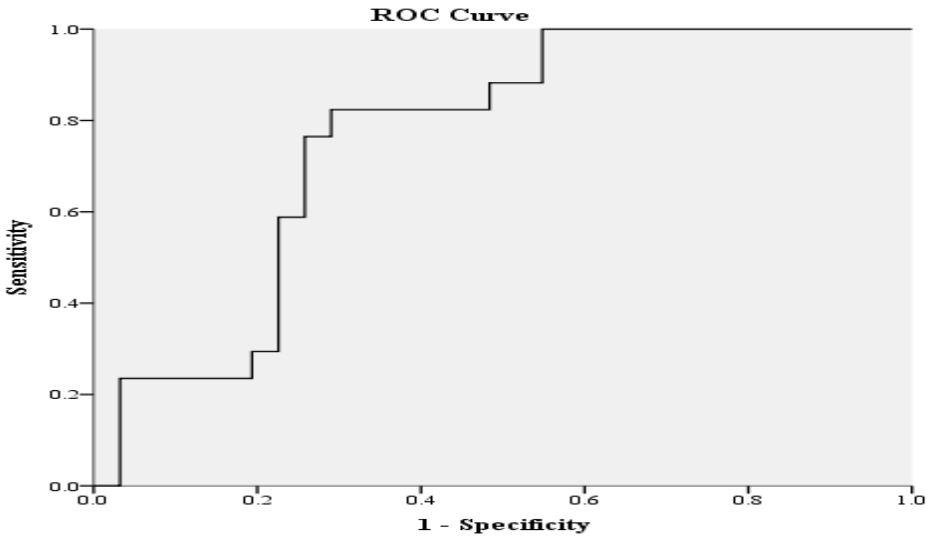

Figure 4.15 Area under the ROC curve of TNF-α in the three groups MDR-TB, TB, No TB
(AUC= 0.759, p=0.003)

TABLE 4.4 Area under the ROC curve for individual cytokines in relation to the three population groups (MDR-TB, TB, Negative controls).

AUC data regarding MDT-TB, Active TB and Negative controls			
Cytokines	**AUC**	**95% CI**	**p**
IL-4	0.827	0.670 - 0.985	**0.009**
TNF-α	0.759	0.623 - 0.895	**0.003**
IFN-γ	0.481	0.310 - 0.651	0.828
IL-17A	0.610	0.373 – 0.847	0.455
IL-12p70	0.606	0.449 - 0.762	0.221
IL-10	0.364	0.068 - 0.659	0.554
IL-6	0.548	0.405 - 0.690	0.537
Granzyme B	0.728	0.587 - 0.869	**0.009**

The area under the ROC curve for all cytokines in relation to MDR-TB and No TB were evaluated and shown in Figures 4.9-4.12. The summary of all corresponding p-values are shown in Table 4.4.

IL-10

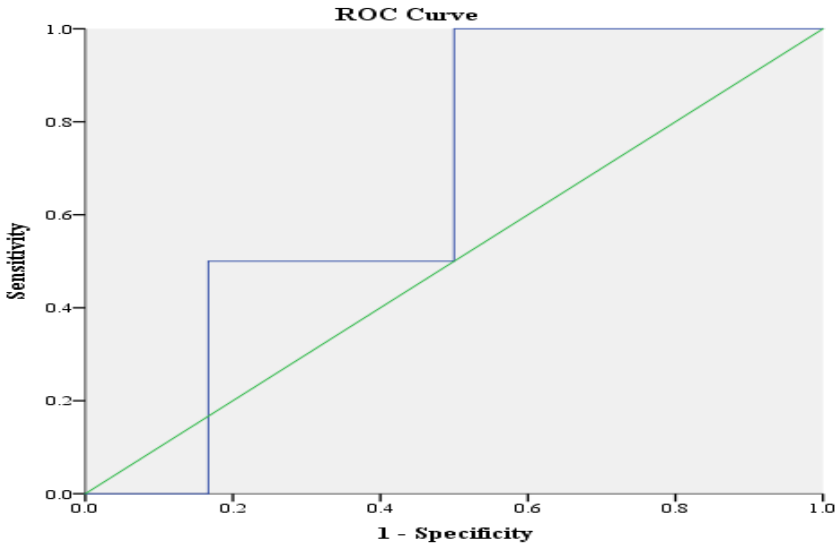

Figure 4.16 Area under the ROC curve ofIL-10 between MDR-TB and No TB (AUC= 0.667, p= 0.505).

IFN-γ

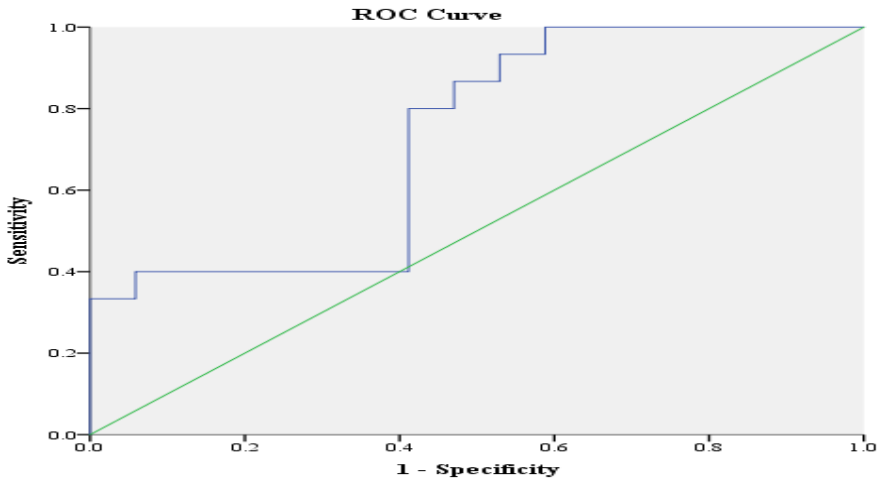

Figure 4.17 Area under the ROC curve of IFN-γ between MDR-TB and No TB (AUC=0.725, p=0.03).

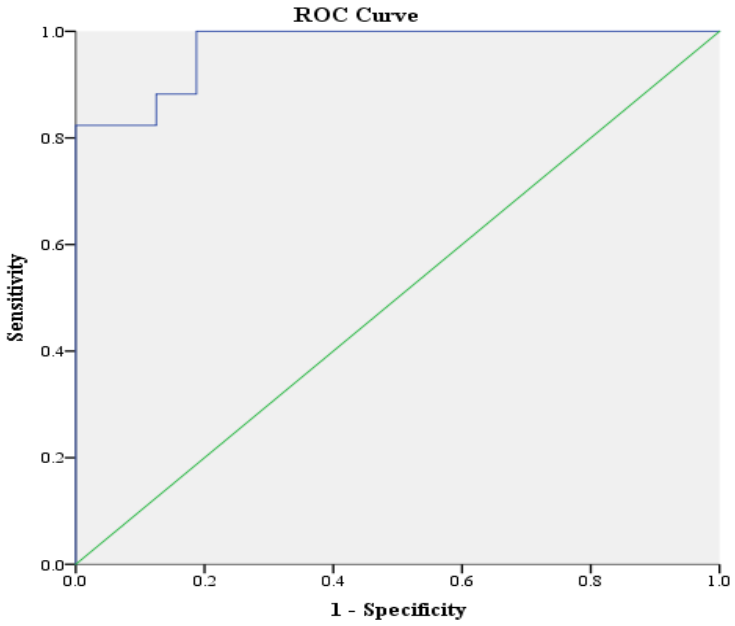

Figure 4.18 Area under the ROC curve of TNF-α between MDR-TB and No TB (AUC= 0.971, p=0.0001).

