THE ROLE OF CYTOKINE IMMUNE RESPONSES IN PULMONARY TUBERCULOSIS IN PATIENTS CO-INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS

BY

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DECLARATION

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DEDICATION

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ABSTRACT

The gold standard for pulmonary tuberculosis (PTB) diagnosis is either isolation of *Mycobacterium tuberculosis* (*Mtb*) by culture or detection of *Mtb*-specific nucleic acids by molecular methods. However, apart from laboratory infrastructure, culture take longer time to get results, while molecular methods are expensive and require a lot of human expertise. Despite its limitation of lower specificity and sensitivity, Acid Fast Bacilli (AFB) microscopy smear remains the most widely used and cost effective laboratory diagnostic technique for PTB diagnosis in low-and-middle income (LMIC) countries. The use of Interferon Gamma Release Assays (IGRAs) have shown promise in diagnosis of TB in immune-competent as compared to immunocompromised individuals. However, IGRA is limited in distinguishing between active and latent TB. Therefore, measuring of *Mtb* antigen-specific immune responses and cytokines levels produced by T cell after exposure to the pathogen would be useful. As such, the overall aim of this study was to evaluate pulmonary tuberculosis smear status with cellular immune profile and cytokine response in adult patients with pulmonary tuberculosis (PTB) co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital (MTRH). The specific objectives were to: compare Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine responses with culture status in patients with PTB co-infected with HIV; determine diagnostic accuracy of Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine responses, and to assess the cofounding effect of lymphocytes in the production of these cytokines, in AFB microscopy smear negative patients with PTB co-infected with HIV and relate T cells (CD3/CD4/CD8), B cells (CD 19), NK cells (CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) cytokines with sputum smear status in patients with PTB co-infected with HIV in AMPATH, MTRH. Study participants were adults with HIV attending TB clinic within AMPATH. In a longitudinal study, a total of 86 study participants were recruited: 46 culture-negative and 40 culture-positive. Blood and sputum samples were collected from all the study participants. The blood samples were analyzed using a four color FACSCalibur flow cytometer to determine immunophenotypic characteristics, and the same samples cultured and supernatant (plasma) harvested to evaluate cytokine profiles using Enzyme-Linked Immunosorbent Assay (ELISA). Sputum samples were analyzed to determine AFB smear status using direct microscopy and Lowenstein Jensen medium. Statistical analyses were performed using R software. The overall mean age of the participants was 39 years (SD=12), with 48.8% being male. Although, lymphocytes counts were higher in culture-positive relative to culture-negative, the CD8, CD19, and CD16/CD56 were comparable in the two groups. The CD4 counts differed between the two groups (P=0.012). The Th1 cytokines showed a better discrimination between culture-positive and culture-negative PTB individuals; IFN-γ (P=0.001), TNF-α (P=0.001), IL-2 (P=0.001) and IL-12(p70) (P=0.016). The Th2 cytokines (IL-4, IL-6 and IL-10) were comparable between the culture-positive and culture-negative groups. When, PTB culture-positive (AFB microscopy smear negative) and PTB culture-negative (AFB microscopy smear negative) cytokines median levels were compared; IFN-γ (P<0.001), TNF-α (P=0.004), IL-2 (P=0.004) and IL-4 (P=0.009) were elevated. Lastly, when Th1 cytokines (IFN-γ, TNF-α and IL-2), Th2 cytokines (IL-6 and IL-10) and T cells were included in the logistic regression fit for PTB outcome, the predictive power of discriminating between those who were AFB smear negative in the diagnosis of PTB was better with cross-validated area under the curve (AUC) being 0.87 (95% CI: 0.78, 0.96). A direct correlation was reported between, IFN-γ and IL-2 cytokines (r=0.48, $P<0.001$, power = 99.1%); IL-2 and TNF-α (r=0.27, P=0.22, power=62.6%); IL-4 and IL-10 (r=0.29, P=0.015, power= 69.1%) cytokines; and IL-6 and TNF-α cytokines (r=0.68, P<0.001, power=97.3%), a concomitant increase in one cytokine resulted in an increase of the other when analyzed by Pearson correlation. On the contrary, an inverse correlation was observed between median levels of; TNF-α and IL-8 cytokines in (r=-0.41, P=0.022, power=93.9%); and IL-6 and IL-8 cytokines (r=-0.44, P<0.001, power>99.9) demonstrating an antagonistic relationship between the two sets cytokines. This study provided evidence for the ability of combination of Th1 cytokines in discriminating against culture-positive and culture-negative PTB, Th1 and Th2 cytokines to determine PTB status in AFB microscopy smear negative patients co-infected with HIV.

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LIST OFABBREVIATIONS AND ACRONYM

AFB – Acid Fast Bacilli

AIDS – Acquired Immunodeficiency Syndrome

AMPATH – Academic Model for Providing Access to Healthcare

ART – Antiretroviral Therapy

CBC – Complete Blood Count

CD – Cluster of Differentiation of antigen

CFP-10 – Culture Filtrate Antigen

EDTA – Ethylene Diamine-Tetra-Acetic Acid

ELISA – Enzyme-Linked Immunosorbent Assay

ELISPOT – Enzyme-Linked Immunospot

EPTB – Extra Pulmonary Tuberculosis

ESAT-6 – Early Secreted Antigen Target

HIV – Human immunodeficiency Virus

IL – Interleukin

IFN-γ – Interferon gamma

iNOS – Inducible Nitric Oxide Synthase

IREC – Institution of Research and Ethics Committee

MC – Mast Cell

MCP – Macrophage Chemoattractant protein

MGIT – Mycobacterium Growth Indicator Tubes

MHC	–	Major Histocompatibility Complex
Mtb	–	Mycobacterium tuberculosis
MTRH	–	Moi Teaching and Referral Hospital
NK	–	Natural Killer Cell
OI	–	Opportunistic Infection
PCR	–	Polymerase Chain Reaction
PTB	–	Pulmonary Tuberculosis
RANTES	–	Regulated upon Activation Normal T cell Expressed and Presumably Secreted Chemokine
RNI	–	Reactive Nitrogen Intermediates
TB	–	Tuberculosis
Th1	–	Type 1 helper T cell
Th2	–	Type 2 helper T cell
TNF	–	Tumour Necrosis Factor
TST	–	Tuberculosis Skin Test
UNAIDS	–	United Nation Programme on HIV/AIDS
WHO	–	World Health Organization
XDR	–	Extensively Drug Resistant Tuberculosis
α	–	Alpha
β	–	Beta
δ	–	Delta

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) is the most common opportunistic infection in persons with human immunodeficiency virus or acquired immunodeficiency syndrome (HIV/AIDS) (Badri, Ehrlich, Wood, Pulerwitz, & Maartens, 2001). TB is the leading cause of mortality and morbidity in persons with HIV/AIDS (WHO, 2015). Previous studies have revealed that one in every three person who died of AIDS was due to TB infection (UNAIDS/WHO). Therefore delayed diagnosis of TB and initiation of treatment after more than three weeks of presentation is responsible for 45-85% of deaths reported among HIV infected patients (Barnes, Bloch, Davidson, & Snider, 1991). It is thus important that emphasis is put on early detection and accurate diagnosis of the *Mycobacterium tuberculosis (Mtb)* among HIV-infected patients. This will go a long way in assisting in better clinical management and control of this disease and eventually improvement on the survival rates among HIV/TB co-infected patients.

TB presentation in HIV-infected individuals with well-preserved immunity is similar to that in immune-competent individuals without HIV. The progression of immunodeficiency, attenuation of host tissue-damaging responses and failure of MTB containment result in an increased likelihood of atypical presentation, and greater proportions of extra-pulmonary and disseminated disease (Lawn, Butera, & Shinnick, 2002). Sputum samples should be examined even if the chest radiograph appears normal. This is because pulmonary cavitations are less common in HIV-positive patients, leading to significantly reduced sensitivity of sputum microscopy for AFB. Therefore, it is necessary to further examine the sputum samples as culture still remains the mainstay of diagnosis in such setups, with limitation of being very expensive and taking a longer period to get results.

Cellular and humoral immune responses play a crucial role in controlling *M. Tuberculosis* replication. The CD4 T cells through interferon gamma (IFN-γ) responses facilitate the cytotoxic functions of CD8 T cells in the host to prevent the development of *M. Tuberculosis* disease (Green, Difazio, & Flynn, 2013). The Th1 CD4 T cells play an important role in protection against TB by promoting activation of macrophages through the production of IFN-γ, IL-2 and Tumor Necrosis Factor alpha (TNF-α) (Caruso *et al*., 1999; Zhu, Yamane, & Paul, 2010). Thus, it has been postulated that perturbation in Th1 cytokines activation mechanism may lead to enhanced development of TB (Ottenhoff, Kumararatne, & Casanova, 1998).

The CD4 depletion during the course of HIV or functional neutralization of TNF-α as a result of anti-TNF-α antibodies increases the risk of an individual to develop TB (Keane *et al*., 2001). In addition, the differential expression of the Th1 cytokines (such as TNF-α, IFN-γ, IL-2, IL-8 and IL-12p70) as well as Th2 cytokines (such as IL-10, IL-6 and IL-4) are associated with active TB (Cooper, 2009). Therefore, there is need to have distinct cytokine profiles in order to diagnose active TB. Hence, the present study was designed to evaluate the relationship between Th1 [IFN-γ, TNF-α, IL-2, IL-8 and IL-12(p70)] and Th2 (IL-4, IL-6, and IL-10) cytokine responses in culture smear-positive and -negative PTB patients co-infected with HIV.

Molecular assays show promise in the rapid diagnosis of smear-negative disease with high sensitivity (Perkins & Cunningham, 2007). One of these tests is the Interferon Gamma Release Assay (IGRA). The challenge with IGRA is that it is dependent on T cell functionality and it is likely that such an assay might not work accurately in immune-challenged individuals, with infections such as HIV. However, the MTB-specific antigens present in IGRA test have great potential to stimulate T-cells to produce multiple cytokines, which could prove beneficial in the diagnosis of PTB among HIV co-infected patients. In

addition studies have demonstrated the inability of interferon gamma alone to accurately diagnose TB in HIV-infected patients (Huo & Peng, 2016; Syed Ahamed Kabeer *et al*., 2009). Furthermore, other studies have shown that IGRA as currently constituted, cannot be used alone to rule out active TB in HIV individuals, because it doesn't add value to the discriminating ability of TB screening clinical algorithms (Nemeth *et al*., 2012; Rangaka *et al*., 2012). Previous studies have shown the ability of Th1 and Th2 cytokines in discriminating between PTB culture-positive and -negative (Kellar *et al*., 2011; Wallis *et al*., 2010). Hence, the present study was designed to determine diagnostic accuracy of Th1 (IFN-γ, TNF-α, and IL-2) and Th2 (IL-4, IL-6 and IL-10) cytokine response in AFB microscopy smear negative PTB-HIV co-infected patients.

Mycobacteria tuberculosis interaction with the host immune system starts with the process of mycobacteria phagocytosis by macrophages leading to activation of the immune responses through the release of different cytokines and chemokines (Beetz *et al*., 2008; Cooper, 2009) . The interplay of Th1 and Th2 cytokines may indicate the level of host-*Mtb* interaction (e.g., presence or absence of the bacteria)(Cooper, 2009). The presence of *Mtb* will induce inflammatory responses that will be required to be regulated to avoid tissue damage. TNF-α, IFN-γ, IL-12, IL-8, IL-6, IL-4 and IL-10 are known either to potentiate the activities of each other so as to activate the immune response towards TB or providing a negative loop to other cytokines in order to regulate and avoid tissue damage (Caccamo *et al*., 2010; Cooper & Khader, 2008; X. Wang *et al*., 2015). Moreover, these cytokines are associated with bacterial loads as evident by their reduced levels in TB patients after completion of anti-TB treatment (Caccamo *et al*., 2010). Understanding the functions and interactions between cytokines and chemokines is therefore critical to our attempts to limit TB. This therefore presents a unique opportunity in understanding the different cytokine correlations during TB infection. As such, the current study correlated T cell (CD3/CD4/CD8), B cell (CD 19), NK cells (CD16/CD56)

and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) with sputum smear status in patients with PTB co-infected with HIV in AMPATH, MTRH.

1.2 Statement of the Problem

In resource constrained countries there has been emphasis on the use of Direct AFB smear microscopy and mycobacterial culture tests in identifying the presence of pulmonary TB. However, laboratory studies have either shown that these tests have low sensitivity ranging between 31-47 per cent or are expensive (Padmapriyadarsini, Narendran, & Swaminathan, 2011). The failure to rapidly detect or confirm TB in immunocompromised patients has crucial implication for both patient care and disease control. Nearly twenty percent of all patients with TB who have treatment initiated die within the first year, and about two thirds deaths occur within two months. This indicates the advance nature of TB infection at time of diagnosis. Furthermore, a study in AMPATH, MTRH has revealed TB as the leading cause of death in patients who die with HIV co-infection (Siika *et al.,* 2008). This also shows that the accuracy of initial diagnosis may be poor, therefore the need for a rapid point-of-care diagnosis and treatment initiation. In response to emergence of extensively drug resistant TB (XDR), strikingly early mortality due to TB in patients with HIV co-infection and the poor performance of microscopy, medical practitioners have resorted to presumptive treatment as a diagnostic method. The danger of this approach is wastage of drug resources, overburdened treatment programs, and mistreatment of many patients with other diseases rather than TB. Poor detection of tuberculosis infection and inefficient prevention of reactivation are major impediments to improved tuberculosis control. Hence there is need for improved screening tests with greater sensitivity and specificity for tuberculosis infection that do not require multiple patient encounters and that significantly improve TB control efforts. Therefore this study investigated pulmonary tuberculosis smear status with cellular immune profile and cytokine responses in adult patients with pulmonary tuberculosis co-infected with HIV in

AMPATH, MTRH. In order to provide one way of strengthening diagnosis of TB with the help of immune responses, in determination of smear status in pulmonary TB patient co-infected with HIV. The current findings would further contribute to a deeper understanding of immunological mechanisms involved in TB/HIV co-infection and thus facilitating efforts toward development of a better point-of-care diagnostic tests for PTB.

1.3 Objectives

1.3.1 Broad Objective

To investigate the relationship between pulmonary tuberculosis smear status with cellular immune profile and cytokine responses in adult patients with pulmonary tuberculosis co-infected patients with HIV in AMPATH, MTRH.

1.3.2 Specific Objectives

i. To compare Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine response with culture-positive and -negative in patients with PTB co-infected with HIV in AMPATH, MTRH.

ii. To determine the diagnostic accuracy of Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine responses, and to assess the cofounding effect of lymphocytes in the production of these cytokines, among AFB microscopy smear negative patients with PTB co-infected with HIV in AMPATH, MTRH.

iii. To correlate T cells (CD3/CD4/CD8), B cells (CD 19), NK cells (CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) within sputum smear status with PTB co-infected with HIV in AMPATH, MTRH.

1.4 Research Questions

1. What are the immune profile T cell profiles, B cells, NK cells, Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) cytokine in culture-positive and -negative patient co-infected HIV?
2. What is the diagnostic accuracy of Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine responses in AFB microscopy smear negative patients with PTB co-infected with HIV in AMPATH, MTRH?
3. What are the correlations between T cell (CD3/CD4/CD8), B cell (CD 19), NK cells (CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) and sputum smear status in patients with PTB co-infected with HIV in AMPATH, MTRH?

1.5 Significance of the Study

Increased TB case rates have been reported over the past decade in many countries in sub-Saharan Africa and this is largely attributable to the HIV epidemics thus, the need for better point-of-care diagnostic tests (Bernal-Fernandez *et al.,* 2006).

A test to rapidly diagnosis PTB in patients co-infected with HIV from an easily accessible compartment like peripheral blood will greatly improve the control and management of the infection. This will ensure efficient and affordable diagnostic tool to aid in the identification of TB disease in acid-fast bacillus smear negative patients and will help initiate targeted therapy in affected patients increasing the cost effectiveness. Therefore, the diagnostic methods of smear status in HIV/PTB co-infection was evaluated in relation to immune status (T cell profiles, B cells, NK cells) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) cytokine responses.

The findings from this study have demonstrated the crucial role of the immune responses in the pathogenesis of TB and hence provides an important link which could be explored in the diagnosis of PTB at the point of care.

CHAPTER TWO

LITERATURE REVIEW

Mycobacterium tuberculosis complex is the causative agent of tuberculosis (TB), one of the oldest diseases known to affect humans (Koch, 1882). It is classified in Mycobacteriaceae family and the order Actinomycetales (Brennan & Nikaido, 1995). Of the pathogenic species belonging to this complex, the most frequent and important agent of human disease is *Mtb.* The complex includes *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium pinnipedii, Mycobacterium caprae and Mycobacterium cannetti*. Although all *Mtb* complex members are obligate pathogens and cause TB, they exhibit distinct phenotypic characteristics and host range (M. Pai, Zwerling, & Menzies, 2008). *Mtb* is a rod-shaped, non-spore-forming, thin aerobic bacterium measuring about 0.5 µm by 3µm and it does not stain readily and are often neutral on Gram's staining. However, once stained, the bacilli cannot be decolorized by acid alcohol, a characteristic justifying their classification as acid-fast bacilli (AFB) (Brennan & Nikaido, 1995). Acid fastness is mainly due to the organisms' high content of long-chain cross-linked fatty acids, mycolic acid and other cell-wall lipids. Transmission usually takes place through the airborne spread of droplet nuclei produced by patients with infectious pulmonary tuberculosis (Raviglione, Gupta, Dye, & Espinal, 2001).