IL-6

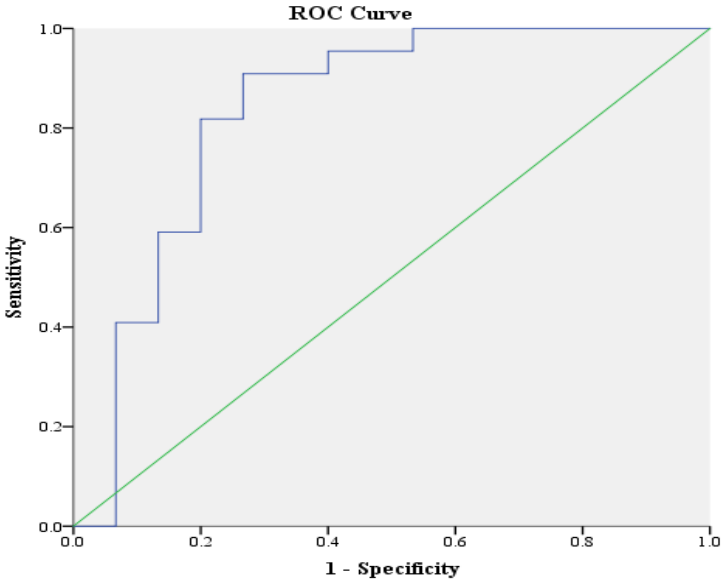

Figure 4.19 Area under the ROC curve of IL-6 between MDR-TB and No TB (AUC=0.836, p=0.001).

IL-12p70

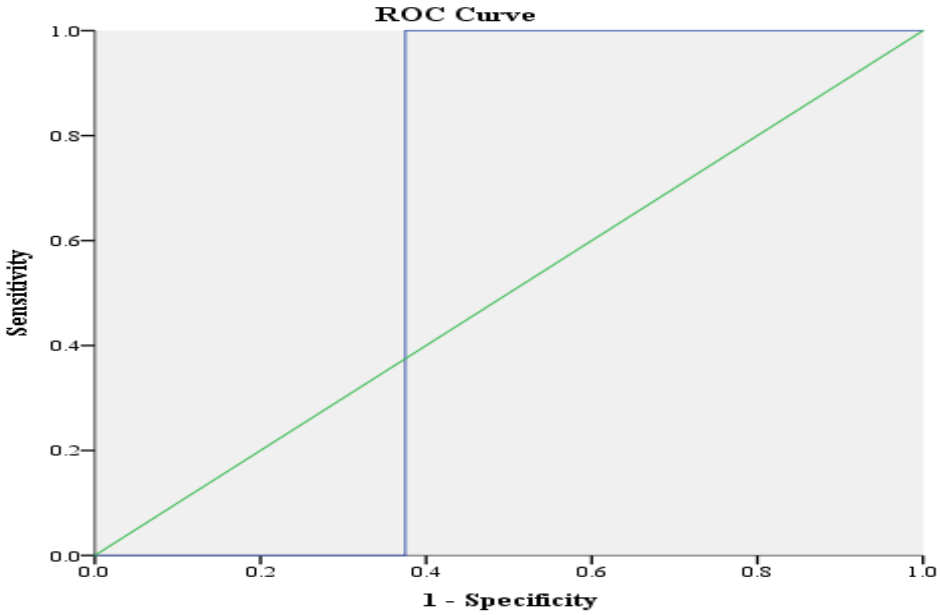

Figure 4.20 Area under the ROC curve of IL-12p70 between MDR-TB and No TB (AUC=0.625, p=0.317)

IL-17A

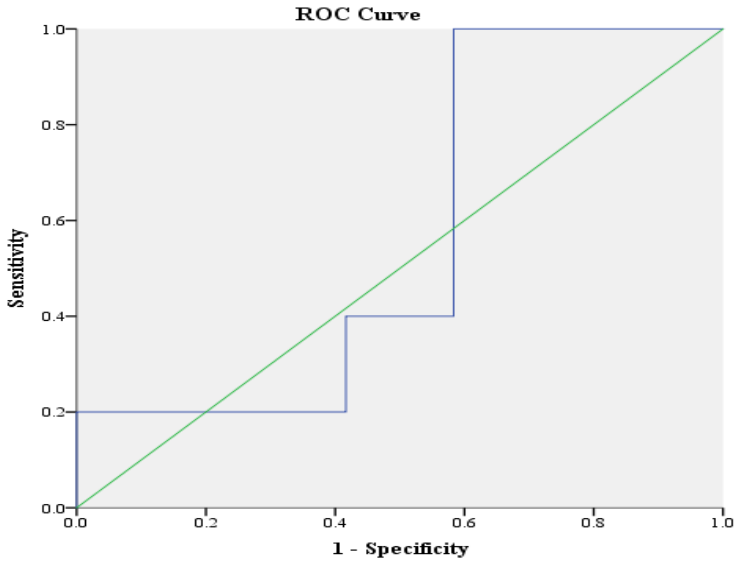

Figure 4.21 Area under the ROC curve of IL-17A between MDR-TB and No TB (AUC=0.567, p=0.151)

IL-4

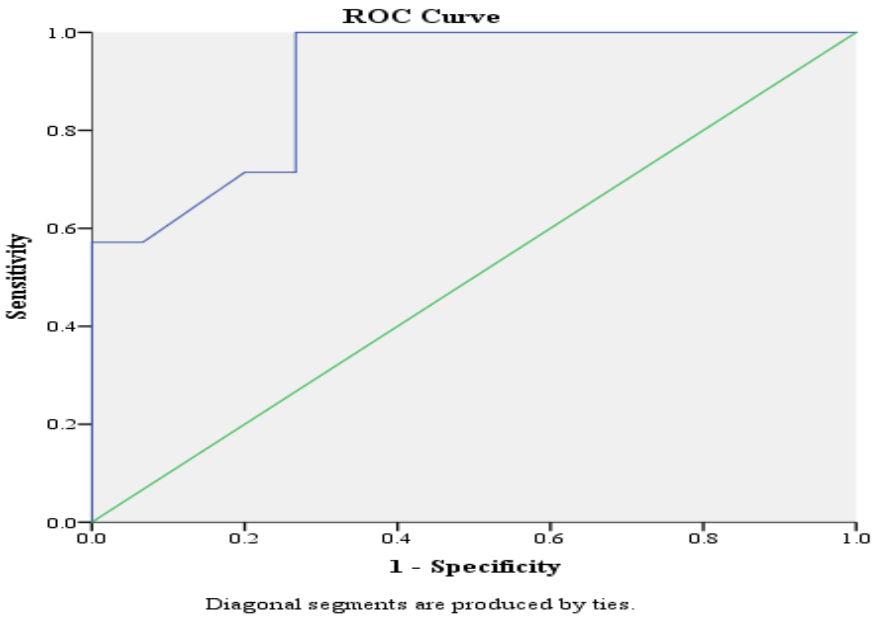

Figure 4.22 Area under the ROC curve of IL-4 between MDR-TB and No TB (AUC=0.905, p=0.003)