2.1 Epidemiology of Tuberculosis and HIV Infection

Tuberculosis is a major public health problem particularly in resource constraint countries. Each year, TB kills more than 2 million people (WHO, 2015). TB infection is estimated at 2 billion people worldwide. It is important to note that in 2017, a best estimate of 10.4 million incident cases of TB occurred (range 9.0-11.1 million) as previously reported (WHO, 2018). This large discrepancy between the number of infected individuals (on the order of billions) and the numbers of annual cases resulting in death (on order of millions) points to an

important fact about tuberculosis. Most individuals infected with *Mtb* do not progress to active disease. It is believed that only 5-10% of infected individuals develop active disease within two years of initial infection (Comstock, 1982). The vast majority are able to contain infection but not to clear it, achieving latent tuberculosis through mounting a successful cellular immune response (Singer & Kirschner, 2004). Reactivation occurs when the immune system is compromised in some way. This might be as a result of co-infection with HIV, abuse of alcohol or drugs, or simply waning immunity due to aging and exogenous re-infection (Singer & Kirschner, 2004).

The highest number of deaths due TB is in the WHO Africa region with estimated TB incidence on the raise. This is attributable to the high prevalence of human immunodeficiency virus (HIV) infection particularly in sub-Saharan Africa (WHO, 2018). An estimated 1.3 million deaths occurred among HIV negative incident cases of TB (20 per 100 000 population) in 2017, while an additional 300 000 deaths among incident TB cases among HIV were reported. This represents 23% of HIV-positive incident cases of TB. Although prevalence and mortality rates of tuberculosis is reducing globally in the six WHO regions. The targets of halving prevalence and death rates by 2015, compared with the baseline of 1990 have not achieved. Kenya is ranked 13th (WHO, 2018) with 0.13 million cases. The number of new sputum smear positive was 158 cases per 100 000 population per year in 2017, with 45 cases per 100 000 population per year recorded among TB co-infected with HIV (WHO, 2018). The mortality rate was 50% and 37% in all forms of TB (deaths per 100 000 pop/year) and TB co-infected with HIV, respectively.

2.2 Pathogenesis of *Mycobacterium tuberculosis*

The *Mtb* infection occurs mainly at the lung through the respiratory route, after successful penetration of the mucosal barrier. The bacteria associate with intraepithelial leukocytes from which it enters the draining lymph nodes then spreads from the initial site of infection in

the lung through the lymphatics or blood to other parts of the body (Fratti, Vergne, Chua, Skidmore, & Deretic, 2000; Pieters, 2001). Alveolar macrophages are the effector cells present at alveolar spaces in the lung (Frieden, Sterling, Munsiff, Watt, & Dye, 2003). Through the process of phagocytosis, macrophages are able to destroy the *Mtb* and prevent infection (van Crevel, Ottenhoff, & van der Meer, 2002).

The complement system also plays a role in the phagocytosis of the bacteria (Li, Petrofsky, & Bermudez, 2002). The complement protein C3 binds to the cell wall and enhances recognition of the mycobacteria by macrophages. Opsonization by C3 is rapid, even in the air spaces of a host with no previous exposure to *Mtb* (Ferguson, Weis, Martin, & Schlesinger, 2004*)*. The subsequent phagocytosis by macrophages initiates a cascade of events that results in either successful control of the infection, followed by latent tuberculosis, or progression to active disease (Frieden et al., 2003). The outcome is essentially determined by the quality of the host immune system and the balance that occurs between host immune mechanism and the invading mycobacterial pathogen (Ferguson *et al*., 2004; van Crevel *et al*., 2002). After being ingested by macrophages, the mycobacteria continue to multiply slowly, with bacterial cell division occurring every 25 to 32 hours (Ferguson *et al*., 2004). Irrespective of whether the infection is controlled or progresses, initial development involves production of proteolytic enzymes and cytokines by macrophages in an attempt to degrade the bacteria (Nicod, 2007; van Crevel *et al*., 2002). Released cytokines attract T lymphocytes to the site, the cells that constitute cell-mediated immunity (Ferguson *et al*., 2004).

Macrophages then present mycobacterial antigens on their surface to the T cells (van Crevel *et al*., 2002), as Mycobacteria continues to multiple within the host to a point where they can elicit a cell-mediated immune response. In immune-competent individuals the next defensive mechanism is the formation of granulomas around the *Mtb* bacteria (Rosenkrands *et al*., 2002). This is characterized by the accumulation of activated T lymphocytes and

macrophages, which creates an inhibitory micro-environment that restricts the replication and spread of the mycobacterial (Frieden *et al*., 2003; Nicod, 2007). The microenvironment facilitates the destruction of macrophages and produces an early solid necrosis at the center of the lesion often referred as caseous necrosis, however, the bacilli are able to adapt and survive by altering the phenotypic expression (Dheda *et al*., 2005; Li *et al*., 2002). Caseous necrosis is characterized by low oxygen levels, low pH, and limited nutrient which restricts further growth and establishes latency. In immunocompetent individual, the lesion will undergo fibrosis and calcification leading to control of infection (Dheda *et al*., 2005), while in the immune-compromised individuals, disease progression will likely to occur due to unsuccessful granuloma formation (Dheda *et al*., 2005; Li *et al*., 2002). The necrotic tissue will undergo liquefaction with the fibrous wall losing its structural integrity. Leading to the release of semiliquid necrotic material, which may drain into a bronchus or nearby blood vessel, leaving an air-filled cavity at the original site.

Mycobacterium is an intracellular pathogen that inhabits the host macrophages. Its success as a pathogen is due to the ability to survive within the macrophage. In these cells, it resides within early endosome-like phagosomes, which makes it resistant to the effect from the cell microbicidal activities and allowing it multiply (Pieters, 2001). The phagosomes containing mycobacterium are hampered in maturation and fail to fuse with lysosomes, which enable the cells to kill the bacillus (Fratti *et al*., 2000). The intralysosomal acidic hydrolases act when they are released from lysosomes to degrade the phagocytized microorganism only upon phagolysosome fusion. This is the reason why prevention of phagolysosomal fusion by mycobacterium has been one of the hypothesized mechanism deployed by the *Mtb* so as to survive within the macrophages (Raja, 2004).

2.3 Host responses against *Mycobacterium tuberculosis*

Humans exhibit a wide range of responses to *Mtb* (Frieden *et al*., 2003, Flynn, 2004). The main host immune response components against *Mtb* involves innate immunity (mainly macrophages and dendritic cells (DCs), T-cell mediated adaptive immunity (including $CD4^+$ and $CD8^+$ T cells) and production of cytokines (pro-inflammatory and anti-inflammatory) and chemokines. These responses are directed towards containing or eliminating the tubercle bacillus within the tissue of the host. The series of cytokines are derived from various host cells including mast cells, macrophages and T cells (Flynn, 2004; Kaufmann, 2002).

Mast cells (MC) are inflammatory cells typically found in the mucosa of the respiratory, gastrointestinal, and urinary tracts and near blood or lymphatic vessels (Teitelbaum *et al*., 1999). Since these sites are entry points of infection, mast cells are likely to be among the first inflammatory cells to interact with the invading pathogens (Abraham & Malaviya, 1997; Mekori & Metcalfe, 2000). Following activation, they release a number of pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α)], and interleukin-6 (IL-6), histamines, proteases, and inflammatory mediators (Abraham & Malaviya, 1997; Mekori & Metcalfe, 2000). Studies have shown that apart from well-known functions of mast cells, derived histamine that include inducing broncho-constriction, mucus secretion, increment of vascular permeability and edema production, they also have an important regulatory role in the immune response to *Mtb* (Munoz, Hernandez-Pando, Abraham, & Enciso, 2003). Mast cells are an important source of TNF-*α* following exposure to *Mtb* and they also produces IL-6, which has both pro- and anti-inflammatory properties, after activation by *Mtb* during early infection (Ladel *et al*., 1997; Schindler *et al*., 1990). This cytokine contributes to the host defense by activating neutrophils and stimulating the growth and function of T cells (Munoz *et al*., 2003). These mast cell mediators are crucial for mobilizing and recruiting various other inflammatory cells to the site of infection (Mekori & Metcalfe, 2000). Previous studies

have postulated that *Mtb* recombinant antigens and ESAT-6 have a potential to induce pro-inflammatory cytokines from mast cells during the early stage of the TB infection (Munoz *et al*., 2003). These cytokines (IL-6 and TNF-α) might play a critical role in discriminating between those with and without the disease (Munoz *et al*., 2003).

Another protective immune response is the production of the cytokine, interferon gamma (IFN-γ) by $CD4^+$ T helper 1 (Th1) type cells. $CD8^+$ T cells can also produce IFN-*γ* and TNF-α which work in synergy to activate the microbicidal mechanisms of macrophages (Flynn & Chan, 2001a, 2001b). In humans, some studies have suggested that $CD4^+$ T cells have additional cytolytic function in local immunity within the lung (Collins & Kaufmann, 2001). While innate immune responses predominate initially, subsequent recruitment of T lymphocytes to the lung is necessary in the containment of *Mtb* within granulomas (Giacomini *et al*., 2001; Saunders, Frank, Orme, & Cooper, 2000).

Macrophages infected with mycobacterium promote Th1 lymphocyte activity by inducing the release of IL-12 and IL-18 (Altare *et al*., 1998; Sugawara *et al*., 1999). These Th1 cytokines then drive macrophage activation by inducing the release of IFN-*γ* and TNF-*α* (Flynn *et al*., 1995; Jouanguy *et al*., 1996). Macrophage apoptosis occurs within the granuloma, which results in the extracellular vesicles production consisting of a different antigenic composition, including glycolipids and proteins. They are readily taken up by dendritic cells, which process and present their antigenic material in relation to MHC I and CD1, leading to the activation of CD8 (Kaufmann, 2002). This is essential in the protection against TB. Granuloma is a major histological feature of mycobacterial infection. It is a dynamic entity, with macrophages dying and being replaced by newly activated monocytes. Similarly, lymphocytes are continuously replaced as cell mediated immunity improves the specificity of cells towards a pathogen (Cree, Nurbhai, Milne, & Beck, 1987). Both apoptotic macrophages and lymphocytes can be seen within the granuloma. Macrophage apoptosis is beneficiary to

the host since macrophages undergoing apoptosis kill intracellular mycobacteria (Fairbairn, 2004). A major communication between infected macrophages and the adaptive immunity is via MHC-2 antigen presentation to $CD4^+$ T lymphocytes (Fairbairn, 2004).

2.4 Specific Roles of Immune Cells and Cytokines in *Mycobacterium tuberculosis*

Studies in animal models and in humans have demonstrated a wide range of immune components involved in an effective immune response against *Mtb*. These include macrophages and dendritic cells, B cells, α/β-T cells (both $CD4^+$ and $CD8^+$), CD1 restricted T cells, $\gamma\delta$-T cells, cytotoxic T cells, and cytokines that are produced by these immune cells (Beetz *et al*., 2008).

The role of B cells against tuberculosis infection is less clear, and there are few studies describing their function in TB. The B cells play a role in the development of pulmonary granuloma and act as antigen presenting cells during mycobacterial infections, however, the mechanism involved remains unknown (Bosio, Gardner, & Elkins, 2000). B cells are thought to participate in the control of mycobacterial infection by producing natural antibodies (C. M. Johnson et al., 1997). In a study carried out in Mexico, there was a correlation between clinical manifestations of pulmonary TB with low B cell counts. It was proposed that B cell could be a useful marker for pulmonary tuberculosis (Hernandez *et al.*, 2010). Therefore, B-cells might represent an additional target for cellular control of *Mtb* infection and thus additional efforts should be devoted to understanding the role of B cells in immunity to TB.

$CD4^+$ T cells and IFN-γ play a primary role in the immunity against TB. Although $CD4^+$ T cells along with $CD8^+$ T cells and NK cells are the major producers of IFN-γ, studies in $CD4^+$ deficient mice have shown that it is the early production of IFN-γ by $CD4^+$ T cells and subsequent activation of macrophages that determine the outcome of infection (Cooper, 2009). The $CD4^+$ T cells also play other roles in the defense against infection that is independent of IFN-γ production. Depletion of $CD4^+$ T cells is associated with the

reactivation of infection in chronically infected mice resulting in increased pathological features and death, even though IFN-γ levels is still high due to a strong response from $CD8^+$ T cells and normal levels of inducible nitric oxide synthase (iNOS) (Scanga *et al.*, 2000).

$CD4^+$ T cells carry out several functions that are important in the control of TB infection in the granuloma by facilitating apoptosis of infected macrophages through Fas/Fas ligand interaction. They induce the production of pro-inflammatory cytokines such as IL-2, IL-6, IL-8 and TNF-α and immunoregulatory cytokines such as IL-10, IL-12, and IL-15 by other immune cells such as macrophage, this is effected through the direct contact via CD40 ligand (Oddo *et al.*, 1998). $CD4^+$ T cells also appear to be critical in the cytotoxic function of $CD8^+$ T cells which is mediated by IL-15 (Serbina, Lazarevic, & Flynn, 2001). It has also been shown that $CD4^+$ T cells can control the intracellular growth of *Mtb* by a nitric oxide-dependent mechanism that is independent of IFN-γ production (Cowley & Elkins, 2003).

In addition to producing IFN-γ and other cytokines, $CD8^+$ T-cells, are known to be cytotoxic against *Mtb* infected macrophages. $CD8^+$ T-cells can directly kill *Mtb* via granulysin, and facilitate the control of both the acute and chronic infection (Cooper, 2009; Grotzke & Lewinsohn, 2005). The abundance of the presence of *Mtb*-specific $CD8^+$ T cells in latently infected individuals shows that the $CD8^+$ T cells also have a role in the control of latent infection (van Pinxteren, Cassidy, Smedegaard, Agger, & Andersen, 2000). Despite the pivotal role of cell-mediated immune response against *MTb*, involving both the $CD4^+$ and $CD8^+$ T-cell subsets, in the progression and pathogenesis of PTB infection, very few studies have been carried out to establish their profiles in peripheral blood in patients with PTB, with existing studies reporting contradictory findings (van Pinxteren *et al*., 2000). Therefore, the current study was designed to compare Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and

Th2 (IL-4, IL-6 and IL-10) cytokine responses with culture smear-positive and -negative in patients with PTB co-infection.

2.5 Tuberculosis Diagnosis

The most common method of TB diagnosis in LMIC such as Kenya, is the detection of AFB through microscopic examination using diagnostic specimen such as a smear of expectorated sputum or of tissue (for example, a lymph node biopsy or fine needle aspiration) (Hudson, Wood, & Maartens, 2000). However, its specificity and sensitivity is below 50% in these settings (WHO, 2011). Furthermore, this conventional AFB staining method accounts for only 20-30% cases detection among HIV/TB co-infected patients (WHO, 2011). However, the challenges related to this method of detection of MTB in HIV and AIDS patients still remain largely unresolved due to the nature of PTB disease in HIV-infected person lacking conventional symptoms, have negative smears status, atypical chest radiography presentation, high prevalence of extra-pulmonary TB and resemblance with other opportunistic pulmonary infection, causing a great diagnostic challenge (Lawn *et al*., 2002). There is also the rise of non-tuberculous mycobacteria (NTM) as opportunistic infection among HIV/AIDS patients, and AFB microscopy does not have the ability to distinguish between TB and NTM disease (UNAIDS/WHO).

Definitive diagnosis depends on the isolation and identification of *Mtb* from a diagnostic specimen (in most cases a sputum) using Mycobacterial Culture on Lowenstein-Jensen or Middle brook 7H10 media by incubating at 37^0C under 5% CO_2 (Katoch, 2004). The use of liquid media with radiometric growth detection (e.g., BACTEC-460) and mycobacterial growth indicator tubes (MGIT) have replaced the traditional methods of isolation on solid media (Katoch, 2004). These new methods have decreased the time required for isolation to 2 to 3 weeks as compared to the 8 weeks required for the traditional culture methods. Advances in knowledge about genetic structure of tubercle bacillus has helped develop gene

probes and gene amplification methods for identification and detection of tubercle bacillus, from culture or directly from clinical specimens and molecular detection of drug resistance. While the gene probes can help in rapid identification of isolates, gene amplification methods (e.g. PCR) developed for diagnosis of TB is demonstrably highly sensitive and detection can be done within hours (Katoch, 2004), although they require highly skilled personnel and greater capital in terms equipment and infrastructure to set up. Alternative specimens for diagnosis of TB can be aspirated effusions, blood for cultures, early morning urine for TB culture and bone marrow biopsy (Hudson *et al*., 2000).

Skin testing with PPD is most widely used in screening for *Mtb* infection. The test is of limited value in the diagnosis of active TB because of its low sensitivity and specificity (J. L. Johnson & Ellner, 2006). False-negative reactions are common in immunosuppressed patients. Positive reactions are obtained when patients have been infected with *Mtb* but do not have active disease and when persons have been sensitized by non-tuberculous mycobacteria or Bacilli Calmette-Guerin (BCG) vaccination. In the past years, new diagnostic methods like Enzyme-linked immunospot (ELISPOT) and Enzyme-linked immunosorbent assay (ELISA) for the diagnosis of infection with *Mtb* have been developed. The ELISPOT and ELISA such Quantiferon TB Gold-in-Tube-test, can detect the secretion of γ-interferon by mononuclear cells in venous blood, specific for *Mtb* peptides, ESAT-6 and CFP-10. These tests are more sensitive and specific for the diagnosis of *Mtb* infection and are superior to the tuberculin skin test (TST) in immunocompetent patients (Ferrara *et al.,* 2006). ESAT-6 and CFP-10 are peptides that mediate *Mtb* virulence (Brodin *et al.,* 2005).

Radiographic procedures, clinical signs and symptoms can also be used in the process of diagnosing TB but are limited. The initial suspicion of pulmonary TB is often based on abnormal chest radiographic findings in a patient with respiratory symptoms (J. L. Johnson & Ellner, 2006). Of the clinical features**,** cough is reported less frequently in HIV patients,

probably because of weak cough reflex due to debilitated condition of the patients in advanced disease, absence of cavitations, and less endobronchial irritation (MOH, 2005).

In untreated HIV patients or in-patients in whom therapy has not adequately controlled the virus replication, the $CD4^+$ T cell count falls below a critical level after a variable period and the patient becomes highly susceptible to opportunistic infections (OIs) (Fauci, 2001). Different OIs occur at different $CD4^+$ T cell levels in HIV/AIDS patients. Unlike most other OIs associated with AIDS, TB can occur at relatively high $CD4^+$ counts (Morris *et al.*, 2003). TB presentation in HIV-infected individuals with well-preserved immunity is similar to that in immune-competent individuals without HIV. The progression of immunodeficiency, attenuation of host tissue-damaging responses and failure of MTB containment result in an increased likelihood of atypical presentation, and greater proportions of extra-pulmonary and disseminated disease (Lawn *et al*., 2002). Sputum samples should be examined even if the chest radiograph appears normal. This is because pulmonary cavitations are less common in HIV-positive patients, leading to significantly reduced sensitivity of sputum microscopy for AFB. Therefore, it is necessary to further examine the sputum samples as culture still remains the mainstay of diagnosis in our setups, with limitation of being very expensive and taking a longer period to get results.

Molecular assays show promise in the rapid diagnosis of smear-negative disease with high sensitivity (Perkins & Cunningham, 2007). One of these tests is the Interferon Gamma Release Assay (IGRA). The challenge with IGRA is that it is dependent on T cell functionality and it is likely that such an assay might not work accurately in immune-challenged individuals, with infections such as HIV. However, the MTB-specific antigens present in IGRA test have great potential to stimulate T-cells to produce multiple cytokines, which could prove beneficial in the diagnosis of PTB among HIV co-infected patients. In addition, studies have demonstrated the inability of interferon gamma alone to accurately

diagnose TB in HIV-infected patients (Huo & Peng, 2016; Syed Ahamed Kabeer *et al*., 2009). Furthermore, other studies have shown that IGRA as currently constituted, cannot be used alone to rule out active TB in HIV individuals, because it doesn't add value to the discriminating ability of TB screening clinical algorithms (Nemeth *et al*., 2012; Rangaka *et al*., 2012). Previous studies have shown the ability of Th1 and Th2 cytokines in discriminating between PTB culture-positive and -negative (Kellar *et al*., 2011; Wallis *et al*., 2010). Hence, the purpose of this study was to determine diagnostic accuracy of Th1 (IFN-γ, TNF-α, and IL-2) and Th2 (IL-4, IL-6 and IL-10) cytokine response and to assess the cofounding effect of lymphocytes in the production of these cytokines, among AFB microscopy smear negative PTB-HIV co-infected patients.

2.6 The Dynamic Relationship between Th1 and Th2 Immune Responses in *Mycobacterium tuberculosis*

IFN-*γ* is the key cytokine for a protective immune response against *Mtb*. Humans and mice with defective in IFN-*γ* or IFN-*γ* receptor genes are more susceptible to *Mtb* infection (Flynn *et al.,* 1993). IFN-*γ*, produced mainly by $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, synergizes with TNF-*α* and activates macrophages to kill intracellular bacilli. IFN-*γ* also augments antigen presentation, leading to recruitment of $CD4^+$ T-cells and/or cytotoxic $CD8^+$ T-cells. These cells participate in mycobacterial killing and also prevents exhaustion of memory T cells (Russell, Dudani, Krishnan, & Sad, 2009). Furthermore, IFN-*γ* induces the transcription of more than 200 genes in macrophages. It is involved in the upregulation of MHC class II expression and the production of antimicrobial effectors such as oxygen radicals and nitric oxide. A major effector mechanism responsible for the antimicrobial activity of IFN-*γ* in association with TNF-*α* is the induction of the production of nitric oxide and other reactive nitrogen intermediates (RNI) by macrophages via iNOS (Scanga *et al.,* 2001). However, some *Mtb* factor(s), such as the 19-kDa lipoprotein, have the potential to

attenuate the response of macrophages to IFN-γ by blocking the transcription of a subset of IFN-γ-responsive genes (R. K. Pai, Convery, Hamilton, Boom, & Harding, 2003).

The TNF-α, produced by macrophages, dendritic cells, and T-cells, is another cytokine that has a major protective role against *Mtb* infection both in mice and humans (Keane, 2005). Paradoxically, TNF-α also contributes significantly to the development of immunopathology associated with TB (Flynn & Chan, 2005). Mice deficient in TNF-α or TNF-α receptors are more susceptible to mycobacterial infections (Bean *et al.*, 1999). This cytokine is involved in both immune and immunomodulatory responses and acts in synergy with IFN-γ to enhance the expression of iNOS and the antimycobacterial activity of macrophages (Chan & Flynn, 2004). TNF-α also initiates cell migration and formation of microbicidal granulomas while disruption of TNF-α responses leads to overgrowth of the mycobacterial pathogens (Chan & Flynn, 2004; Cooper, 2009). The TNF-α produced by the infected macrophages induces the expression of chemokines, such as IL-8, MCP-1, and RANTES which provide signals for migration of immune cells to the sites of *Mtb* infection (Algood, Chan, & Flynn, 2003). Both T cell- and macrophage-derived TNF-α is required for sufficient and long-term protection against *Mtb* infection (Saunders *et al.*, 2005). The phenolic glycolipid, a virulence factor in the cell wall of a hyper virulent strain of *Mtb* (W-Beijing family) inhibits the release of pro-inflammatory cytokines TNF-α, IL-6 and IL-12 by macrophages (Reed *et al.*, 2004). The importance of IL-12 is also evident from increased susceptibility of mice and humans deficient in IL-12 responses to mycobacterial infections (Jouanguy *et al.*, 1999). Individuals with defects in the production of IL-12 or its receptor are highly susceptible to active TB disease (Lichtenauer-Kaligis *et al.*, 2003). The T-cell-derived cytokines, IFN-γ and TNF-α, are produced abundantly by activated $CD4^+$ T cells under the influence of IL-12. The role of IFN-γ and TNF-α in activating and augmenting the microbicidal effector functions of

phagocytic cells is well established. They confer a protective immune response against *Mtb* infection (Chan & Flynn, 2004; Cooper, 2009; Scanga *et al*., 2001).

One immune limiting mechanism is the inhibitory and anti-inflammatory cytokine IL-10. It is known that IL-10 is not only made by Th2 cells but also produced by most if not all $CD4^+$ T cell subsets. This includes Th1 and Th17 cells, B cells, neutrophils, macrophages and some dendritic cells subsets. Its major role is to suppress macrophages and dendritic cell functions, which are necessary for the capture, control and initiation of immune responses to pathogen such as *Mtb* (Redford, Murray, & O'Garra, 2011). IL-10 inhibits the protective immune response to pathogens by blocking the production of pro-inflammatory cytokines, such as TNF-α and the Th1-polarizing cytokines IL-12, by directly acting on antigen-presenting cells such as macrophage and dendritic cells (Redford *et al*., 2011). IL-10 can also inhibit phagocytosis and microbial killing through limiting the production of reactive oxygen and nitrogen intermediates in response to IFN-γ. All of which are important for mediating immunity to intracellular pathogens (Redpath, Ghazal, & Gascoigne, 2001). IL-10 is thus associated with the ability of *Mtb* to evade immune responses. Thus mediate long-term infection in the lung. IL-6 is a B cell growth and differentiation factor that induces immunoglobulin production in activated B cells. IL-6 may mediate hyperglobulinemia that is a feature of TB. IL-4 is known to deactivate macrophages and block T cells proliferation by downregulating IL-2 receptor expression and inhibiting the transcription of IL-2 gene. IL-4 therefore inhibits the immune response to *Mtb*. TB patients have been found to have specific Immunoglobulin E (IgE) and IgG4 antibodies, which are IL-4 dependent (Sharma & Bose, 2001). However, the role of Th1 and Th2 type cytokines interactions in the peripheral immune response and delayed type hypersensitivity to *Mtb,* remains undetermined. As such, the current study was designed to relate T cell (CD3/CD4/CD8), B cell (CD 19), NK cells

(CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) with sputum smear status with PTB co-infected with HIV in AMPATH, MTRH.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Setting

The USAID – AMPATH partnership serves a catchment population of 4 million in western Kenya. AMPATH is a collaboration between Moi University School of Medicine (MUSoM), MTRH and a consortium of North American Universities led by Indiana University. Since its inception in 2001, AMPATH has enrolled over 150,000 HIV infected patients of whom over 90,000 have ever been initiated on Antiretroviral Therapy (ART).

AMPATH has a TB clinic where patients are diagnosed with *Mtb* and treated. It is also equipped with three laboratory facilities that is; the *Mtb* laboratory that carries out the various TB diagnosis ranging from microscopy to culturing. There is also a reference laboratory that that facilitate research related tests and a Care laboratory that runs patient care related tests.

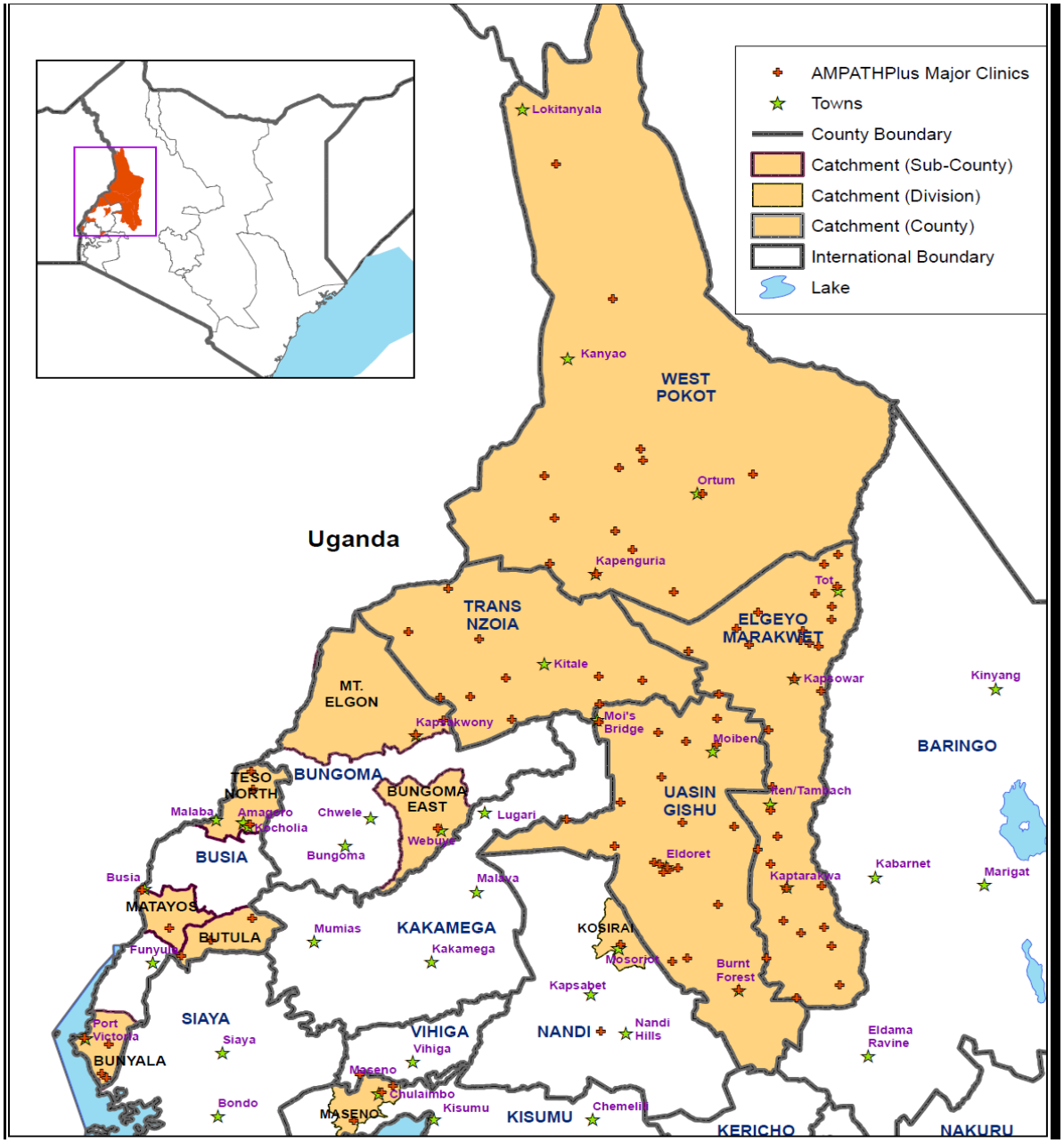

Figure 3.1: AMPATH catchment area

3.2 Study Design

A longitudinal study was conducted at AMPATH and Medical Wards at MTRH situated in Eldoret, Kenya. Patients who had one or more TB specific symptoms and signs based on 2013 WHO guidelines for management of TB and leprosy in Kenya, were eligible for the study (Divison of Leprosy, 2013). The patients were requested to provide a sputum sample, which was first screened by direct smear microscopy and subsequently subjected to mycobacterial culture to confirm the smear status as indicated in Figure 1. They were followed up after 6 months upon TB treatment to ascertain their clinical status.

3.3 Study Algorithm

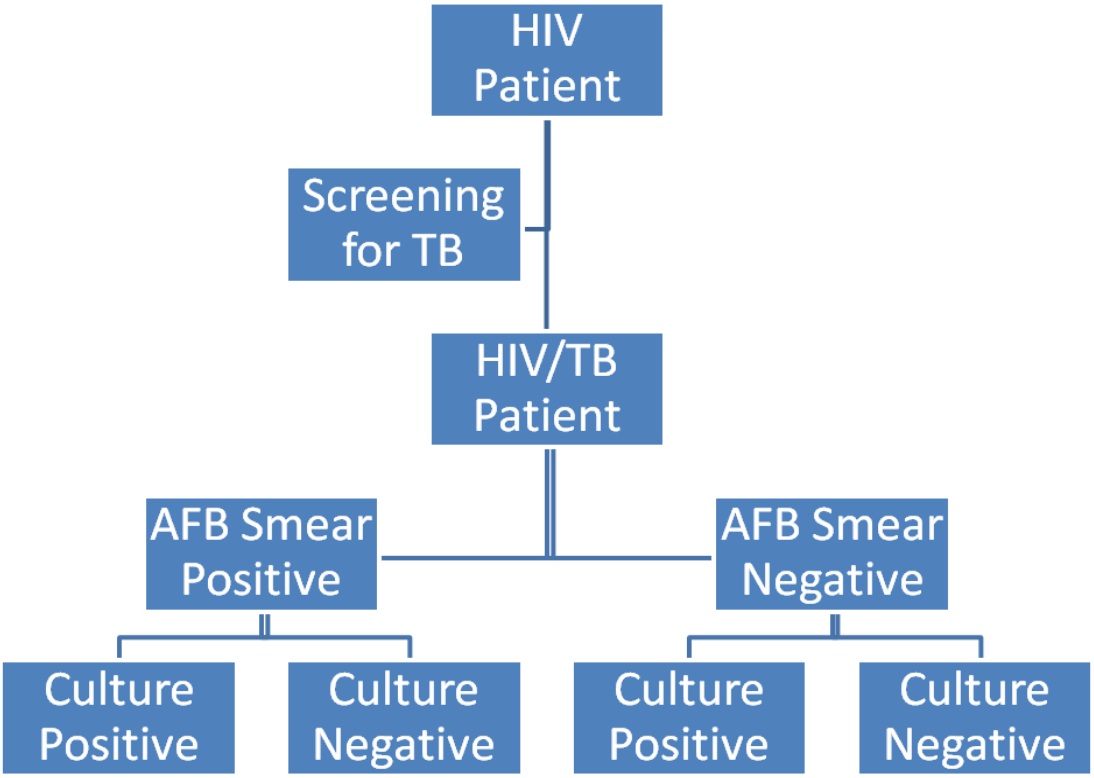

Figure 3.2: Study algorithm

3.4 Study Participants

Study participants were adults who attend TB clinic within AMPATH. Patients who had one or more TB specific symptoms and signs based on 2013 WHO guidelines for management of TB and leprosy in Kenya, were eligible for the study (Divison of Leprosy, 2013).

3.5 Sample Size Calculation

The total sample size required to attain a power of 80% to detect an effect size of 0.67, where the estimated IFN-γ mean for the culture negative is 12.99 ±5.7 pg/ml, and estimated IFN-γ mean for the culture positive is 48.69 ± 28.78 (Hussain *et al.*, 2010), is a minimum of 70 i.e. 35 culture-positive and 35 culture-negative. The sample size was determined using a previous formula for such studies (Hulley, 2007).

$$n = 2\left(\frac{Z_{1-\alpha/2} + Z_{\beta}}{d}\right)^2$$

$$= 2\left(\frac{Z_{1-0.05/2} + Z_{0.8}}{d}\right)^2$$

$$= 2\left(\frac{Z_{0.975} + Z_{0.8}}{d}\right)^2$$

$$= 2\left(\frac{1.96 + 0.84}{0.67}\right)^2$$

$$= 35$$

When *n* is the sample size for one arm,

Therefore; 2*n* = 2 X 35, hence a minimum of 70 study participants (35 culture-positive and 35 culture-negative).

$Z_{1-\alpha/2}$ is the quantile of the standard normal distribution corresponding to $(1 - \alpha/2) \times 100\%$ percentile, Z_{β} is the quantile of the standard normal distribution corresponding to $\beta \times 100\%$ percentile, α is the type I error, β is the power of the study, *d* is the effect size.

3.6 Sampling Technique

The study participants who met the inclusion criteria were consecutively sampled until the required sample size was attained.

3.7 Inclusion and Exclusion Criteria

3.7.1 Inclusion Criteria

i. Those who were 18 years and above

ii. Participants who voluntary accepted to participate in the study.

iii. Participants who were HIV Positive, naïve for highly active antiretroviral therapy (HAART) and anti-TB treatment.

iv. Patients who submitted complete samples; a blood and sputum samples.