Granzyme B

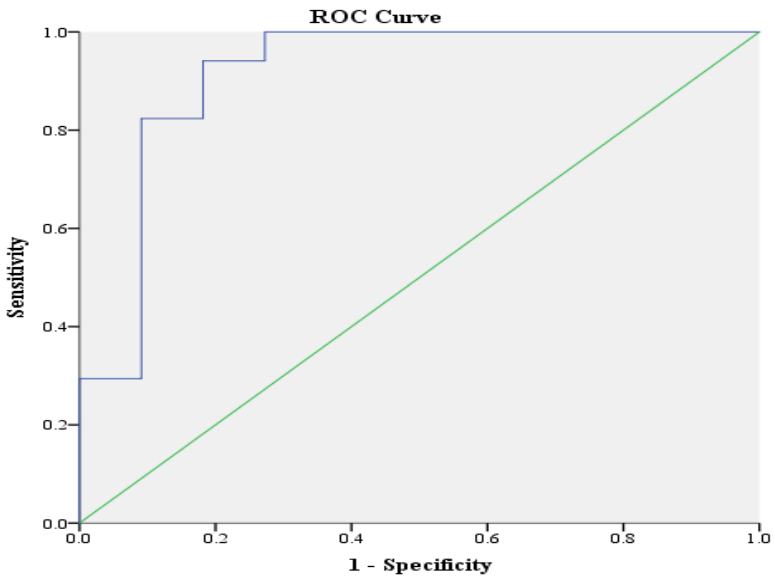

Figure 4.23**Area under the ROC curve of Granzyme B between MDR-TB and No TB** (AUC= 0.914, p=0.0001)

TABLE 4.5 Area under the ROC curve for individual cytokines in relation to MDR-TB, and No TB.

AUC data regarding MDR-TB and Negative controls			
Cytokines	**AUC**	**95% CI**	**p**
IL-4	0.905	0.778 - 1.000	**0.0030**
TNF-alpha	0.971	0.924- 1.000	**0.0001**
IFN-gamma	0.725	0.546- 0.905	**0.0300**
IL-17A	0.567	0.271 – 0.862	0.1510
IL-12p70	0.625	0.290 - 0.960	0.3170
IL-10	0.667	0.267 – 1.000	0.5050
IL-6	0.836	0.685 - 0.988	**0.0010**
Granzyme B	0.914	0.787 – 1.000	**0.0001**

The area under the ROC curve for all cytokines in relation to TB and No TB were evaluated and shown in Figures 4.13-4.16. The summary of all corresponding p-values is shown in Table 4.

TNF-α

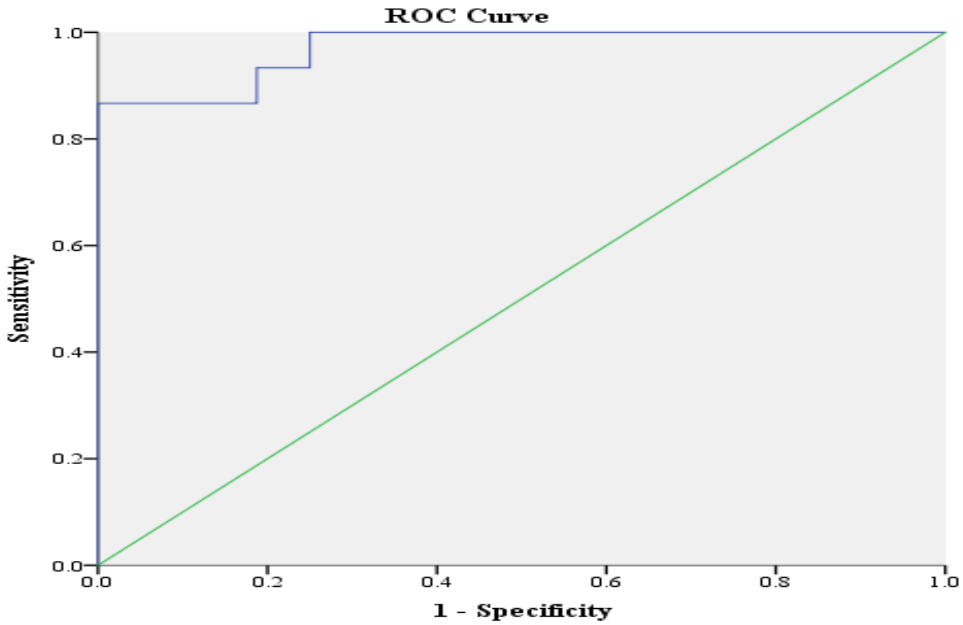

Figure 4.24 Area under the ROC curve of TNF-α between TB and NO TB (AUC=0.971,p=0.0001).

IL-6

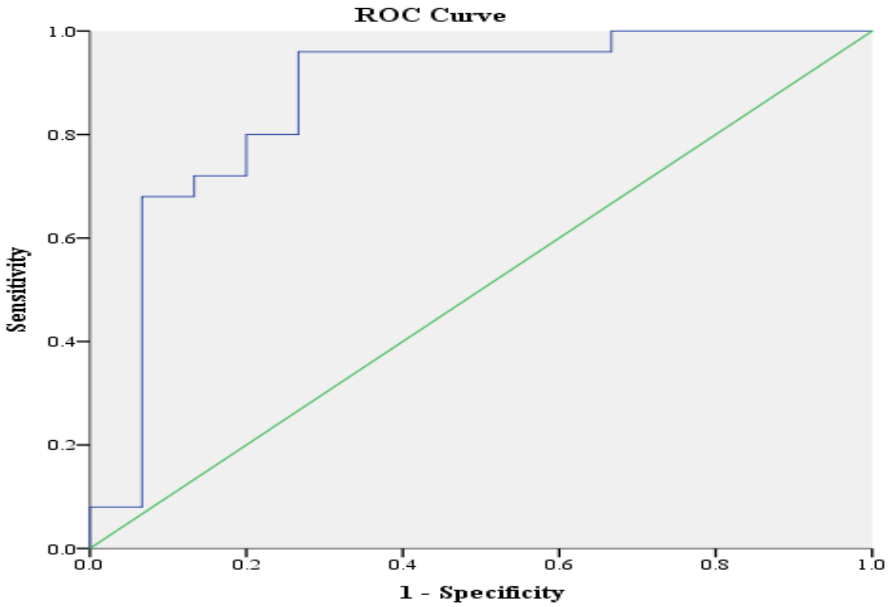

Figure 4.25 Area under the ROC curve of IL-6 between TB and NO TB (AUC=0.869, p=0.0001).

IL-10

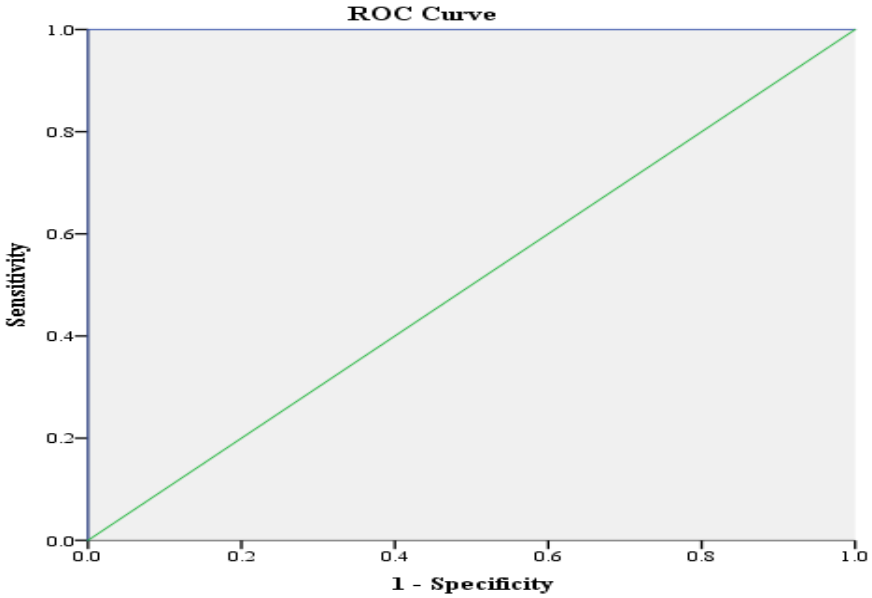

Figure 4.26 Area under the ROC curve of IL-10 between TB and NO TB (AUC=1.000, p=0.006).

IFN-γ

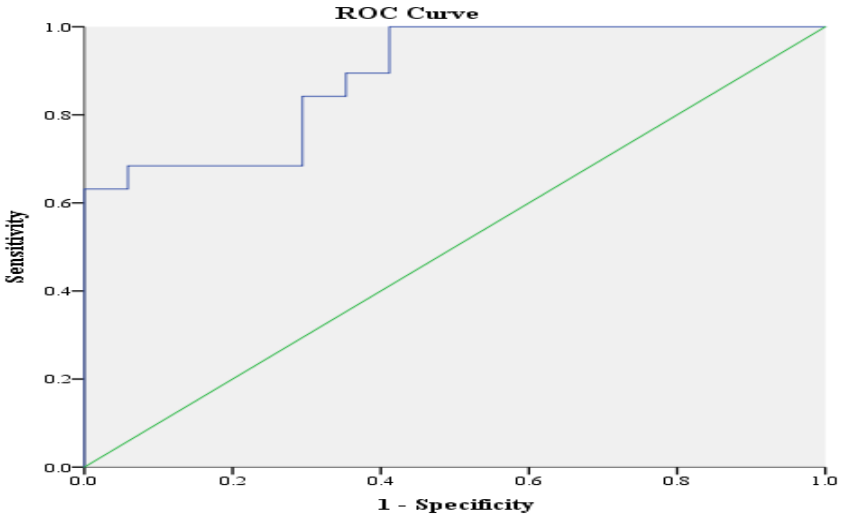

Figure 4.27 Area under the ROC curve of IFN-γ between TB and NO TB (AUC=0.889, p=0.0001).

IL-4

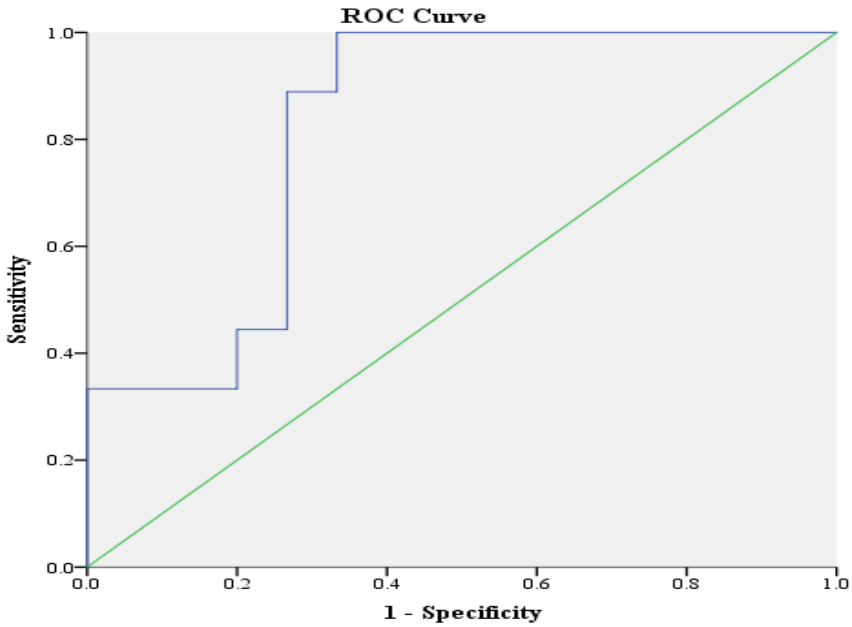

Figure 4.28 Area under the ROC curve of IL-4 between TB and NO TB (AUC=0.822, p=0.009).

IL-17A

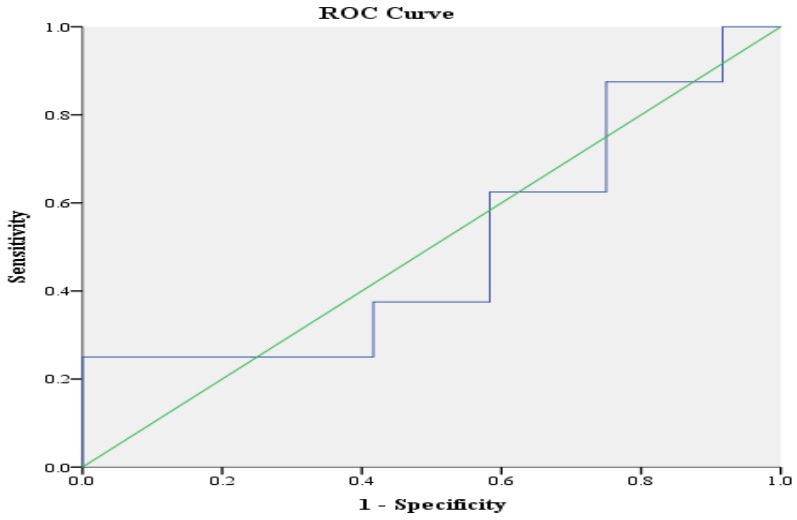

Figure 4.29 Area under the ROC curve of IL-17A between TB and NO TB (AUC=0.500, p=1.00).

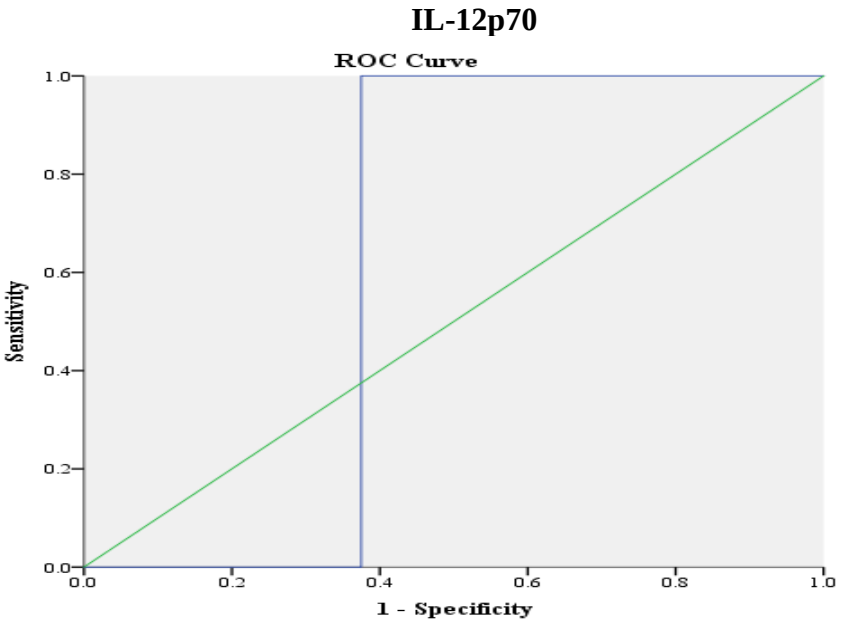

Figure 4.30 Area under the ROC curve of IL-12p70 between TB and NO TB (AUC=0.625, p=0.299).