3.7.2 Exclusion Criteria

Participants who;

i. had febrile illness

ii. were pregnant, diabetic, or otherwise immunologically-challenged or harboring an autoimmune disease were excluded from the study.

The above-mentioned diseases and medical conditions are associated with the modification of immune responses, and therefore could alter the study's findings and conclusions, by selective depression of cellular mechanism and physiological process.

3.8 Sample Collection and Procedures

The participants were physically examined and clinical histories collected by medical personnel. Those diagnosed for PTB and consented, a questionnaire (as shown in appendix I) was filled to collect the demographic data, and then blood sample was drawn into ethylene diamine-tetra-acetic acid (EDTA) and Lithium Heparin tubes. Blood samples collected were used for lymphocyte immunophenotyping, cytokines profiles, and Quantiferon-TB Gold in Tube method (QGIT Test). Each participant enrolled into the study was given instruction and a bottles to provide sputum samples for direct microscopy, and culture. Samples were collected in the morning from 9:00 AM to 1200 noon and kept at room temperature, then transferred to reference and *Mtb* laboratories for analysis within 24 hours of collection.

3.8.1 Immunophenotyping

Whole blood samples were also collected into 4mL EDTA-containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Two combinations of 4 monoclonal antibodies were used (anti-CD3/CD8/CD45/CD4 and anti-CD3/CD16+56/CD45/CD19 Catalogue # 342416) and TruCOUNT tubes (Becton Dickinson, NJ, USA). About 20µL of MultiTEST CD3FITC/CD8PE/CD45PERCP/CD4APC (Becton Dickson, NJ, USA, Catalogue # 342417) reagent was pipetted into the bottom of one tube while another 20µL of MultiTEST CD3FITC/CD16+CD56PE/CD45PERCP/CD19APC into the bottom of a second tube. About 50 µL of well-mixed, anti-coagulated whole blood sample was pipetted into the bottom of each tube. The tubes were capped and vortex gently to mix, then incubated for 15 minutes in the dark at room temperature (20-25°C). Following this step, 450 µL of the Lysing Solution (Becton Dickson, NJ, USA, Catalogue # 349202) was added into each tube. The tubes were again capped and vortexed gently to mix and incubated for 15 minutes in the dark at room temperature (20-25°C). Determination of lymphocyte subsets was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) on single technology platform. At least 10^5 cells were analyzed on a FACSCalibur equipped with a two-laser system (488- and 635-nm wavelength, respectively). Lymphocyte subsets were acquired using MultiSET software (Becton Dickinson, NJ, USA) as indicated in Appendix II and analyzed using Flowjo (Tristar Inc, Herzenberg, Stanford, US). The absolute count of the cell population (A) was calculated using the equation below as per BD Multitest kit manufacturer instruction:

$A = X/Y \times N/V$, where:

X is the number of positive cell events

Y is the number of bead events

N is the number of beads per test

V is the sample volume (50µL)

The CD45 and side scatter (SSC) was used for gating. Leucocyte populations were identified by their SSC and CD45+ characteristics. Lymphocytes were identified and gated by drawing a gate on all bright CD45+ cells with low SSC. The lymphocytes were further analyzed by using SSC against CD3 to isolate T lymphocytes. The CD4/CD8 T lymphocytes were easily distinguished from non-CD4+ T-cells /non-CD8+ T cells and their respective percentage obtained as a percentage of total lymphocytes (Figure 3).

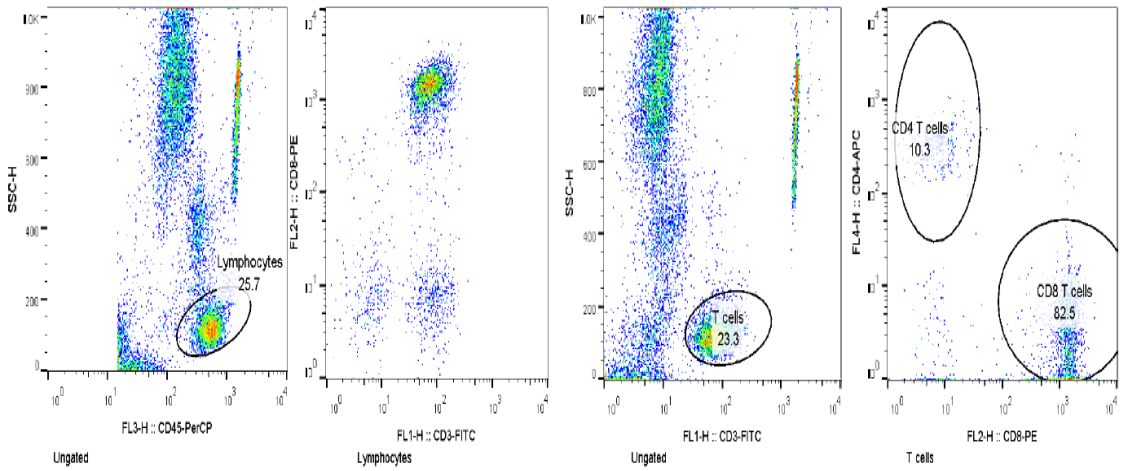

Figure 3.3: CD45 and SSC gating strategy

3.8.2 Quantiferon- TB Test

Blood was cultured using the Quantiferon TB Gold-in-Tube-test (QFT-GIT) (Cellestis, Qiagen GmBH, Carnegie, Victoria, Australia, Cat #05900301) kit as per the manufacturer's instructions. Briefly, one mL of blood was placed into each of the three tubes that were pre-coated with either TB antigen (peptide cocktail of ESAT-6, CFP-10 and TB7.7 proteins), phytohemaglutinin for positive control or nil antigen for negative control. The tubes were then incubated for 16-24h at 37°C and the supernatant (plasma) were harvested after centrifugation, and were snap-frozen at -80°C until use.

3.8.3 Measurement of Cytokines Profiles

The frozen aliquots of cultured QFT-GIT supernatant from the three tubes namely TB antigens, Control tube and Nil tubes from the each study participants, were thawed and the cytokine levels for IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α and IFN-γ in QFT-GIT supernatants were measured by standard sandwich ELISA technique using GenWays Biotech Kit (Greenways Biotech Inc., San Diego, CA, USA; Catalogue #s GWB-ZZD007, GWB-ZZD002, GWB-ZZD006, GWB-ZZD013, GWBZZD005, GWBZZD009, GWBZZD003 and GWB-ZZD004) as per the manufacturer's instructions. In brief, the test cytokine-specific monoclonal antibodies were pre-coated to the plate and the human-specific detection polyclonal antibodies were biotinylated. The QFT-GIT supernatant and biotinylated detection antibodies were added to the wells subsequently and followed by washing with 0.01M Phosphate Buffered Solution (PBS). Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with 0.01M PBS. Horseradish peroxidase substrate (HRP) 3,3',5,5'-tetramethylbenzidine (TMB) was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding hydrochloric acid as a stop solution. The Optical Density (OD) of each well was measured within 5 minutes of stopping the reaction using an ELISA microplate reader. Standard curves were plotted to determine the respective cytokines concentrations (as measured by OD).

3.8.4 Sputum Analysis

3.8.4.1 Ziehl-Nielsen Direct Microscopy: The two sputum samples were collected, designated as S_1, and M_1. S_1 – 1^{st} sample the patient produces when he / she visited the clinic on the 1^{st} day and M, early morning sample before he / she takes breakfast on the following day. All Sputum samples collected from the study participants, were smeared, stained by

Ziehl-Nielsen (ZN), and observed for the presence or absence of acid-fast bacilli under a light microscope with a 100X objective lens under oil immersion (Appendix III).

3.8.4.2 Culturing-(Lowenstein-Jensen Medium) and ZN Staining: All sputum samples collected from study participants were cultured in Lowenstein Jensen (LJ) Medium for 42 days to confirm the presence or absence of acid fast bacilli (Appendix IV). This was done irrespective of their initial ZN direct microscopy smears outcome. Positive cultures were confirmed by ZN staining and species determined by capilia (FIND and Tauns Co. Ltd) according to manufacturer's instructions. A definite TB case was defined as a positive culture confirmed by speciation. The study participants were partition into PTB culture-positive and -negative based on post-culture staining outcome. Culture was chosen, because of its sensitivity and being the gold standard in the diagnosis of TB.

3.9 Quality Control

In additional to commercial control run daily with each of the three instruments, the laboratory routinely participates (3 times per year) in external proficiency testing panels distributed by the United Kingdom National Quality Control Assurance Service for lymphocyte immunophenotyping, and College of American Pathologist for haematology and clinical chemistry. In the event that the daily commercial controls failed, testing was suspended.

3.10 Minimization of Error/Bias

The following was done to reduce error and bias:

i. Double entry – The data collected was double entered into computer using Epi-data. This was done to ensuring accuracy of the data being captured; by correct any wrong entry and omissions made when matching both process and reconciling any difference that arise.

ii. Blood sample was collected from 9:00am to 12:00pm this was to reduce diurnal variation during immunophenotyping.

iii. Samples were collected by one person, and analyzed by one person so as to minimize intra-person errors. Then the results were reviewed by the laboratory manager to ensure accuracy and validity of the results.

3.11 Data Management and Analysis

Data analysis was performed using software for statistical computation (R Core Team, 2016). Categorical variables such as gender were summarized as frequencies and their corresponding percentages. Continuous variables were assessed for Gaussian assumptions using Shapiro Wilks test and normal probability plots. Those that met the Gaussian assumptions were summarized as mean and the corresponding standard deviation (SD), while those that violated the assumptions were summarized as median and their corresponding interquartile range (IQR).

The medians Th1 and Th2 cytokine levels were compared between culture-positive and -negative using two-sample Wilcoxon rank sum test.

Means between two groups; culture-positive AFB microcopy (smear negative) and culture-negative AFB microscopy (smear negative) were compared using independent sample t-test. The medians were compared using two-sample Wilcoxon rank sum test. Pearson's Chi-square test was used to compare the distribution of male and female participants across the culture and AFB microscopy smear status. In order to assess the predictive ability of Th1 and Th2 in the diagnosis of the PTB status (culture status used as the confirmed outcome), binary logistic regression models were fitted for Th1 [IFN-γ, TNF-α, IL-2 and IL-12(p70)] and Receiver Operating Characteristic (ROC) curves plotted. The area under the curve (AUC) was calculated to assess the predictive power of the model in giving us the correct diagnosis. Statistical significance was assessed at $P \leq 0.05$.

The variables included in the models for the combined ROC curves were selected based on their association with the culture status findings. The Th1 cytokines that were significantly associated with the culture status were included in the model for predicting Th1 cytokines ROC curve. The Th2 cytokine that was significantly associated with the culture status was IL-4, although it was eliminated due to inherent multicollinearity measured using variance inflation factor that was >5 units. The IL-6 and IL-10 were not associated with the culture status but were included since they improved the predictive power of the model. All models were assessed for multi-collinearity. There was no evidence of multi-collinearity since the variance inflation factor was 1 for all the cytokines included in the models.

In the analysis of the predictive power of the cytokines on PTB, the AUC and the corresponding 95% confidence intervals from cross validated data set, were included. Using the R packages "cvAUC" and "ROCR", 10 validation folds were used to cross validate the AUC. For each validation fold, predicted values from trained and tested logistic regression fit, were generated. The sensitivity and specificity was derived from the ROC curve.
In general, an AUC of 0.5 suggests no discrimination (i.e., ability to diagnose patients with and without the disease or condition based on the test), 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding (Mandrekar, 2010a, 2010b).

The medians lymphocyte levels were compared between culture-positive and -negative using two-sample Wilcoxon rank sum test. Pearson's correlation coefficient test was used to determine the relationship within Th1/Th2 and between Th1 and Th2 cytokines.

3.12 Ethical Consideration

The research proposal was approved (**Approval number 0001163**) by the Institutional Research and Ethics Committee (IREC) based at Moi Teaching and Referral Hospital (Appendix V) and Maseno University, School of Graduate Studies for approval (Appendix VI). Written informed consent was obtained from participant prior to participation in the study (as shown in informed consent form (Appendix VII).

Blood collection through venipuncture from the cubital vein can cause temporary discomfort, bruises and pain. To minimize these risks, venipuncture was carried out by trained and qualified phlebotomist from the Ministry of Health. Furthermore, sterile blood collection needles were used and all sharps were stored in the appropriate biohazard sharps' containers before disposal. The participation in the study was voluntary, the participants had a right to withdraw at any time during the study, and access to health care was not dependent on participation. All the samples collected were coded for identification by use of a unique study identifier, and investigators controlled access to the data. Data was kept under lock and key and the database was pass coded.

CHAPTER FOUR

RESULTS

4.1 To determine and compare Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine response with culture smear positive in patients with PTB co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital.

4.1.1 Demographic characteristics

A total of 86 participants were enrolled into the study. These were partitioned as follows: PTB culture-positive (n=40) and PTB culture-negative (n=46). The overall mean age was 39 (SD=12) years. Almost half of the males (45.7%) were culture-negative and more than half (54.3%) of the females were culture-negative. The demographic characteristics, age and gender were comparable between PTB-positive and PTB-negative as indicated in Table 4. 1 below.

Table 4. 1: The comparison of demographic characteristic with AFB microscopy smear status

		Culture		
		Negative (N=46)	**Positive (N=40)**	
	N	**n (%)**	**n (%)**	***Test-statistic (P*-value)**
Age (years)	86	41.2 (12.3)	36.6(11.1)	1.790 (0.078[a])
Gender				
Male	86	21 (45.7%)	21 (52.5%)	0.402 (0.526[b])
Female		25 (54.3%)	19 (47.5%)	

[a] statistical analysis determined by independent sample t-test, [b] statistical analyses determined by Pearson's Chi-square test. The smaller *Test-statistics* indicated that there was no difference in the variables assessed.

There was no difference in the demographic characteristics (age and gender) between those who were confirmed to be PTB culture-positive and those who were confirmed to be culture-negative.

4.1.2 Tuberculosis Diagnoses Results

The following two approaches were used to diagnose for TB; direct sputum staining by ZN and microscopy, culturing of sputum, subsequent staining by ZN and speciation by capila. When, sputum samples were first stained with ZN, 16 individuals were diagnosed as positive, while 70 were diagnosed as negative for AFB. Consequently, all sputum samples were cultured in *Lowenstein-Jensen Medium, confirmed with ZN* and speciated with capila, 40 participants tested culture -positive while 46 were culture-negative for *Mtb*. Finally, based on these results, study participants were categorized to be *Mtb* positive or negative based on the gold standard culture and subsequent staining (40 positive and 46 negative) (Table 4. 2).

Table 4. 2: Tuberculosis diagnosis

Test/Results	Sputum ZN staining	Culture and staining
Positive	16	40
Negative	70	46
Total	86	86

4.1.3 Comparison between Th1 Cytokines and PTB culture status of study participants

To further extend these studies, a comparison between Th1 cytokines and PTB culture status was performed (Table 4.3). Results revealed that Th1 cytokines levels, INF-γ (*P*=0.001), TNF-α (*P*=0.001), IL-2 (*P*=0.001) and IL-12p70 *P*=0.016 were elevated in culture-positive participants compared to culture-negative participants, however, IL-8 (*P*=0.337) were non-significantly higher in PTB culture-positive (Table 4. 3).

Table 4. 3: Comparison between Th1 cytokines and PTB culture status of study participants

	N	Culture Results		
		Negative (N=46)	**Positive (N=40)**	
		Median (IQR)	**Median (IQR)**	***Test-statistic (P-value)***
IFN-γ	86	7.6 (5.6, 16.9)	56.0 (29.8, 167.0)	339.5 (0.001)
TNF -α	86	16.1 (14.4, 19.2)	21.0 (16.8, 24.6)	469.0 (0.001)
IL-2	86	15.3 (8.4, 26.9)	59.6 (23.9, 145.6)	389.0 (0.001)
IL-12(p70)	82	2.1 (0.7, 3.5)	3.1 (1.9, 5.0)	580.0 (0.016)
IL-8	82	154.2 (50.1, 204.5)	126.6 (72.5, 167.6)	905.0 (0.337)

Two-sample Wilcoxon rank sum test was used determine the difference between two groups. Test-statistics was used to determine variation difference within the two group means.

Participants who were culture-positive had a significantly higher ($P< 0.05$) Th1 cytokine profiles [IFN-γ, TNF-α, IL-2, and IL-12(p70)] compared to those who were culture negative. Statistical significance determined by two-sample Wilcoxon rank sum test, data presented as median (IQR).

4.1.4 Comparison between Th2 cytokines and PTB culture status of study participants

In order to compare Th2 cytokines and PTB, a two-sample Wilcoxon rank sum test was performed. Results presented here show that the Th2 cytokines median levels between the culture-positive and culture-negative participants were comparable, IL-4 median level of 8.6 (IQR1.1, 26.0) vs. 18.9 (IQR 3.4, 52.7), IL-6 median level of 10.1 (IQR 34.0, 44.0) vs. 12.1 (3.9, 27.6), and IL-10 median level of 10.8 (IQR 8.6, 13.7) vs. 12.8 (IQR 8.4, 15.7) (Table 4. 4)

Table 4. 4: Comparison between Th2 cytokines and PTB culture status participants

	N	Culture Results		
		Negative (N=46) Median (IQR)	Positive (N=40) Median (IQR)	*Test-statistic (P*-value)
IL-4	86	8.6 (1.1, 26.0)	18.9 (3.4, 52.7)	706.0 (0.063)
IL-6	86	12.1 (3.9, 27.6)	10.1 (34.0, 44.0)	904.0 (0.891)
IL-10	86	12.4 (8.4, 15.7)	10.8 (8.6, 13.7)	1073.0 (0.187)

Two-sample Wilcoxon rank sum test was used determine the difference between two groups. Data presented as Median with their respective IQR. Test-statistics was used to determine variation difference within the two group means.

The IL-4, IL-6, and IL-10 between the culture-positive and culture-negative participants were comparable. Statistical significance determined by two-sample Wilcoxon rank sum test.

4.2 To determine diagnostic accuracy Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine response and to assess the cofounding effect of lymphocytes in the production of these cytokines, among AFB microscopy smear negative patients with PTB co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital.

4.2.1 Lymphocyte characteristic, Th1 and Th2 cytokines levels in PTB culture-negative and -positive AFB Microscopy smear negative status

A two-sample Wilcoxon rank sum test was performed to determine the comparison between immunophenotypic characteristic in PTB culture-negative and -positive AFB microscopy smear negative status. The median levels of CD4 and CD19 counts were elevated in PTB culture-positive AFB microscopy smear negative compared to PTB culture-negative AFB microscopy smear negative status, CD4 206.5 cells/µL (101.0, 315.5) vs 67.5 cells/µL (22.5, 192.5), *P=0.033* and CD19 96.5 cells/µL (29.5, 143.3) vs 46 cells/µL (15.5, 99.30), *P=0.048* (Table 4.5). Although the median levels of CD8 and CD16/CD56 were lower in PTB culture-negative AFB microscopy smear negative compared to PTB culture-positive AFB microscopy smear negative 473.0 cells/µL (157.8, 1166.0) vs 582.5 cells/µL (217.5, 945.5), *P=0.797* and 63.0 cells/µL (33.3, 113.5) vs 107.5 cells/µL (56.6, 152.3), *P=0.123*, respectively, they were comparable between the two groups (Table 4.5).

Table 4. 5: Comparison between Lymphocyte characteristics, Th1 and Th2 cytokines in PTB culture-negative and -positive of AFB microscopy smears negative status

	N	Culture Results		
		Negative (N=46)	Positive (N=24)	
		Median (IQR)	Median (IQR)	*T-statistic (P-value)*
CD8 cells/µL	70	473.0 (157.8, 1166.0)	582.5 (217.5, 945.5)	531.0 (0.797)
CD4 cells/µL	70	67.5 (22.5, 192.5)	206.5 (101.0, 315.5)	380.5 (0.033)
CD16/CD56 cells/µL	70	63.0 (33.3, 113.5)	107.5 (56.6, 152.3)	393.0 (0.123)
CD19 cells/µL	70	46.0 (15.5, 99.3)	96.5 (29.5, 143.3)	393.0 (0.048)
IFN -γ pg/mL	70	7.6 (5.6, 16.9)	70.6 (8.6, 202.7)	256.5 (<0.001)
TNF - α pg/mL	70	16.1 (14.4, 19.2)	19.7 (15.8, 22.9)	326.0 (0.004)
IL-2 pg/mL	70	15.3 (8.4, 26.9)	53.1 (14.1, 122.1)	321.0 (0.004)
IL-8 pg/mL	67	154.2 (50.1, 204.5)	118.1(73.8, 158.6)	574.5 (0.174)
IL-12p70 pg/mL	67	2.1 (0.7, 3.5)	2.7 (1.7, 5.0)	373.0 (0.079)
IL-4 pg/mL	70	8.6 (1.1, 26.0)	39.5 (4.5, 56.5)	345.5 (0.009)
IL-6 pg/mL	70	12.1 (3.9, 27.6)	14.5 (7.6, 44.0)	509.0 (0.598)
IL-10 pg/mL	70	12.4 (8.4, 15.7)	10.8 (7.1, 13.7)	688.0 (0.092)

Statistical analysis was performed using Wilcoxon rank sum test, data presented as median (IQR) and with respective T-statistic. Test-statistics was used to determine variation difference within the two group means.

The participants who had PTB culture-positive AFB microscopy smears negative had significantly higher CD4 and CD19 counts compared to those who were PTB culture-negative AFB microscopy smear negative. However, CD8 and CD16/56 were higher but not significant in PTB culture-positive AFB microscopy smears negative compared to PTB culture-negative AFB microscopy smears negative. Statistical significance was determined by two-sample Wilcoxon rank sum test.

PTB culture-positive AFB microscopy smear negative had significantly higher IFN-γ, TNF-α, and IL-2 profiles compared to those were PTB culture-negative AFB microscopy smear negative (Table 4.5). There was no statistically significant difference in IL-12p70 between the PTB culture-positive AFB microscopy smears negative and culture-negative AFB microscopy smears negative. A two-sample Wilcoxon rank sum test was to compare between

the two groups. The study participants who were culture-positive AFB microscopy smear negative had a significantly higher IL-4 profile compared to those who were culture-negative AFB microscopy smear negative, (*P=0.009*). Statistical significance was determined by two-sample Wilcoxon rank sum test. The study further compared Th1 cytokines in PTB culture-negative with culture-positive AFB microscopy smear negative status by performing a two-sample Wilcoxon rank sum test. The data presented here demonstrate that Th1 cytokines IFN-γ (*P*<0.001), TNF-α (*P*=0.004) and IL-2 (*P*=0.004) were elevated in PTB culture-positive AFB microscopy smear negative as compared to PTB culture-negative AFB microscopy smear negative. However, IL-12p70 (*P*=0.079) was not significantly elevated in PTB culture-positive AFB microscopy smear negative. The median level for IL-8 (*P*=0.174) was comparable between the two groups (Table 4. 5).

In order to compare Th2 cytokines with PTB, a two-sample Wilcoxon rank sum test was performed. The results revealed that IL-4 levels (*P*=0.009) were elevated in PTB culture-positive AFB microscopy smear negative compared to PTB culture-negative AFB microscopy smear negative. However, the median levels of IL-6 (*P*=0.598) and IL-10 (*P*=0.092) were comparable between the two groups as indicated in (Table 4. 5).

4.2.2 Comparisons of Th1 and Th2 Cytokines levels and PTB culture-negative and -positive AFB microscopy smear negative study participants stratified by CD4 and CD8 levels

In order to investigate cofounding effect of CD4 and CD8 on Th1 and Th2 cytokine levels among PTB culture-negative and -positive AFB microscopy smear negative, study participants were stratified by CD4 and CD8 levels and a two-sample Wilcoxon rank-sum test was performed and adjustment of the p-value for multiple comparison was done using Bonferroni method. Statistical significance was assessed at *P*<0.0127. For the study participants with CD4≤ 200, the levels of Th1 cytokines [IFN-γ (*P*=0.062), TNF-α

(*P*=0.579), IL-2 (*P*=0.362), IL-8 (*P*=0.756) and IL-12p70 (*P*=0.460) and Th2 cytokines [IL-4 (*P*=0.663), and IL-6 (*P*=0.579) were comparable between PTB culture-negative and -positive AFB microscopy (both smear negative). However, IL-10 levels *(P=0.027)* were elevated in culture-negative AFB microscopy (smear negative) as compared to culture-positive AFB microscopy (smear negative).

The data presented for the study participants with CD4>200 demonstrated elevated median Th1 cytokine levels IFN-γ 11.2pg/mL (6.4,15.4) vs. 164.0pg/mL (38.8, 240.8), *P*=0.002, TNF–α 14.8pg/mL (13.5, 16.5) vs. 22.2pg/mL (18.9, 28.3), *P*<0.001, IL-2pg/mL 13.7 (10.7, 37.3) vs 80.9pg/mL (24.9, 153.3), *P*=0.015 and IL-12p70 1.2pg/mL (0.5, 1.9) vs. 3.8pg/mL (2.3, 5.0), *P*=0.001 in PTB culture-positive AFB microscopy (smear negative) as compared to PTB culture-negative AFB microscopy (smear negative). Only IL-4 median levels, 1.8pg/mL (0.0, 13.0) vs. 54.3 (22.6, 71.2) *P*=0.001, were higher in PTB culture-positive AFB microscopy (smear negative) as compared to PTB culture-negative AFB microscopy (smear negative) (Table 4. 6).

Table 4. 6: Comparison of Th1 and Th2 cytokines between culture negative AFB microscopy smear negative and culture positive AFB smear negative participants stratified by CD4 and CD8 levels

Strata	Cytokines (pg/mL)	Culture negative AFB microscopy smear negative	Culture Positive AFB microscopy smear negative	*Test-statistic (P-*value)
		Median (IQR)		
	Th1 cytokines	N = 34	N = 11	
	IFN -γ	6.8 (5.6, 17.8)	23.6 (7.6, 92.8)	116.0 (0.062)
	TNF - α	17.1 (14.8, 19.7)	15.2 (15.0, 18.7)	208.5 (0.579)
CD4 ≤ 200	IL-2	15.9 (7.1, 25.7)	31.2 (5.0, 56.6)	152.0 (0.362)
	IL-8	127.5 (47.1, 202.5) ₮	119.4 (112.7, 149.0) §	149.0 (0.756)
	IL-12p70	2.5 (1.4, 3.6) ₮	1.3 (0.6, 3.2) §	185.5 (0.460)
		N = 12	N = 13	
	IFN -γ	11.2 (6.4, 15.4)	164.0 (38.8, 240.8)	21.5 (0.002)
	TNF - α	14.8 (13.5, 16.5)	22.2 (18.9, 28.3)	8.0 (<0.001)
CD4 > 200	IL-2	13.7 (10.7, 37.3)	80.9 (24.9, 153.3)	33.0 (0.015)
	IL-8	197.8 (144.0, 204.9)	135.2 (21.3, 168.4)	111.0 (0.077)
	IL-12p70	1.2 (0.5, 1.9)	3.8 (2.3, 5.0)	16.0 (0.001)
	Th2 cytokines	N = 34	N = 11	
	IL-4	10.7 (3.2, 28.7)	4.1 (1.8, 54.0)	204.0 (0.663)
CD4 ≤ 200	IL-6	14.9 (6.1, 52.4)	10.9 (6.4, 35.4)	208.5 (0.579)
	IL-10	13.4 (10.0, 19.8)	8.1 (7.0, 13.7)	271.0 (0.027)
		N = 12	N = 13	
	IL-4	1.8 (0.0, 13.0)	54.3 (22.6, 71.2)	16.0 (0.001)
CD4 > 200	IL-6	7.5 (3.1, 11.9)	16.9 (7.7, 42.1)	46.5 (0.092)
	IL-10	8.1 (7.6. 10.5)	11.6 (8.7, 13.3)	57.5 (0.276)
	Th1 cytokines	N = 31	N = 19	
	IFN -γ	8.4 (5.0, 26.4)	52.0 (16.2, 170.0)	131.5 (0.001)

	TNF - α	16.5 (14.6, 18.5)	21.4 (16.2, 25.5)	138.5 (0.002)
CD8 ≤1000	IL-2	12.3 (6.2, 25.1)	50.5 (19.8, 77.9)	146.0 (0.003)
	IL-8	154.0 (50.6, 206.1) †	119.4 (72.5, 151.5) ħ	299.5 (0.406)
	IL-12p70	1.9 (0.7, 3.4) †	2.3 (1.2, 5.6) ħ	196.5 (0.161)
		N = 15	N = 5	
	IFN -γ	6.8 (6.0, 11.2)	200.8 (5.2, 240.8)	28.5 (0.457)
CD8 >1000	TNF - α	14.8 (14.2, 19.7)	18.1 (14.0, 18.2)	39.5 (0.896)
	IL-2	16.6 (11.5, 28.9)	157.3 (0.5, 158.8)	30.0 (0.541)
	IL-8	194.7 (39.9, 202.4)	164.9 (111.1, 168.4)	42.0 (0.727)
	IL-12p70	2.3 (1.1, 3.3)	3.4 (3.4, 3.8)	17.5 (0.088)
	Th2 cytokines	N = 31	N = 19	
	IL-4	8.4 (0.2, 21.0)	38.4 (8.1, 57.4)	183.0 (0.026)
CD8 ≤1000	IL-6	8.7(2.8, 19.3)	9.2 (5.5, 52.5)	252.0 (0.401)
	IL-10	12.7 (8.4, 20.7)	11.6 (7.1, 15.3)	365.5 (0.159)
		N = 15	N = 5	
	IL-4	8.9 (1.8, 27.9)	54.3 (2.6, 55.9)	23.0 (0.221)
CD8 >1000	IL-6	22.0 (8.3, 50.1)	15.6 (14.9, 16.9)	40.0 (0.861)
	IL-10	10.4 (8.2, 14.9)	8.7 (7.2, 10.7)	51.5 (0.238)

Ŧ N = 32; § N = 10; † N = 29; ħ N = 18, p-value < 0.0127 (Bonferroni corrected *P*-value for multiple comparisons). Data presented as Median (IQR). Test-statistics was used to determine variation difference within the two group means.

The interaction between CD4 or CD8 and the TB status in comparison to cytokines levels was further investigated within each group [culture-positive AFB microscopy (smear negative) and culture -negative AFB (smear negative)] by performing a two sample Wilcoxon rank-sum test. The data demonstrated that median Th1 and Th2 cytokine levels were comparable within each respective group (Table 4. 6).

When further stratified into those with CD4≤ 200 and CD4>200, and comparison made between culture-negative AFB microscopy smear negative and culture-positive AFB microscopy smear negative, there was no significant difference in levels of Th1 cytokines (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 cytokines (IL-4, IL-6 and IL-10) in study participants with CD4≤ 200. However, in the study participants with CD4>200, there was significant difference in Th1 cytokines between culture-negative AFB microscopy smear negative and culture-positive AFB microscopy smear negative, IFN-γ (P=0.002), TNF-α (P<0.001), IL-2 (P=0.015), and IL-12p70, P=0.001). Only IL-4 median levels were significantly different (P=0.001) between culture-negative AFB microscopy smear negative and culture-positive AFB microscopy smear negative among the study participants with CD4>200.

The study participants were stratified further into those with CD8≤ 1000 and CD8>1000 and comparison made between PTB culture-negative and -positive AFB microscopy smear negative. Among study participants who had CD8≤ 1000, IFN-γ (P=0.001), TNF-α (P=0.002), IL-2 (P=0.003), and IL-4 (P=0.026) were significantly different between culture-negative AFB microscopy smear negative and culture-positive AFB microscopy smear negative. However, the study participants with CD8>1000, there was no difference in Th1 or Th2 cytokines levels evaluated between PTB culture-negative and -positive AFB microscopy smear negative.

Table 4. 7: Comparison of Th1 & Th2 cytokine levels by CD4 and CD8 levels (or groups) within the PTB culture-negative and -positive AFB microscopy smear negative.

	Culture Negative AFB microscopy smear -ve			Culture Positive AFB microscopy smear -ve		
Cytokines (pg/mL)	CD4 ≤ 200 N = 34	CD4 > 200 N = 12	*Test-statistic (P-value)*	CD4 ≤ 200 N = 11	CD4 > 200 N = 13	*Test-statistic (P-value)*
	Median (IQR)			Median (IQR)		
Th1 cytokines						
IFN -γ	6.8 (5.6, 17.8)	11.2 (6.4, 15.4)	179.0 (0.539)	23.6 (7.6, 92.8)	164.6 (38.8, 240.8)	40.5 (0.077)
TNF - α	17.1 (14.8, 19.7)	14.8 (13.5, 16.5)	283.5 (0.048)	15.2 (15.0, 18.7)	22.2 (18.9, 28.3)	19.0 (0.003)
IL-2	15.9 (7.1, 25.7)	13.7 (10.7, 37.3)	175.0 (0.476)	31.2 (5.0, 56.6)	80.9 (24.9, 153.3)	38.0 (0.056)
IL-8	127.5 (47.1, 202.5)[₮]	197.8 (144.0, 204.9)	142.0 (0.192)	119.4 (112.7, 149.0)[§]	135.2 (21.3, 168.4)	72.0 (0.687)
IL-12p70	2.5 (1.4, 3.6)[₮]	1.2 (0.5, 1.9)	266.5 (0.051)	1.3 (0.6, 3.2) [§]	3.8 (2.3, 5.0)	36.0 (0.077)
Th2 cytokines						
IL-4	10.7 (3.2, 28.7)	1.8 (0.0, 13.0)	268.0 (0.110)	4.1 (1.8, 54.0)	54.3 (22.6, 71.2)	31.0 (0.020)
IL-6	14.9 (6.1, 52.4)	7.5 (3.1, 11.9)	269.0 (0.107)	10.9 (6.4, 35.4)	16.9 (7.7, 42.1)	63.5 (0.664)
IL-10	13.4 (10.0, 19.8)	8.1 (7.6, 10.5)	301.0 (0.016)	8.1 (7.0, 13.7)	11.6 (8.7, 13.3)	59.5 (0.505)
Cytokines (pg/mL)	CD8 ≤ 1000 N = 31	CD8 > 1000 N = 15	Test-statistic (P-value)	CD8 ≤ 1000 N = 19	CD8 > 1000 N = 5	Test-statistic (P-value)
	Median (IQR)			Median (IQR)		
Th1 cytokines						
IFN -γ	8.4 (5.0, 26.4)	6.8 (6.0, 11.2)	250.5 (0.681)	52.0 (16.2, 170.0)	200.8 (5.2, 240.8)	43.5 (0.803)
TNF - α	16.5 (14.6, 18.5)	14.8 (14.2, 19.7)	248.5 (0.716)	21.4 (16.2, 25.5)	18.1 (14.0, 18.2)	76.0 (0.046)
IL-2	12.3 (6.2, 25.1)	16.6 (11.5, 28.9)	173.5 (0.170)	50.5 (19.8, 77.9)	157.3 (0.5, 158.8)	41.0 (0.670)
IL-8	154.0 (50.6, 206.1)[†]	194.7 (39.9, 202.4)	216.0 (0.980)	119.1 (72.5, 151.5) [ħ]	164.9 (111.1, 168.4)	34.0 (0.434)
IL-12p70	1.9 (0.7, 3.4) [†]	2.3 (1.1, 3.3)	191.0 (0.519)	2.3 (1.2, 5.6) [ħ]	3.4 (3.4, 3.8)	35.5 (0.502)
Th2 cytokines						
IL-4	8.4 (0.2, 21.0)	8.9 (1.8, 27.9)	221.5 (0.805)	38.4 (8.1, 57.4)	54.3 (2.6, 55.9)	51.0 (0.831)
IL-6	8.7 (2.8, 19.3)	22.0 (8.3, 50.1)	147.0 (0.046)	9.2 (5.5, 52.5)	15.6 (14.9, 16.9)	40.0 (0.619)
IL-10	12.7 (8.4, 20.7)	10.4 (8.2, 14.9)	261.0 (0.512)	11.6 (7.1, 15.3)	8.7 (7.2, 10.7)	61.0 (0.355)

[₮] N = 32; [§] N = 10; [†] N = 29; [ħ] N = 18, Test-statistics was used to determine variation difference within the two group means.