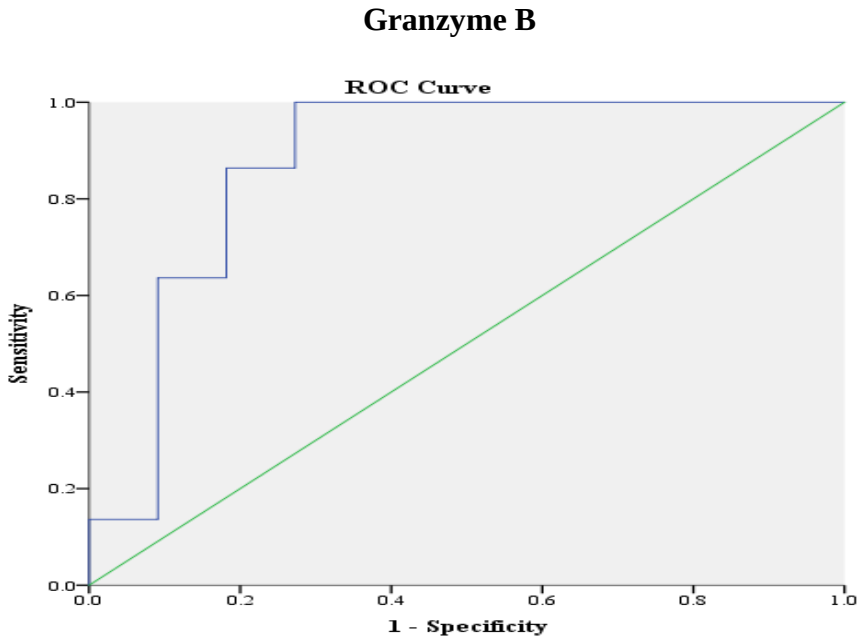

Figure 4.31 Area under the ROC curve of Granzyme B between TB and NO TB (AUC=0.875, p=0.001).

TABLE 4.6 Area under the ROC curve for individual cytokines in relation to Active-TB cases and Negative controls.

AUC data regarding Active-TB and No TB			
Cytokines	**AUC**	**95% CI**	**p**
IL-4	0.822	0.653–0.922	**0.0090**
TNF-alpha	0.971	0.922 - 1.000	**0.0001**
IFN-gamma	0.889	0.786 - 0.992	**0.0001**
IL-17A	0.500	0.224 – 0.776	1.0000
IL-12p70	0.625	0.290 - 0.960	0.2990
IL-10	1.000	1.000 – 1.000	**0.0060**
IL-6	0.869	0.738 – 1.000	**0.0001**
Granzyme B	0.876	0.719 – 1.000	**0.0010**

The area under the ROC curve for all cytokines in relation to MDR-TB and TB were evaluated as shown in Figures 4.15-4.16. TNF-a, IL-10, IFN-g and IL-6 are shown on the same graph likewise that of IL-12p70, Granzyme B, IL-17A and IL-4. The respective AUROC and p-values have also been shown (Figures 4.15-4.16).

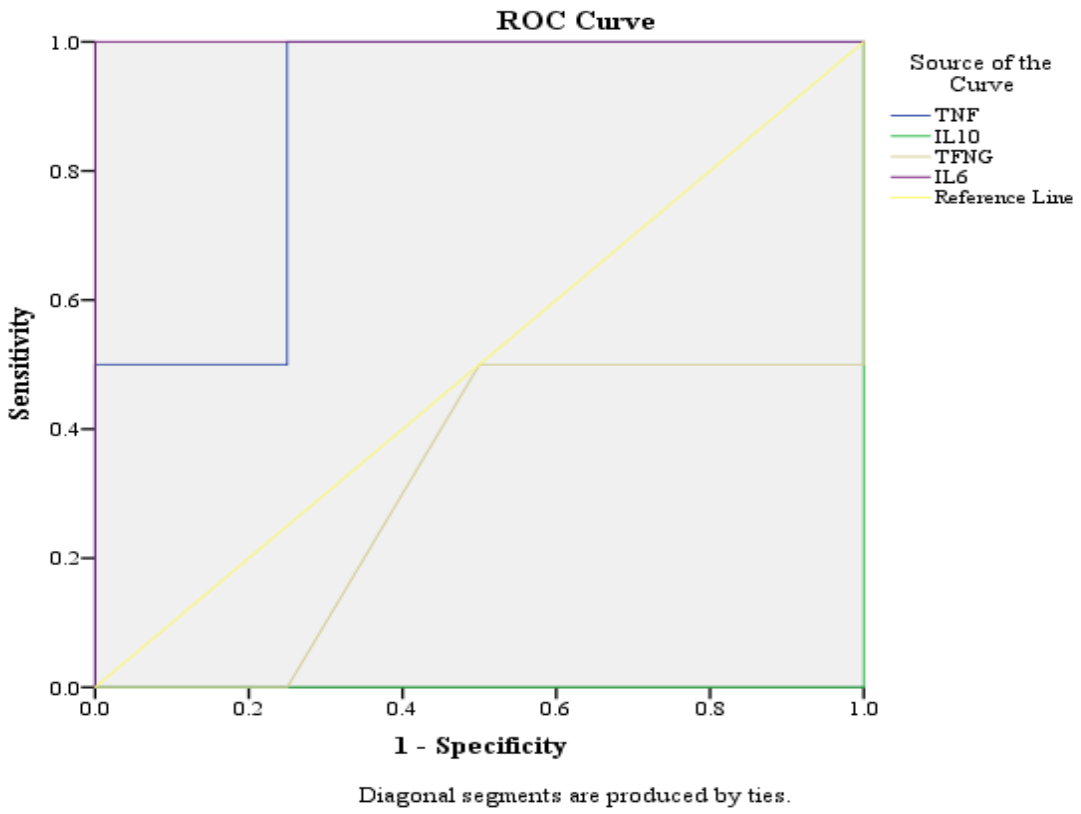

Figure 4.32 Area under the ROC curve IL-6, IL-10, IFN-γ, TNF-α between MDR-TB and TB

Table 4.7 Summary of AUC and p-values between MDR-TB and TB

Cytokine	TNF-α	IL-10	IFN-γ	IL-6
AUC	0.875	0.002	0.313	1.000
p-value	0.165	0.064	0.487	0.064

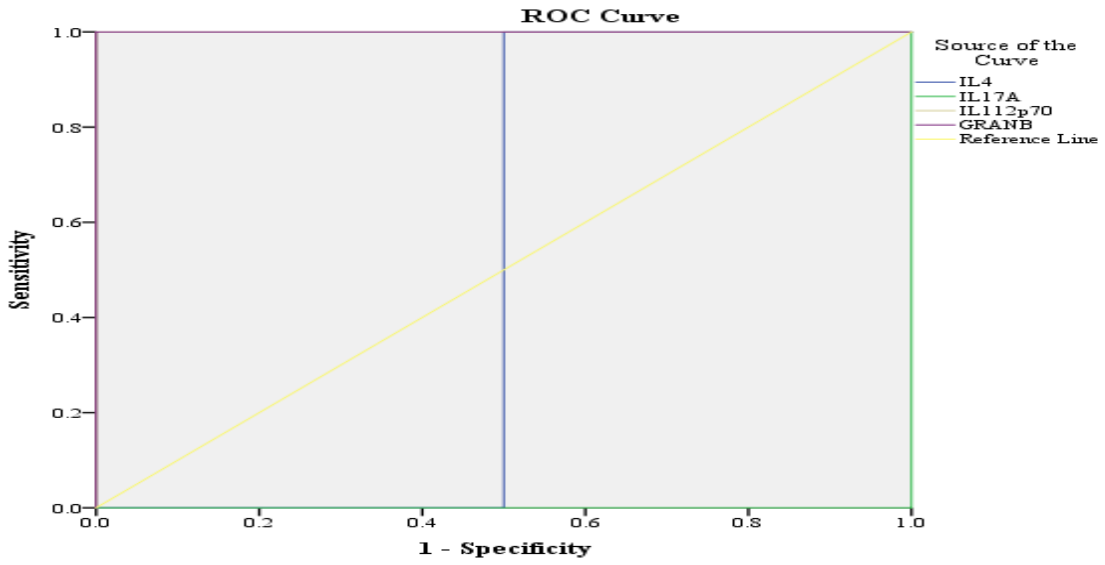

Figure 4.33 Area under the ROC curve for IL-17A, IL-4, IL-12p70 and Granz B (MDR-TB and TB).

Table 4.8 Summary of AUC and p-values between MDR-TB and TB

Cytokine	IL-4	IL-17A	IL-12	Granz B
AUC	**0.500**	**0.001**	**1.000**	**1.000**
p-value	**1.000**	**0.221**	**0.221**	**0.221**

4.3 Assessing the degree of relationship between cytokines

To assess the relationship between different cytokines, a Pearson test of correlation between the cytokines was performed. In general, there were significantly positive correlations between the pro-inflammatory mediators TNF-α and IFN-γ. IL-12 p70 had a virtually negative correlation with all other cytokines. Nonetheless there were other exceptions as shown in Table 4.6

TABLE 4.9 Spearman rho correlations between cytokines.

Correlations

		TNF-a	IL6	IL10	IFN-g	IL4	IL17A	IL112p70	GRANB
TNF	Pearson Correlation	1	.415**	-.013	.357*	.434*	-.046	-.072	.075
	Sig. (2-tailed)		.004	.969	.020	.019	.842	.684	.653
	N	48	47	12	42	29	21	34	38
IL6	Pearson Correlation	.415**	1	.258	.189	-.084	.032	-.036	.058
	Sig. (2-tailed)	.004		.395	.194	.659	.886	.808	.690
	N	47	62	13	49	30	23	49	49
IL10	Pearson Correlation	-.013	.258	1	.398	.139	.538	-.033	.619*
	Sig. (2-tailed)	.969	.395		.178	.683	.462	.920	.024
	N	12	13	13	13	11	4	12	13
IFNG	Pearson Correlation	.357*	.189	.398	1	.148	.814**	-.164	.329*
	Sig. (2-tailed)	.020	.194	.178		.436	.000	.338	.038
	N	42	49	13	51	30	23	36	40
IL4	Pearson Correlation	.434*	-.084	.139	.148	1	-.005	-.128	.312
	Sig. (2-tailed)	.019	.659	.683	.436		.984	.613	.168
	N	29	30	11	30	31	18	18	21
IL17A	Pearson Correlation	-.046	.032	.538	.814**	-.005	1	-.152	.036
	Sig. (2-tailed)	.842	.886	.462	.000	.984		.638	.898
	N	21	23	4	23	18	25	12	15
IL112p70	Pearson Correlation	-.072	-.036	-.033	-.164	-.128	-.152	1	-.062
	Sig. (2-tailed)	.684	.808	.920	.338	.613	.638		.684
	N	34	49	12	36	18	12	49	46
GRANB	Pearson Correlation	.075	.058	.619*	.329*	.312	.036	-.062	1
	Sig. (2-tailed)	.653	.690	.024	.038	.168	.898	.684	
	N	38	49	13	40	21	15	46	50

*. Correlation is significant at the 0.05 level (2-tailed).

CHAPTER FIVE

DISCUSSION

5.1 Factors associated with MDR TB

The increasing rate of MDR-TB is a subject of concern for global TB control. This surge is seen among both new and previously treated cases (Faustini et al., 2005). Low socio-economic indicators have been implicated as risk factors for tuberculosis but their role in MDR-TB is not clear (Flora et al., 2013). None of the socio-economic variables (educational status, marital status, smoking, alcohol intake, and occupation) varied significantly between TB and MDR-TB cases. A study to compare MDR-TB risk factors in a tertiary hospital in Peshawar (Pakistan), also reported similar results (Wahab et al., 2013). In a study in Thailand, improvement in educational level positively correlated with a decrease in MDR-TB but no significant association was observed (Anunnatsie et al., 2005). In contrast to our results, majority of MDR-TB cases in Scotland were unemployed (Anderson et al., 2009) indicating an association between MDR-TB and educational status. In this study younger age groups (Average age=46 years) were more likely to have MDR-TB than their older counterparts similar to a study by Flora et al., (2013) in Bangladesh.

The numbers of MDR-TB cases under DOTS were significantly higher (85.7%) as compared to TB cases (32%). It can therefore, by inferred that MDR-TB patients because of their status, tend to receive strict DOTS care. By extension it can also be explained that the relatively higher (68%) TB cases who are not under DOTS may serve a contributory factor to developing MDR-TB. Hence strict adherence to the DOTS guidelines right from the first day of diagnosis would help reduce the rate of MDR-TB (Rahman et al., 2005, Banu et al., 2010). A meta-analysis of MDR-TB risk

factors in Africa showed low prevalence in higher DOTS coverage areas compared to those countries with poor DOTS coverage (Viswanathan et al., 2008).

From this study, it is clear that previous treatment is a significant determinant of MDR-TB as all the MDR-TB cases had previously been treated for TB. This finding lends credence to the view that MDR-TB is predominantly caused by the selection of strains induced by previous treatment (Faustini et al., 2006). This view is also supported by a study in Burkina Faso which demonstrated that inadequate or inappropriate previous treatment promotes survival and increase in resistant strains within the quasi species (Diande et al., 2006).

Multivariate biomarkers have been investigated over the years from high-throughput data with the aim of discovering a potential biosignature with the ability to shorten clinical trials of new TB vaccines, monitor treatment outcomes as well as provide the basis for better diagnostic tools to determine TB progression (Ansari et al., 2009, Djoba et al., 2009, Chegou et al., 2009). There are diverse schools of thought regarding the variations of cytokine profiles in various stages of TB pathogenesis, thus the discovery of suitable and validated biomarkers in that regard ought to be further investigated. To this end, this study sought to assess the variations in cytokine profiles among active TB, multi-drug resistant and individuals with no TB.

5.2 Dynamics of cytokine profiles in different TB population groups

The results from this study have revealed several differential patterns in TNF-α, IFN-γ, IL-17A, IL-4, IL-6, IL-10, Granzyme B & IL-12 p70 profiles that merit attention. In general, the results indicate that serum cytokine levels for each cytokine vary among active tuberculosis, MDR-TB and the control groups. The results show significantly increased levels of TNF-α, IFN-γ, IL-10, IL-4 and IL-6 between active TB and healthy controls, and between MDR-TB and healthy

controls. There was no significant variation among the three different population groups with respect to Granzyme B, IL-12p70 and IL-17A. In addition, no significant difference is observed for all the eight cytokines between active TB and MDR-TB cases.