The top left panel of Table 4.7 presents the comparison of Th1 & Th2 cytokine levels between the participants with CD4 ≤ 200 cells/µl and those with CD4 > 200 cells/µl among those who were culture-negative AFB microscopy smear negative. The findings do not demonstrate strong evidence of a difference in the cytokine levels by the CD4 levels. Similarly, the top right panel of Table 4.7 presents the comparison of Th1 & Th2 cytokine levels between the participants with CD4 ≤ 200 cells/µl and those with CD4 > 200 cells/µl among those who were culture-positive AFB microscopy smear negative.

Similar comparisons of cytokine levels by CD8 levels was done among those who were culture-negative AFB microscopy smear negative (bottom left panel of Table 4.7), and culture-positive AFB microscopy smear negative (bottom right panel of Table 4.7). There was no evidence from the findings to suggest an effect of CD8 levels on the cytokine levels within the specific culture status among the AFB smear negative participants.

The lack of difference in Table 4.7 is a statistical indication of lack of interaction between CD4 or CD8 and the TB status to influence the levels of the cytokines.

4.2.3 Predictive power of Th1 cytokines on PTB culture status among those who screened negative for AFB microscopy smears

To demonstrate the predictive power of Th1 cytokine on culture status, a Receiver Operating Curve (ROC) analysis was performed. The area under the curve (AUC) was 0.76 (95% CI: 0.61, 0.90) (Figure 4), thus indicating a good predictive power of the ROC model for Th1 (IFN-γ, TNF-α, and IL-2), CD4 (<200 vs. ≥200), and CD8 (<1000 vs. ≥1000) covariates in discriminating between culture-positive and culture-negative in PTB patients who tested negative for AFB microscopy smears.

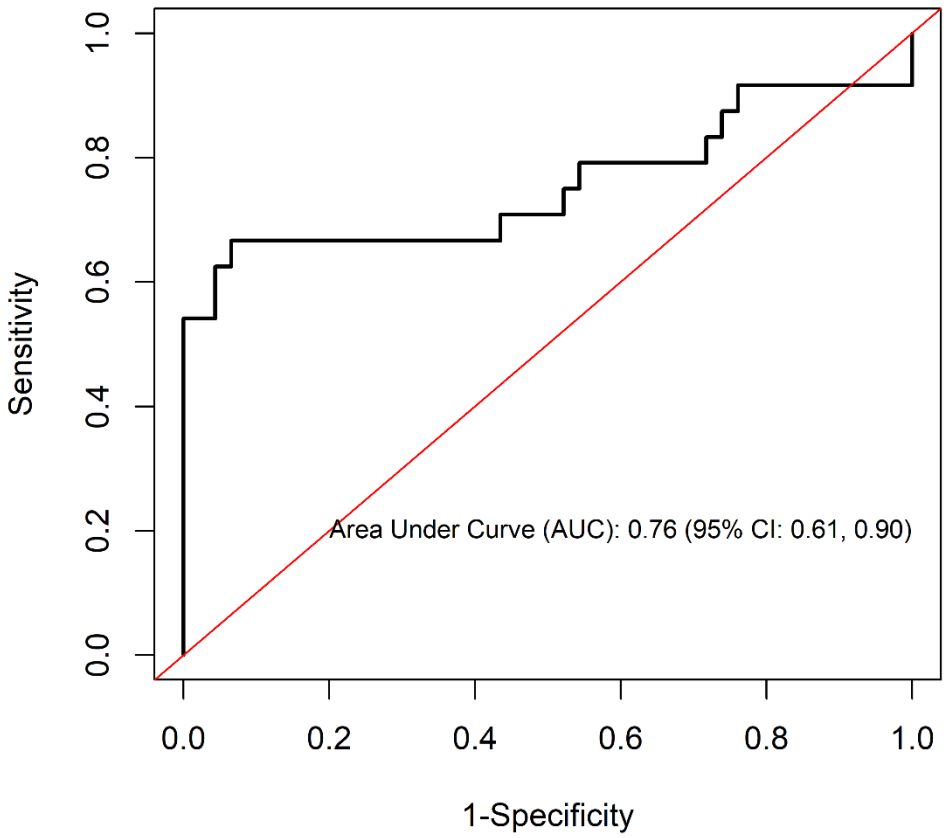

Figure 4: Predictive power of Th1 cytokines on PTB culture status among AFB microscopy smear negative.

The model included IFN- γ, TNF - α, IL-2, CD4 (<200 vs. ≥200), and CD8 (<1000 vs. ≥1000).

4.2.4 Predictive power of Th2 cytokines on PTB culture status among those who screened negative for AFB microscopy smears

In order to determine the predictive power of Th2 cytokines on PTB culture status among those who screened negative for AFB microscopy smears, a ROC analysis was performed. The area under the curve (AUC) was 0.69 (95% CI: 0.56, 0.81) (Figure 5) implying that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group 69% percent of the times.

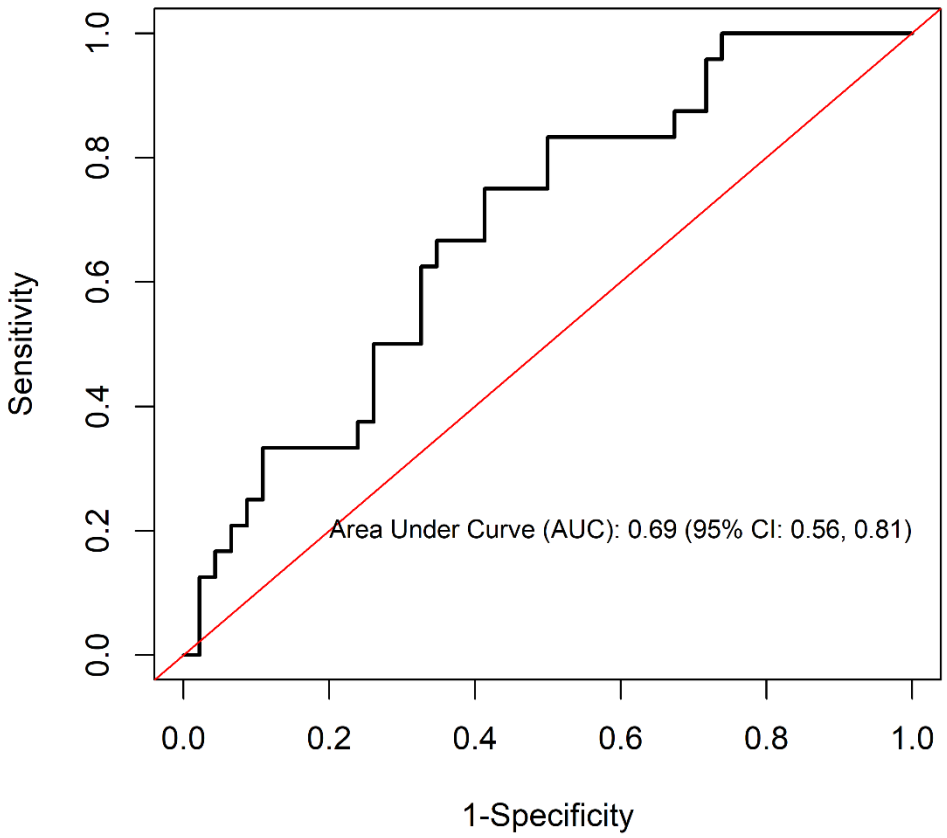

Figure 5: Predictive power of Th2 cytokines on PTB culture status among AFB microscopy smear negative.

The model include IL-4, IL-6, IL-10, CD4 (<200 vs. ≥200), and CD8 (<1000 vs. ≥1000).

4.2.5 Predictive power of Th1 cytokines, Th2 cytokines and T-cells (CD4, and CD8) on PTB culture status among AFB microscopy smear negative samples

In order to assess the predictors of positive culture results among the AFB smear negative participants, binary logistic regression model was used. The inclusion of Th1 and the Th2 cytokines in the models was done in two steps. First, bivariate analyses were performed to assess the relationship between each of the cytokines on the culture positive test result. If the relationship was statistically significant, the variable was included in the multivariable logistic regression model. The variables in the logistic regression model were assessed for multi-collinearity using the variance inflation factor (VIF) approach. The variables in the models had VIF that were less than 2, an evidence of lack of multi-collinearity. The predictive power of discriminating between those who were AFB-smear negative in the diagnosis of PTB was good with cross-validated area under the curve (AUC) being 0.87 (95% CI: 0.78, 0.96) (Figure 6).

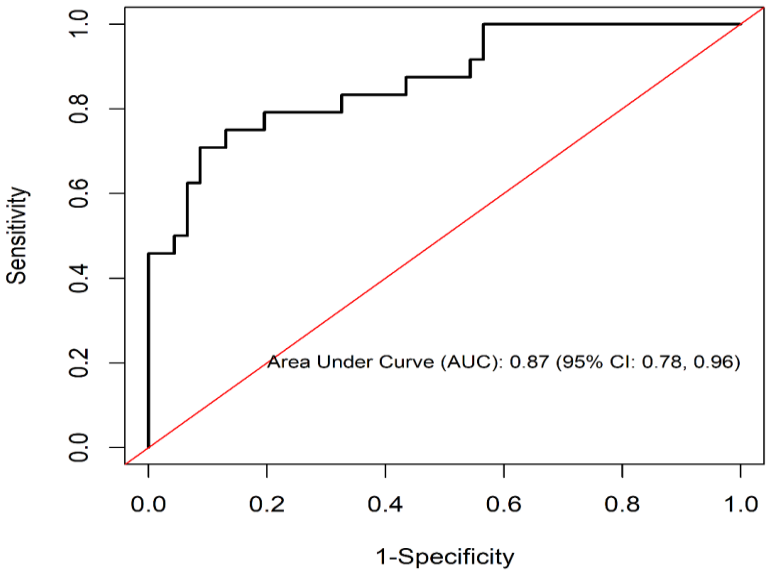

Figure 6: Predictive power of Th1 cytokines, Th2 cytokines and T-cells (CD4, and CD8) on PTB culture status among AFB microscopy smear negative.

The model included IFN-γ, TNF-α, IL-2, IL-6, IL-10, CD4 (<200 vs. ≥200), and CD8 (<1000 vs. ≥1000), but IL-4 was eliminated due to inherent multicollinearity measured using variance inflation factor that was >5 units.

4.3 To relate T cell (CD3/CD4/CD8), B cell (CD 19), NK cells (CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) with sputum smear status in patients with PTB co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital.

4.3.1 Lymphocyte characteristics between the PTB culture-positive and -negative of study participants

The present study further sought to compare lymphocyte counts with PTB culture status. The results presented here demonstrated that CD8 (P=0.979), CD19 (P=0.346), and CD16/CD56 (P=0.637) were comparable between the two groups. Although, in general the median counts of T lymphocytes were higher in culture-positive as compared to culture-negative participants, it was only median CD4 count of 504.5 (IQR: 217.5, 907.5) *vs.* 473.0 (IQR: 157.8, 1166.0), respectively, that differed (P=0.012) between the groups as shown in Table 4. 8.

Table 4. 8: Lymphocyte characteristics between the PTB culture-positive and -negative of study participants

	N	Culture Results		
		Negative (N=46) Median (IQR) or n (%)	**Positive (N=40) Median (IQR) or n (%)**	***Test-statistic (P-value)***
CD8	86	473.0 (157.8, 1166.0)	504.5 (217.5, 907.50)	923.0 (0.979[c])
CD4	86	67.5 (22.5, 192.5)	210.5 (97.3, 309.5)	633.0 (0.012[c])
CD16/CD56	86	63.0 (33.3, 113.5)	87.0 (46.5, 138.0)	810.5 (0.346[c])
CD19	86	46.0 (15.5, 99.3)	46.0 (21.8, 129.8)	865.0 (0.637[c])

[c]Statistical analysis was performed using Wilcoxon rank sum test. Data presented as median (IQR) and with respective T-statistic. Test-statistics was used to determine variation difference within the two group means.

4.3.2 Correlation among Th1 and Th2 Cytokine Levels and between Th1 and Th2 cytokines levels

The correlation among Th1 cytokines levels data demonstrated a concurrent increase in the median levels of IFN-γ and IL-2 cytokines (r=0.48, *P<0.001*, power = 99.1%) and of IL-2 and TNF-α (r=0.27, *P*=0.22, power=62.6%) indicating a synergistic relationship between the two cytokines when analyzed by Pearson correlation. On the contrary, it was evident that while median levels of TNF-α increased, that of IL-8 cytokine reduced (r=-0.41, *P*=0.022, power=93.9%) indicating an antagonistic relationship between the two cytokines.

Similarly, among the Th2 cytokines, the data indicated an increase in both the median levels of IL-4 and IL-10 (r=0.29, *P*=0.015, power=69.1%) cytokines indicating a positive correlation between the two cytokines (Table 4.9).

The results presented provide evidence of a synergistic relationship between Th2 cytokine, IL-6 and Th1 cytokine, TNF-α (r=0.68, *P*<0.001, power=97.3%), as an increase in one cytokine resulted in a concomitant increase in the other cytokine.

However, there was an inverse relationship between Th2 cytokine, IL-6 and Th1 cytokine, IL-8 (r=-0.44, *P*<0.001, power>99.9), since an increase in IL-6 resulted in a decrease of IL-8 cytokine (Table 4.9).

Table 4. 9: Correlation among Th1 and Th2 Cytokine Levels and between Th1 and Th2 cytokines levels.

	Pearson Correlation coefficient (r) (P-value)							
	IFN-γ (pg/mL)	**TNF - α** (pg/mL)	**IL-2** (pg/mL)	**IL-8*** (pg/mL)	**IL-12*** (pg /mL)	**IL-4** (pg/mL)	**IL-6** (pg/mL)	**IL-10** (pg/mL)
IFN-γ (pg/mL)	1.00							
TNF-α (pg/mL)	0.16 (0.178)	1.00						
IL-2 (pg/mL)	**0.48 (<0.001)**	**0.27 (0.022)**	1.00					
IL-8* (pg/mL)	-0.10 (0.405)	**-0.41 (<0.001)**	-0.08 (0.513)	1.00				
IL-12p70* (pg/mL)	-0.06 (0.634)	0.17 (0.173)	0.05 (0.701)	-0.16 (0.205)	1.00			
IL-4 (pg/mL)	-0.02 (0.880)	0.08 (0.513)	0.04 (0.752)	-0.07 (0.589)	-0.09 (0.487)	1.00		
IL-6 (pg/mL)	-0.10 (0.456)	**0.68 (<0.001)**	0.14 (0.208)	**-0.44 (<0.001)**	0.05 (0.701)	-0.09 (0.480)	1.00	
IL-10 (pg/mL)	-0.19 (0.119)	0.11 (0.346)	-0.12 (0.357)	0.23 (0.058)	0.01 (0.920)	**0.29 (0.015)**	0.03 (0.746)	1.00

*n = 67
Statistical analysis was performed by Pearson correlation

There was positive correlation between INF-γ and IL-2 (r = 0.45, p <0.001, power = 99.3%) demonstrating a synergistic relationship; an increase in the secretion of one of the cytokines was associated with an increase in the secretion of the other cytokine. Similarly, there was evidence of a moderate negative correlation between TNF - α and IL8 (r = -0.45, *P*<0.001, power = 99.3%) indicating an antagonistic relationship; where an increase in the secretion of one of the cytokines was associated with a decrease in the secretion of the other cytokine, while the rest of the relationships between the Th1 cytokines were comparable (*P*>0.05). Among the Th2 cytokines, there was a positive relationship between IL-4 and IL-10 (r = 0.28, *P*=0.008, power = 75.0%), although not strong, an increase in the secretion of one of the two Th2 cytokines was associated with an increase in the other.

CHAPTER FIVE

DISCUSSION

5.1 To determine and compare Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine response with culture smear positive in patients with PTB co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital.

The present study sought to evaluate the role of *Mtb* antigens-specific cytokines in determination of smear status in pulmonary tuberculosis co-infected with HIV. This approach was in an attempt to develop a diagnostic technique that utilizes a combination of these cytokines in rapidly and accurately detecting the presence or absence of TB. The most commonly available methods used in the detection of the bacilli have been shown to be less sensitive, less accurate or takes a longer period to carry out a diagnosis (WHO, 2015).

Similar to other studies carried out, CD4 T-lymphocytopenia was observed in TB-HIV co-infected patients as compared to HIV-infected individuals. (Al-Aska *et al.,* 2011). The CD4 T lymphocytes are known to confer protection against *Mtb* through induction of IFN-γ and TNF-α by recruiting monocytes and granulocytes and their anti-microbial activities (Cooper & Khader, 2008; Walzl, Ronacher, Hanekom, Scriba, & Zumla, 2011). Thus, this observation did not show a direct correlation of HIV-TB co-infection disease progression with CD4 levels in an individual, which further supports argument that HIV disease progression monitoring on the account of CD4 counts, might be misleading in HIV-TB co-infection.