5.2.1 Interferon gamma (IFN-γ)

Amongst the most extensively studied cytokines with respect to TB pathogenesis is IFN-γ. Recent studies on IFN-γ have reported its proinflammatory role in addition to it serving as the major activator of macrophages towards protective immune response (Sauzuloo et al., 2016, da Silva et al., 2013, Cavalcanti et al., 2012). Immunodiagnostic tests for tuberculosis are based on the estimation of IFN-γ as in IFN-γ release assays (Sarkar et al., 2016).The study reveals that, serum levels of IFN-γ are higher in active TB and MDR-TB cases compared to healthy controls. This difference in serum cytokine concentration level is significant between healthy controls and active TB cases ($p=0.0001$) as well as MDR-TB cases ($p=0.01$). The level of significance is higher with the active TB cases than the MDR-TB cases as evident from their p-values. Similarly, Fatima et al. (2016) reported a significantly higher IFN-γ level in MDR-TB cases compared to healthy controls ($p=0.001$). In another study using serum samples from TB patients, a statistically significant difference between the levels of IFN-γ of TB patients and healthy controls were observed (Chadrashakara et al., 2015). Increased levels of IFN-γ in acute and chronic TB patients (Handzel et al., 2007) relative to healthy controls have also been reported from a study in Ethiopia. These and many other studies explain and further affirm the protective inflammatory role of IFN-γ in TB pathogenesis.

In contrast, results from other groups have demonstrated that cell-stimulated IFN-γ production from peripheral blood mononuclear cells (PBMCs) extracted from active TB cases secrete lower levels of IFN-γ following stimulation with mycobacterial antigens (Silva et al., 2013, Sahiratmadja

et al., Pereira et al., 2004, Vankayalapati et al., 2003). Lee and his colleagues showed that IFN-γ production was significantly reduced in MDR-TB cases compared to healthy tuberculin reactors following stimulation with purified protein derivative (PPD). This reduced IFN-γ production by PBMC is likely to be due to T cell exhaustion or due to sequestration of IFN-γ positive CD4 and CD8 T cells to the sites of infection (the lungs) during the acute phase of the disease. The significant increase in IFN-γ levels between active TB, MDR-TB and healthy controls in this study could probably be as a result of hyperactivation of macrophages to contain the bacilli in the face of high mycobacterial burden.

Our findings further suggest no significant difference between the IFN-γ levels in active TB and MDR-TB cases which is in conformity with a study by Shahemabadi and colleagues. This was also reflected in a study with whole blood after stimulation with culture filtrate protein of Mtb (Eum et al., 2008).

5.2.2 Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor alpha has been shown to act in synergy with IFN-γ to provide protective immunity to TB infection. Results from this study show elevated levels of TNF-α in active TB and MDR-TB cases compared to healthy controls. The variation in TNF-α levels between active TB and healthy controls is highly significant ($p=0.00001$), likewise the difference between the MDR-TB cases and healthy controls ($p=0.0001$). Similar findings were reported from a study in India (Fatima et al., 2016). This may be attributed to the fact that active diseased state under drug pressure may have necessitated elevated TNF-α level in the quest to control disease progression by activating more immune cells (macrophages, T. lymphocytes, dendritic cells) to the focal point of infection (Mohan et al., 2001; Sanga et al., 2000). However, a similar study in India reported

no significant variation in TNF-α level between TB patients and healthy controls (Chandrashekara et al., 2015).

5.2.3 Interleukin-6 (IL-6)

In this study, high levels of IL-6 were observed in sera of active TB and MDR-TB cases compared to healthy controls. There was a significant difference in IL-6 levels between active TB cases and healthy controls (p=0.001). The findings are consistent with studies by Correia et al (2009). However, Handzel et al found no significant difference in IL-6 levels between chronic and acute TB patients as well as between TB contacts and healthy controls. This may have resulted from the difference in comparable groups. Interestingly, the difference in serum IL-6 levels between MDR-TB cases and healthy individuals is not statistically significant as observed by Correia et al (2009) between drug susceptible TB and MDR-TB groups. Thus elevated IL-6 levels in pulmonary TB may be independent of multi-drug resistance. The robust increase observed in this study suggests that IL-6 contributes to the inflammatory activity in the TB patients, in accordance with its proinflammatory potential in experimental models of acute infection (Poveda et al., 2001).

5.2.4 Interleukin-10 (IL-10)

IL-10 is critical for the regulation of host defense against Mtb infection. There is conflicting evidence regarding the immunomodulatory role of IL-10 and its potential to favour disease progression by antagonizing Th1 cytokine production such as IFN-g and TNF-a (Jankovic et al., 2007, Mocellin et al., 2004). This study reports significantly elevated serum IL-10 level in active TB cases compared with levels in healthy individuals (p=0.01). It has been reported that patients with advanced disease have increased IL-10 levels (Handzel et al., 2007, Vankayalapati et al., 2003). A report by Fatima et al made an interesting contribution in this regard (Fatima et al., 2016). However from this study, no significance variation is observed between MDR-TB cases and

healthy individuals as well as between active TB and MDR-TB cases. Although statistically insignificant, the levels of IL-10 in this study are lower in MDR-TB compared to active TB cases This confirms a recent report from Ukraine indicating significantly lower serum IL-10 levels in MDR-TB group than that in the healthy controls (Butov et al., 2016).

The observed increase in IL-10 levels in active diseases state from this study suggests probable immunoregulatory role against the deleterious effect of the elevated IFN-γ and TNF-α levels through inflammatory reactions. Thus buttressing the argument by Silva et al (2015) that the relationship between IL-10 (anti-inflammatory cytokine) and IFN-γ, TNF-α (pro-inflammatory cytokine) is not necessarily an antagonistic one as already believed but rather appears to act in complementary fashion (Jankovic et al., 2007).

5.2.5 Interleukin-4 (IL-4)

IL-4 is a Th2 cytokine believed to be an indicator of weak cytotoxic activity detected in active and MDR-TB patients (Geffner et al., 2009).The study reports a significance difference in IL-4 levels between active TB and healthy controls ($p=0.01$). The study results also shows a highly significant difference in IL-4 levels between MDR-TB and healthy controls ($p=0.0001$) with no statistically significant difference observed between active and MDR-TB cases. This elevation between MDR-TB and healthy controls relative to active TB and healthy individuals point to the fact that high IL-4 levels favour disease progression. A report from India investigating serum concentration of IL-4 showed significant elevation in MDR-TB cases compared to healthy controls($p<0.001$) as observed in our study and other studies by Rook et al (2005) and Smith et al (2004). Considering these changes, we can assume a decreased Th1-lymphocyte activity in these groups (active TB and MDR-TB). It may also indicate certain stabilization in patients with pulmonary TB taking into account the fact that serum IL-4 level is still significantly higher compared to healthy subjects.

Notably, however, recent work by Chandrashekara and colleagues observed no significant difference with respect to IL-4 serum levels among TB patients, TB patient contacts and healthy controls.

5.2.6 Interleukin-12p70, Interleukin-17A, Granzyme-B

In this study, there is no significant difference betweenIL-12p70, IL-17A and Granzyme B in any of the three groups. Granzyme B mediates cytolytic activity of CD8+ T cells aimed at killing mycobacterial infected cells (Afonina et al., 2010). It initiates apoptosis through a perforin-dependent mechanism. Our results did not show any significant difference between any two of the three groups. A recent study suggests that granzyme B levels may not be the best proxy for estimation of cytolytic activity because a moderate decrease in granzyme B did not correlate with reduced in-vivo killing (Booty et al., 2016). Also in line with our results, Garcia-Laorden et al. (2015) and Brahmbhatt et al. (2006) reported that TB patients had a slightly elevated expression of granzyme B compared to healthy controls but no statistical significance was observed. Jiang et al. (2017) showed a similar result that a slight increase in the expression of granzyme B in CD8 + T cells from TB patients compared with healthy controls but no statistical significance was observed. However, contradictory reports have also been published showing statistically significant granzyme B downregulation in active TB disease compared to healthy controls (Silva et al., 2014).

Activation of naïve T cells, in the presence of IL-6 and TGF-b directs the differentiation into Th17 cells which in-turn produces IL-17A (Yang et al., 2011). It has been suggested that the Th17 response from which IL-17 emanates, is indispensable for protection against Mtb infection (Khader et al., 2005). IL-17 triggers recruitment of neutrophils to the lung and facilitate granuloma formation. Hence it is expected that the high level of IL-6 observed in active and MDR-TB patients

compared to healthy controls would directly correlate with the level of IL-17A. However this was not the case in this study as no significance difference is observed between any of two of the three groups. It has been reported that cross-regulation of Th1 and Th17 populations is essential is crucial for conferring a protective effect against Mtb without excessive damage (Wonziak et al., 2014). This was supported by Nandi et al. (2011) who stated that IFN-γ inhibits IL-17 production. Therefore, by extension, the no significance difference in IL-17A levels may be probably explained as due to the inhibitory effect of elevated IFN-γ levels.

5.3 Predictive ability (sensitivity and specificity) of cytokines as biomarkers in MDR

Mtb infection triggers a complex interaction of inflammatory mediators such as cytokines. Cytokine specific immune responses to different categories Mtb antigens have been demonstrated in several reports (Zyl-Smith et al., 2009, Djoba et al., 2009). Muller et al 2010 reported the utility of Interferon Gamma Release Assay (IGRA) for the rapid diagnosis of latent tuberculosis (LTBI) as encouraging. Although the usefulness of IGRA is traditionally ascertained and recommended, it cannot be used to distinguish LTBI from active disease or MDR-TB (Dheda et al., 2009).

Cytokines can be useful in the design of rapid immunodiagnostic tool for MDR TB but there is the need to assess the potential of many different cytokines in different combinations to discover a cytokine profile with a high predictive value. Such studies ought to be conducted in different parts of the world such as any population based differences would be uncovered. In this study, Granzyme B, IL-4 and TNF alpha singly show a relatively greater potential (AUC 72.8% p=0.009, 82.7% p=0.009, 75.9% p=0003) to discriminate among the different population groups. IL-10 had the lowest (AUC 36.6% p=0.554) predictive potential. This observation is partly attributed to the least number of readable values recorded (Table 4.3).

5.4 Degree of relatedness among cytokines

Cytokines are interrelated, some are pleomorphic whiles the presence of others may either stimulate or inhibit the activities of others. Due to these dynamics of synergistic and antagonistic effect of cytokines, we investigated the mutual relatedness of the cytokines. In general, there were significantly positive correlations between the cytokines except for IL-12p70 which shows no positive correlation with other cytokines. Nagabhushaman and colleagues also identified strong positive correlation between IL-6 and IL-4 (Nagabhushaman et al., 2003) and IFN-γ and TNF alpha (Geffner et al., 2009). The study also shows a negative correlation between IL-10 and TNF, IL-6 and IL-4, IL-10 and IL-12p70 thus indicating the antagonistic role of the pairs of cytokines. In addition, a significant positive correlation is observed within pro-inflammatory cytokines and anti-inflammatory cytokines. Moreover, whiles some pro-inflammatory and anti-inflammatory cytokines records negative correlation, others show a rather positive correlation. This indicates and supports the assertion by Silva et al., 2015 that contrary to what is generally believed, cytokine interplay in TB pathogenesis appear to act in complementary form with reference to the specific clinical state of the individual. The observed dynamics in the correlation of cytokines indicates that combination of cytokines may be useful in partitioning the population groups.

The degree of the sensitivity of a specific cytokine as observed from the area under ROC curve varies greatly between any two of the three groups and among all the groups. Statistically significant sensitivity patterns are observed for all the cytokines comparing MDR-TB and negative controls except for IL-17A, IL-10 and IL-12p70. The AUC of active TB and negative controls follows a similar pattern excluding IL-17A and IL-12p70. Similar findings were reported by Nazish et al., (2015).In Mtb infection, production of IL-12p70 is one of the earliest events in the activation of cell mediated immunity (Torres et al., 1998) hence may not be an excellent predictor

in advanced stages of Mtb infection. In MDR-TB and active TB, all cytokines show no significant sensitivity (p=0.0001) although the AUC for IL-12p70A, TNF alpha, IL-6 and Granzyme B were very high (AUC 100%, 87.5%, 100%, 100% respectively).

5.5 Limitations of the study

There are some limitations of the study that merits discussion. These include the small sample size (n=62, MDR-TB=21, TB=25, Negative control=18) coupled with the uneven number in each group. In addition, despite diluting the samples prior to analysis, some of the cytokine concentrations were still above detectable limits of the kits making direct comparison of results challenging. It would have been interesting to follow and take samples from one participant several times throughout the defined period of treatment (longitudinal study). However, due to time constraints this could not be done.

Despite these shortcomings, the results indicate that cytokine profiles could offer baseline information needed to assess treatment response, manage and monitor therapeutic success.

CHAPTER SIX

6.0 CONCLUSION

It is evident that Mtb infection is a dynamic process with wide range of pathology. The balance between resistance and susceptibility to infection with Mtb comes with diverse controversies over the cytokine profiles of T helper cell populations. Although the complete mechanism of immune response to TB infection is complex and poorly understood, an improved understanding of the immunopathogenesis of TB would facilitate the drive towards development of rapid diagnostic tools for monitoring and management of treatment. In order to avoid increasing number of MDRT-TB cases, there is an urgent need for monitoring throughout the treatment course.

The altered stability in the level of serum cytokine concentrations observed in this study among different groups demonstrate that measuring cytokines may be useful as potential biomarker for MDR-TB and TB diagnosis as well as for the management and monitoring of differential treatment responses. The distinct serum IFN-γ, IL-4, IL-6, IL-10 and TNF-α levels in MDR-TB/TB and negative controls suggest their utility in TB diagnosis and management. The findings of this study further reveals that the diagnostic performance of Granzyme B, IFN-γ, IL-4, IL-6, and TNF-α to discriminate between MDR-TB and No TB were similar to that of TB and No TB. In addition, the utility of multiple cytokine responses in discriminating among MDR-TB, TB, and No TB individuals may be more sensitive compared to the diagnostic value of a single cytokine.

6.1 RECOMMENDATIONS

Given the considerable variations in cytokines amongst individuals as has been observed in this study, using cytokines as diagnostic biomarkers for MDR or to monitor anti-TB treatment would require studies in large populations with a wider range of cytokines. Future studies should consider longitudinal studies with larger sample size for biomarker identification. This would provide a clear picture of the cytokine dynamics in the face of anti-TB treatment.

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