In the current study, the levels of IFN-γ were significantly higher in PTB culture-positive as compared to PTB culture-negative study participants. These findings corroborate the findings of a previous study in which it was indicated that patients with culture-confirmed PTB positives had higher levels of IFN-γ (Kellar *et al*., 2011). This was irrespective of HIV status of an individual. These observations indicated the potential of IFN-γ to discriminate

between PTB culture-positive and PTB culture-negative after stimulation with *Mtb*-specific antigens.

The association between TNF-α and PTB culture status was further assessed in this study. TNF-α is a Th1 cytokine known to play a key role in the formation of granuloma for the containment and protection against *Mycobacterium*. Reports from previous studies have shown elevated levels of TNF-α in culture supernatants from stimulated whole blood with TB-specific antigens (ESAT-6, CFP-10 and TB7.7 present in QFT-IT assay), and this was in agreement with the current study (Al-Attiyah, El-Shazly, & Mustafa, 2012). In the current study, there were variations in TNF-α levels between PTB culture-positive and PTB culture-negative, with higher levels of the cytokine being observed in PTB culture-positive. These results suggested that TNF-α levels were elevated during TB infection. It is plausible that the enhanced production contributes to the formation of granuloma, an important immunological process for the containment and protection against *Mycobacterium*. Consequently, other studies in western Africa and India have demonstrated that the TNF-α levels can be used to distinguish between individuals with and without TB infection (Frahm *et al*., 2011; Prabhavathi, Kabeer, Deenadayalan, & Raja, 2015).

IL-2 plays a critical role in the activation, proliferation and clonal expansion of T lymphocyte. The results from the current study indicate that IL-2 levels were higher in culture-positive in relation to the culture-negative individuals. Previous investigations supported observations made in this study (Prabhavathi *et al*., 2015). It has been shown, that IL-2 reduces the replication of *Mycobacterium* through activation of CD8 cytotoxic T lymphocytes and macrophages to release interferons (Bermudez, Stevens, Kolonoski, Wu, & Young, 1989). In this regard, it was hypothesized that higher levels of IL-2 reported in

culture-positive individuals could be due to presence of the *Mtb* antigens, hence, the differing levels of this cytokine between the two groups.

Moreover, a study carried out in India reported lower levels of IL-12p70 in HIV patients when compared to TB-HIV co-infected individuals (Benjamin *et al.*, 2013), an observation that was consistent with findings of the present study. Although the difference in IL-12(p70) cytokines levels between groups was small in the present study, they were still statistically significant. It is important to note that IFN-γ and IL-12(p70) mutually influence their own production, which indicate the ability of IL-12(p70) to discriminate between culture-positive and -negative individuals.

It is also important to note that combination of the Th1 cytokines [IFN-γ, TNF-α, IL-2 and IL-12(p70)] showed an excellent predictive power in discriminating between culture-confirmed pulmonary-tuberculosis patients against culture-negative individuals. Similar results were reported by a study carried out in China, although the AUC was slightly higher compared to the current study (Qiao *et al.*, 2011). This could be attributed to the differences in the methods used, for example, in the previous study, pleural effusion mononuclear cells were stimulated with *Mtb*-specific antigen while in the present study, and whole blood was stimulated with TB-specific antigens. Despite the differences in the methodology, it is still evident that Th1 cytokines are predominant in discriminating between the two groups.

5.2 To determine diagnostic accuracy of T cells Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) and Th2 (IL-4, IL-6 and IL-10) cytokine response and to assess the cofounding effect of lymphocytes in the production of these cytokines, among AFB microscopy smear negative patients with PTB co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital.

The current study revealed that among the AFB microscopy smear negative PTB individuals, the median levels of CD4 counts were significantly higher in those who were culture-positive as compared to culture-negative individuals. Similar results were reported of higher levels of CD4 counts in study participants with PTB culture positive as compared to PTB culture negative (Sester *et al.,* 2011). Therefore, the higher levels of CD4 T cells observed in culture-positive AFB microscopy smear negative study participants were possibly an indication of activation of immune responses in reaction to proteins of phagocytosed bacilli, which were presented as peptides associated with class II major histocompatibility complex. This argument was in line with previous studies that have demonstrated that the sites of active HIV/TB coinfection are key in the contribution to systemic immune activation (Meng *et al.,* 2016).

Significantly elevated levels of $CD19^{+}$ B cell in culture-positive AFB microscopy smears negative participants compared to culture-negative AFB microscopy smears negative participants were reported in this study. This finding was similar to that of other studies that demonstrated increased antibody levels in culture-positive as compared to culture-negative individuals when their peripheral blood were stimulated with TB-specific antigens (Abebe, Holm-Hansen, Wiker, & Bjune, 2007; Raja, Uma Devi, Ramalingam, & Brennan, 2002). Collectively, these underscored the important role of antibody-mediated immunity resulting from Class II MHC restricted T cell-B cell collaboration driven by antigen-receptor interactions, which caused the activation of both B and T cells. In the present study, the levels of IFN-γ were significantly higher in culture-positive (AFB microscopy smear negative) as compared to culture-negative (AFB microscopy smear negative). This finding

was in agreement with a previous study that indicated higher levels of IFN-γ in culture-positive as compared to culture-negatives patients' peripheral blood stimulated with TB-specific antigens (Winkler *et al.,* 2005). It was also important to note that IFN-γ levels were high in the study participants in that study irrespective of low or high CD4 counts. Taken together, these findings indicated that IFN-γ production in PTB patient was as a result of activation of *Mycobacterium*-specific T cells used in the stimulation of the whole blood. Moreover, elevated levels of IFN-γ in HIV-TB co-infection with significant raise in IFN-γ/IL-10 ratio have been known to alter the impact of IL-10 inhibitory function in HIV infection, thus leading to a shift towards a more dominant pro-inflammatory reaction (Th1 cytokine mediated), potentially resulting from *Mtb*-induced pathogenesis (Jamil *et al.,* 2007). Similar to other studies carried out, elevated levels of TNF-α in culture-positive (AFB microscopy smear negative) as compared to culture-negative (AFB microscopy smear negative) individuals was reported. Higher levels of TNF-α have been reported in culture supernatants of peripheral blood in patients with PTB stimulated with mycobacterial antigens, suggesting a role with other cytokines such as IFN-γ in the control of *Mycobacterial tuberculi* multiplication (Al-Attiyah *et al*., 2012; C. H. Wang *et al*., 2001).

A previous study reported significantly increased levels of IL-2 in TB patients after stimulation with ESAT-6 (Caccamo *et al*., 2010). This was in line with findings from the current study that reported higher levels of IL-2 in culture-positive (AFB microscopy smear negative) as compared to culture-negative (AFB microscopy smear negative) individuals. Furthermore, it has been reported that measurement of multiple cytokines would be important in determining PTB smear status especially among TB-HIV co-infection patients (Biselli *et al*., 2010; Boaz, Waters, Murad, Easterbrook, & Vyakarnam, 2002). Similarly, it has been demonstrated in other studies that there were elevated levels of IL-2 and IFN-γ during active TB when the bacilli load is high, an observation which was supporting the observation made

in the current study (Kerry A. Millington, 2007). The IFN-γ plays a critical role in the activation of macrophages and thus the control of *Mtb* bacilli whereas IL-2 is known to stimulate the proliferation of T cell. Increased levels of these two cytokines in culture-positive (AFB microscopy smear negative), demonstrated the presence of *Mtb* infection through their involvement in initiation of immune response against the bacilli.

The comparison between Th2 cytokines and AFB microscopy smears status was further assessed. Significant differences were reported in median levels of IL-4 in culture-positive (AFB microscopy smear negative) as compared to culture-negative (AFB microscopy smear negative). This corroborate the findings from a study that reported increased expression of IL-4 to be associated with the virulence factor, leading to the promotion of anti-inflammatory functions and the ability to cause tissue damage in conjunction with TNF-α (Seah & Rook, 2001).

The present study evaluated further if the low levels of Th1 and Th2 cytokines in AFB smear negative PTB-HIV co-infected study participants were due to depleted CD4 and CD8 T cells. The study participants were stratified into categories of CD4 ≤ 200 cells/µl vs. CD4 > 200 cells/µl, and CD8 ≤ 1000 cells/µl vs. CD8> 1000 cells/µl. In CD4 > 200 cells/µl category, there was significant difference in Th1 [IFN-γ, TNF-α, IL-2 and IL-12(p70)] and Th2 (IL-4) cytokines observed between culture-positive (AFB microscopy smear negative) and culture-negative (AFB microscopy smear negative) study participants. Similar findings were reported in another study carried out in India (Benjamin *et al*., 2013). However, among study participants with CD4 ≤ 200 cells/µl, there were no significant differences reported in Th1 and Th2 cytokines between the two groups, except IL-10. This was similar to previous findings (Benjamin *et al*., 2013) that demonstrated that despite low CD4 ≤ 200 cells/µl HIV-TB patients had significantly elevated Th2 (IL-4 and IL-10) cytokines as compared to HIV

patients. This can be attributed to the extent and interaction of the immune system in response to HIV and/or TB co-infection.

With respect to CD8 T cells, the data revealed significant difference in Th1 cytokines (IFN-γ, TNF-α, IL-2) and Th2 cytokine (IL-4) among study participants who had CD8≤ 1000 when comparing AFB smear negative culture negative and AFB smear negative culture positive. However, there was no difference observed in Th1 and Th2 cytokine levels between AFB smear negative culture negative and culture positive individuals with CD8>1000. The role of CD8 T cells remains unclear, with some studies reporting that CD8 lymphocytes might have an important function in conferring immunity against TB through the secretion of cytolysins or perforins (Brighenti & Andersson, 2010; Ottenhoff & Kaufmann, 2012), while others reported the existence of CD8 lymphocytes might be harmful, since CD8 lymphocytes levels were elevated in AFB smear negative culture positive as compared to AFB smear negative culture negative individuals.

In the current study, the highest AUC after ROC analysis to differentiate between culture-positive AFB smear negative and culture-negative AFB smear negative was observed in combined cytokine measurements as compared to single cytokine measurements. Similar findings have been reported in other studies proposing the use of either multiple cytokines measurements or cytokine ratios would be more useful in diagnosis of PTB. This was due to inter-individual variability when only one cytokine was used making these immune-diagnostic tests unreliable especially in immune-compromised individuals (Nemeth *et al*., 2012; Rangaka *et al*., 2012).

5.3 To correlate T cells (CD3/CD4/CD8), B cell (CD 19), NK cells (CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) with sputum smear status in patients with PTB co-infected with HIV in AMPATH, Moi Teaching and Referral Hospital.

The current study sought to investigate the relationship between T cells (CD3/CD4/CD8), B cells (CD 19), NK cells (CD16/CD56) and Th1 (IFN-γ, TNF-α, IL-2, IL-8 and IL-12p70) /Th2 (IL-4, IL-6 and IL-10) cytokines with sputum smear status in patients with PTB co-infected with HIV. It was demonstrated that there was a synergistic relation between IFN-γ and IL-2, such that an increase in one resulted to an increase of the other in culture-positive compare to culture-negative. Similar findings have been reported in a study carried out by Millington *et al.*, which demonstrated a co-dominance of IL-2 and IFN-γ in participants diagnosed with *Mtb*. This suggested the roles of the two cytokines as important immunological biomarkers in determining the mycobacterial load in PTB co-infected with HIV (Millington *et al.*, 2007).

Several studies, (Dlugovitzky *et al*., 1997; North, 1998; Oswald, Gazzinelli, Sher, & James, 1992) have reported the synergistic relationship of IL-4 and IL-10. These were similar to the present study. Low levels of both cytokines are known to suppress *Mtb* killing by IFN-γ induced macrophage cytotoxicity and nitric oxide (NO) production. NO, a molecule that plays a crucial role in microbicidal activities and pathophysiological functions resulting in inflammation and tissue damage. IL-4 and IL-10 upregulation are also known to provide a negative loop towards Th1 cytokines mediated responses.

A direct correlation between TNF-α and IL-6 has been demonstrated (Juarez *et al.*, 2012), with associated significant increase of both cytokines in culture-positive compared to culture-negative. This corroborated with the findings from the present study which indicate the importance of the two cytokines acting synergistically in the control of intracellular growth of virulent *Mtb*.

5.4 Limitation of the study

The study was limited in that it was conducted in a single locality, and therefore the findings from the study provided preliminary information and insight that would culminate in designing larger multicenter studies that will yield more details.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

The overall mean age of the participants was 39 years (SD=12), 42% being male. Although, lymphocytes counts were higher in culture-positive relative to culture-negative, the CD8, CD19, and CD16/CD56 were comparable in the two groups. The CD4 counts differed between the two groups (P=0.012). The Th1 cytokines showed a better discrimination between culture-positive and -negative PTB individuals; IFN-γ (P=0.001), TNF-α (P=0.001), IL-2 (P=0.001) and IL-12(p70) (P=0.016). The Th2 cytokines (IL-4, IL-6 and IL-10) were comparable between the culture-positive and -negative groups. However, when the combination of Th1 cytokines [IFN-γ, TNF-α, IL-2 and IL-12(p70)] was fitted in binary logistic regression models, the predictive power was high with area under curve (AUC) being 89.7% in discriminating PTB. IFN-γ (P<0.001), TNF-α (P=0.004), IL-2 (P=0.004) and IL-4 (P=0.009) median levels were elevated in PTB culture-positive (AFB microscopy smear negative) as compared to PTB culture-negative (AFB microscopy smear negative) participants. Finally, when Th1 cytokines (IFN-γ, TNF-α and IL-2), Th2 cytokines (IL-6 and IL-10) and T cells were included in the logistic regression fit for PTB outcome, the predictive power of discriminating between those who were AFB smear negative in the diagnosis of PTB was good with cross validated area under the curve (AUC) being 0.87 (95% CI: 0.78, 0.96).

A concurrent increase in the median levels of IFN-γ and IL-2 cytokines (r=0.48, *P<0.001*, power = 99.1%) and of IL-2 and TNF-α (r=0.27, P=0.22, power=62.6%) was reported indicating a synergistic relationship between the two cytokines when analyzed by Pearson correlation. On the contrary, the median levels of TNF-α increased, while that of IL-8 cytokine decreased (r=-0.41, P=0.022, power=93.9%) demonstrating an antagonistic relationship between the two cytokines.

Similarly, in Th2 cytokines, there was an increase in the median levels of both IL-4 and IL-10 (r=0.29, *P*=0.015, power= 69.1%) indicating a positive correlation between the two. A synergistic relationship between the Th2 cytokine IL-6, and the Th1 cytokine TNF-α (r=0.68, *P*<0.001, power=97.3%) was evident, demonstrating that an increase in one cytokine resulted in a concomitant increase in the other cytokine. However, there was an inverse relationship between the Th2 cytokine IL-6, and the Th1 cytokine IL-8 (r=-0.44, *P*<0.001, power>99.9), therefore an increase in IL-6 resulted in a decrease in IL-8.

6.2 Conclusions

1. The study provided evidence that the combination of Th1 cytokines [IFN-γ, TNF-α, IL-2 and IL-12(p70)] had a potential to discriminate between culture-positive and -negative PTB participants with HIV co-infection.

2. A combination of multiple cytokines, both Th1 (IFN-γ, TNF-α & IL-2) and Th2 (IL-6, and IL-10) could discriminate between culture-positive AFB smear negative and culture-negative AFB smear negative status among HIV patients.

3. A synergistic relationship was observed amongst the Th1 cytokines (between IFN-γ and IL-2 and between IL-2 and TNF-α); amongst Th2 cytokines (between IL-4 and IL-10), and between Th1 (TNF-α) and Th2 (IL-6) cytokines. However, an antagonist relationship was observed between IL-6 and IL-8 cytokines in culture-positive when compared to culture-negative PTB participants with HIV co-infection.

6.3 Recommendations from Current Study

1. Blood test (cytokines) should be used as alternative to sputum cultures in the diagnosis of PTB since the turnaround is shorter.
2. A combination of multiple Th1 and Th2 cytokines could be useful in diagnosis of PTB in HIV co-infected patients, who initially tested negative for AFB microscopy.

3. Targeted immunotherapy for PTB could be developed by either blocking or activating the different immune mechanisms during TB pathogenesis.

6.4 Recommendations for Future Studies

1. It would be important to extend this study to a larger cohort in order to validate the diagnostic accuracy of both Th1 (IFN-γ, TNF-α & IL-2) and Th2 (IL-6, and Il-10) biomarkers.

2. Finally, molecular studies could be design to further understand the different immunological pathways that are upregulated or down regulated in PTB culture-positive patients.

REFERENCES

Abebe, F., Holm-Hansen, C., Wiker, H. G., & Bjune, G. (2007). Progress in serodiagnosis of Mycobacterium tuberculosis infection. *Scand J Immunol, 66*(2-3), 176-191. doi:SJI1978 [pii]10.1111/j.1365-3083.2007.01978.x

Abraham, S. N., & Malaviya, R. (1997). Mast cells in infection and immunity. *Infect Immun, 65*(9), 3501-3508. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9284112

Al-Aska, A., Al-Anazi, A. R., Al-Subaei, S. S., Al-Hedaithy, M. A., Barry, M. A., Somily, A. M., . . . Al Anazi, N. A. (2011). CD4+ T-lymphopenia in HIV negative tuberculous patients at King Khalid University Hospital in Riyadh, Saudi Arabia. *Eur J Med Res, 16*(6), 285-288. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21810564

Al-Attiyah, R., El-Shazly, A., & Mustafa, A. S. (2012). Comparative analysis of spontaneous and mycobacterial antigen-induced secretion of Th1, Th2 and pro-inflammatory cytokines by peripheral blood mononuclear cells of tuberculosis patients. *Scand J Immunol, 75*(6), 623-632. doi:10.1111/j.1365-3083.2012.02692.x

Algood, H. M., Chan, J., & Flynn, J. L. (2003). Chemokines and tuberculosis. *Cytokine Growth Factor Rev, 14*(6), 467-477. doi:S1359610103000546 [pii]

Altare, F., Lammas, D., Revy, P., Jouanguy, E., Doffinger, R., Lamhamedi, S., . . . Kumararatne, D. S. (1998). Inherited interleukin 12 deficiency in a child with bacille Calmette-Guerin and Salmonella enteritidis disseminated infection. *J Clin Invest, 102*(12), 2035-2040. doi:10.1172/JCI4950

Badri, M., Ehrlich, R., Wood, R., Pulerwitz, T., & Maartens, G. (2001). Association between tuberculosis and HIV disease progression in a high tuberculosis prevalence area. *Int J Tuberc Lung Dis, 5*(3), 225-232. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11326821

Barnes, P. F., Bloch, A. B., Davidson, P. T., & Snider, D. E., Jr. (1991). Tuberculosis in patients with human immunodeficiency virus infection. *N Engl J Med, 324*(23), 1644-1650. doi:10.1056/NEJM199106063242307

Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D., & Britton, W. J. (1999). Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *J Immunol, 162*(6), 3504-3511. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10092807

Beetz, S., Wesch, D., Marischen, L., Welte, S., Oberg, H. H., & Kabelitz, D. (2008). Innate immune functions of human gammadelta T cells. *Immunobiology, 213*(3-4), 173-182. doi:10.1016/j.imbio.2007.10.006S0171-2985(07)00128-3 [pii]

Benjamin, R., Banerjee, A., Sunder, S. R., Gaddam, S., Valluri, V. L., & Banerjee, S. (2013). Discordance in CD4+T-cell levels and viral loads with co-occurrence of elevated peripheral TNF-alpha and IL-4 in newly diagnosed HIV-TB co-infected cases. *PLoS One, 8*(8), e70250. doi:10.1371/journal.pone.0070250 PONE-D-13-08502 [pii]

Bermudez, L. E., Stevens, P., Kolonoski, P., Wu, M., & Young, L. S. (1989). Treatment of experimental disseminated Mycobacterium avium complex infection in mice with recombinant IL-2 and tumor necrosis factor. *J Immunol, 143*(9), 2996-3000. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2553816

Bernal-Fernandez, G., Hermida, C., Espinosa-Cueto, P., Cubilla-Tejeda, A. C., Salazar-Gonzalez, J. F., Ortiz-Ortiz, L., . . . Mancilla, R. (2006). Impact of opportunistic

Mycobacterium tuberculosis infection on the phenotype of peripheral blood T cells of AIDS patients. *J Clin Lab Anal, 20*(3), 80-86. doi:10.1002/jcla.20105

Biselli, R., Mariotti, S., Sargentini, V., Sauzullo, I., Lastilla, M., Mengoni, F., . . . Nisini, R. (2010). Detection of interleukin-2 in addition to interferon-gamma discriminates active tuberculosis patients, latently infected individuals, and controls. *Clin Microbiol Infect, 16*(8), 1282-1284. doi:10.1111/j.1469-0691.2009.03104.x S1198-743X(14)64231-2 [pii]

Boaz, M. J., Waters, A., Murad, S., Easterbrook, P. J., & Vyakarnam, A. (2002). Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. *J Immunol, 169*(11), 6376-6385. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12444145

Bosio, C. M., Gardner, D., & Elkins, K. L. (2000). Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology. *J Immunol, 164*(12), 6417-6425. doi:ji_v164n12p6417 [pii]

Brennan, P. J., & Nikaido, H. (1995). The envelope of mycobacteria. *Annu Rev Biochem, 64*, 29-63. doi:10.1146/annurev.bi.64.070195.000333

Brighenti, S., & Andersson, J. (2010). Induction and regulation of CD8+ cytolytic T cells in human tuberculosis and HIV infection. *Biochem Biophys Res Commun, 396*(1), 50-57. doi:10.1016/j.bbrc.2010.02.141 S0006-291X(10)00375-X [pii]

Brodin, P., de Jonge, M. I., Majlessi, L., Leclerc, C., Nilges, M., Cole, S. T., & Brosch, R. (2005). Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of Mycobacterium tuberculosis, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity. *J Biol Chem, 280*(40), 33953-33959. doi:M503515200 [pii] 10.1074/jbc.M503515200

Caccamo, N., Guggino, G., Joosten, S. A., Gelsomino, G., Di Carlo, P., Titone, L., . . . Dieli, F. (2010). Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. *Eur J Immunol, 40*(8), 2211-2220. doi:10.1002/eji.201040455

Caruso, A. M., Serbina, N., Klein, E., Triebold, K., Bloom, B. R., & Flynn, J. L. (1999). Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol, 162*(9), 5407-5416. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10228018

Chan, J., & Flynn, J. (2004). The immunological aspects of latency in tuberculosis. *Clin Immunol, 110*(1), 2-12. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14986673

Collins, H. L., & Kaufmann, S. H. (2001). The many faces of host responses to tuberculosis. *Immunology, 103*(1), 1-9. doi:imm1236 [pii]

Comstock, G. W. (1982). Epidemiology of tuberculosis. *Am Rev Respir Dis, 125*(3 Pt 2), 8-15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7073104

Cooper, A. M. (2009). Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol, 27*, 393-422. doi:10.1146/annurev.immunol.021908.132703

Cooper, A. M., & Khader, S. A. (2008). The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. *Immunol Rev, 226*, 191-204. doi:10.1111/j.1600-065X.2008.00702.x IMR702 [pii]

Cowley, S. C., & Elkins, K. L. (2003). CD4+ T cells mediate IFN-gamma-independent control of Mycobacterium tuberculosis infection both in vitro and in vivo. *J Immunol, 171*(9), 4689-4699. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14568944

Cree, I. A., Nurbhai, S., Milne, G., & Beck, J. S. (1987). Cell death in granulomata: the role of apoptosis. *J Clin Pathol, 40*(11), 1314-1319. doi:10.1136/jcp.40.11.1314

Dheda, K., Booth, H., Huggett, J. F., Johnson, M. A., Zumla, A., & Rook, G. A. (2005). Lung remodeling in pulmonary tuberculosis. *J Infect Dis, 192*(7), 1201-1209. doi:JID34125 [pii] 10.1086/444545

Divison of Leprosy, T. a. L. D. (2013). Guidelines for Management of Tuberculosis and Leprosy in Kenya. Retrieved from guidelines.health.go.ke:8000/media/TB_Treatment_GUIDELINES_2013.pdf

Dlugovitzky, D., Torres-Morales, A., Rateni, L., Farroni, M. A., Largacha, C., Molteni, O., & Bottasso, O. (1997). Circulating profile of Th1 and Th2 cytokines in tuberculosis patients with different degrees of pulmonary involvement. *FEMS Immunol Med Microbiol, 18*(3), 203-207. doi:S0928-8244(97)00040-0 [pii] 10.1111/j.1574-695X.1997.tb01046.x

Fairbairn, P. I. (2004). Macrophage apoptosis in host immunity to mycobacterial infections. *Biochemical Society Transactions, 32, part 3*.

Fauci, A. S., Lane, H.C. (2001). *Human immunodeficiency virus disease: AIDS and related disorders. In: Harrison's principle of internal medicine* (Vol. 15th edn). New York: McGraw-Hill.

Ferguson, J. S., Weis, J. J., Martin, J. L., & Schlesinger, L. S. (2004). Complement protein C3 binding to Mycobacterium tuberculosis is initiated by the classical pathway in human bronchoalveolar lavage fluid. *Infect Immun, 72*(5), 2564-2573. doi:10.1128/iai.72.5.2564-2573.2004

Ferrara, G., Losi, M., D'Amico, R., Roversi, P., Piro, R., Meacci, M., . . . Richeldi, L. (2006). Use in routine clinical practice of two commercial blood tests for diagnosis of infection with Mycobacterium tuberculosis: a prospective study. *Lancet, 367*(9519), 1328-1334. doi:S0140-6736(06)68579-6 [pii]10.1016/S0140-6736(06)68579-6

Flynn, J. L. (2004). Immunology of tuberculosis and implications in vaccine development. *Tuberculosis (Edinb), 84*(1-2), 93-101. doi:S1472979203000921 [pii]

Flynn, J. L., & Chan, J. (2001a). Immunology of tuberculosis. *Annu Rev Immunol, 19*, 93-129. doi:19/1/93 [pii]10.1146/annurev.immunol.19.1.9

Flynn, J. L., & Chan, J. (2001b). Tuberculosis: latency and reactivation. *Infect Immun, 69*(7), 4195-4201. doi:10.1128/IAI.69.7.4195-4201.2001

Flynn, J. L., & Chan, J. (2005). What's good for the host is good for the bug. *Trends Microbiol, 13*(3), 98-102. doi:S0966-842X(05)00027-2 [pii]10.1016/j.tim.2005.01.005

Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A., & Bloom, B. R. (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med, 178*(6), 2249-2254. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7504064

Flynn, J. L., Goldstein, M. M., Chan, J., Triebold, K. J., Pfeffer, K., Lowenstein, C. J., . . . Bloom, B. R. (1995). Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity, 2*(6), 561-572. doi:1074-7613(95)90001-2 [pii]10.1016/1074-7613(95)90001-2

Frahm, M., Goswami, N. D., Owzar, K., Hecker, E., Mosher, A., Cadogan, E., . . . Stout, J. E. (2011). Discriminating between latent and active tuberculosis with multiple biomarker responses. *Tuberculosis (Edinb), 91*(3), 250-256. doi:10.1016/j.tube.2011.02.006 S1472-9792(11)00029-1 [pii]

Fratti, R. A., Vergne, I., Chua, J., Skidmore, J., & Deretic, V. (2000). Regulators of membrane trafficking and Mycobacterium tuberculosis phagosome maturation block. *Electrophoresis, 21*(16), 3378-3385. doi:10.1002/1522-2683(20001001)21:16<3378::AID-ELPS3378>3.0.CO;2-B [pii] 10.1002/1522-2683(20001001)21:16<3378::AID-ELPS3378>3.0.CO;2-B

Frieden, T. R., Sterling, T. R., Munsiff, S. S., Watt, C. J., & Dye, C. (2003). Tuberculosis. *Lancet, 362*(9387), 887-899. doi:S0140-6736(03)14333-4 [pii] 10.1016/S0140-6736(03)14333-4

Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., . . . Coccia, E. M. (2001). Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response. *J Immunol, 166*(12), 7033-7041. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11390447

Green, A. M., Difazio, R., & Flynn, J. L. (2013). IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during Mycobacterium tuberculosis infection. *J Immunol, 190*(1), 270-277. doi:10.4049/jimmunol.1200061jimmunol.1200061 [pii]

Grotzke, J. E., & Lewinsohn, D. M. (2005). Role of CD8+ T lymphocytes in control of Mycobacterium tuberculosis infection. *Microbes Infect, 7*(4), 776-788. doi:S1286-4579(05)00046-8 [pii]10.1016/j.micinf.2005.03.001

Hernandez, J., Velazquez, C., Valenzuela, O., Robles-Zepeda, R., Ruiz-Bustos, E., Navarro, M., & Garibay-Escobar, A. (2010). Low number of peripheral blood B lymphocytes in patients with pulmonary tuberculosis. *Immunol Invest, 39*(3), 197-205. doi:10.3109/08820130903586346

Hudson, C. P., Wood, R., & Maartens, G. (2000). Diagnosing HIV-associated tuberculosis: reducing costs and diagnostic delay. *Int J Tuberc Lung Dis, 4*(3), 240-245. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10751070

Hulley, S. B., Cumming, S. R., Browner, W. S., Grady D. G., and Newman, T. B. (2007). *Designing Clinical Research* (3rd Edition ed.). USA: Lippincott and Wilkins.

Huo, Z. Y., & Peng, L. (2016). Accuracy of the interferon-gamma release assay for the diagnosis of active tuberculosis among HIV-seropositive individuals: a systematic review and meta-analysis. *BMC Infect Dis, 16*, 350. doi:10.1186/s12879-016-1687-810.1186/s12879-016-1687-8 [pii]

Hussain, S., Afzal, N., Javaid, K., Ullah, M. I., Ahmad, T., & Saleem Uz, Z. (2010). Level of interferon gamma in the blood of tuberculosis patients. *Iran J Immunol, 7*(4), 240-246. doi:IJIv7i4A6 06 [pii]

Jamil, B., Shahid, F., Hasan, Z., Nasir, N., Razzaki, T., Dawood, G., & Hussain, R. (2007). Interferon gamma/IL10 ratio defines the disease severity in pulmonary and extra pulmonary tuberculosis. *Tuberculosis (Edinb), 87*(4), 279-287. doi:S1472-9792(07)00036-4 [pii]10.1016/j.tube.2007.03.004

Johnson, C. M., Cooper, A. M., Frank, A. A., Bonorino, C. B., Wysoki, L. J., & Orme, I. M. (1997). Mycobacterium tuberculosis aerogenic rechallenge infections in B cell-deficient mice. *Tuber Lung Dis, 78*(5-6), 257-261. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10209680

Johnson, J. L., & Ellner, J. J. (2006). *Tuberculosis and atypical mycobacterial infection. In: Tropical infectious disease, principles, pathogenesis and practice, Guerrant RL, Walker DH, Weller PF (eds)* (2nd edn ed.): Churchill living stone.

Jouanguy, E., Altare, F., Lamhamedi, S., Revy, P., Emile, J. F., Newport, M., . . . Casanova, J. L. (1996). Interferon-gamma-receptor deficiency in an infant with fatal bacille

Calmette-Guerin infection. *N Engl J Med, 335*(26), 1956-1961. doi:10.1056/NEJM199612263352604

Jouanguy, E., Doffinger, R., Dupuis, S., Pallier, A., Altare, F., & Casanova, J. L. (1999). IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol, 11*(3), 346-351. doi:imb313 [pii]

Juarez, E., Carranza, C., Hernandez-Sanchez, F., Leon-Contreras, J. C., Hernandez-Pando, R., Escobedo, D., . . . Sada, E. (2012). NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans. *Eur J Immunol, 42*(4), 880-889. doi:10.1002/eji.201142105

Katoch, V. M. (2004). Newer diagnostic techniques for tuberculosis. *Indian J Med Res, 120*(4), 418-428. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15520490

Kaufmann, S. H. (2002). Protection against tuberculosis: cytokines, T cells, and macrophages. *Ann Rheum Dis, 61 Suppl 2*, ii54-58. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12379623

Keane, J. (2005). TNF-blocking agents and tuberculosis: new drugs illuminate an old topic. *Rheumatology (Oxford), 44*(6), 714-720. doi:keh567 [pii] 10.1093/rheumatology/keh567

Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W. D., . . . Braun, M. M. (2001). Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med, 345*(15), 1098-1104. doi:10.1056/NEJMoa011110

Kellar, K. L., Gehrke, J., Weis, S. E., Mahmutovic-Mayhew, A., Davila, B., Zajdowicz, M. J., . . . Mazurek, G. H. (2011). Multiple cytokines are released when blood from patients with tuberculosis is stimulated with Mycobacterium tuberculosis antigens. *PLoS One, 6*(11), e26545. doi:10.1371/journal.pone.0026545 PONE-D-11-09968 [pii]

Kerry A. Millington, J. A. I., Sarah Hackforth, Timothy S. C. Hinks, Jonathan J. Deeks, Davinder P. S, Dosanjh, Valerie Guyot-Revol, Rubamalaar Gunatheesan, Paul Klenerman, Ajit Lalvani. (2007). Dynamic relationship between IFN-γ and IL-2 profile of Mycobacterium tuberculosis-specific T cells and antigen load *Journal of Immunology, 178*(8), 5217-5226.

Koch, R. (1882). Die Aetiologie der Tuberculose. [The aetiology of Tuberculosis.] *In: Berliner Klinische Wochenschrift., Bd.19*(Nr. 15), S. 221–230.

Ladel, C. H., Blum, C., Dreher, A., Reifenberg, K., Kopf, M., & Kaufmann, S. H. (1997). Lethal tuberculosis in interleukin-6-deficient mutant mice. *Infect Immun, 65*(11), 4843-4849. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9353074

Lawn, S. D., Butera, S. T., & Shinnick, T. M. (2002). Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis. *Microbes Infect, 4*(6), 635-646. doi:S1286457902015824 [pii]

Li, Y. J., Petrofsky, M., & Bermudez, L. E. (2002). Mycobacterium tuberculosis uptake by recipient host macrophages is influenced by environmental conditions in the granuloma of the infectious individual and is associated with impaired production of interleukin-12 and tumor necrosis factor alpha. *Infect Immun, 70*(11), 6223-6230. doi:10.1128/iai.70.11.6223-6230.2002

Lichtenauer-Kaligis, E. G., de Boer, T., Verreck, F. A., van Voorden, S., Hoeve, M. A., van de Vosse, E., . . . Ottenhoff, T. H. (2003). Severe Mycobacterium bovis BCG infections in a large series of novel IL-12 receptor beta1 deficient patients and evidence for the existence of partial IL-12 receptor beta1 deficiency. *Eur J Immunol, 33*(1), 59-69. doi:10.1002/immu.200390008

Mandrekar, J. N. (2010a). Receiver operating characteristic curve in diagnostic test assessment. *J Thorac Oncol, 5*(9), 1315-1316. doi:10.1097/JTO.0b013e3181ec173dS1556-0864(15)30604-3 [pii]

Mandrekar, J. N. (2010b). Simple statistical measures for diagnostic accuracy assessment. *J Thorac Oncol, 5*(6), 763-764. doi:10.1097/JTO.0b013e3181dab122 S1556-0864(15)30501-3 [pii]

Mekori, Y. A., & Metcalfe, D. D. (2000). Mast cells in innate immunity. *Immunol Rev, 173*, 131-140. doi:10.1034/j.1600-065x.2000.917305.x

Meng, Q., Sayin, I., Canaday, D. H., Mayanja-Kizza, H., Baseke, J., & Toossi, Z. (2016). Immune Activation at Sites of HIV/TB Co-Infection Contributes to the Pathogenesis of HIV-1 Disease. *PLoS One, 11*(11), e0166954. doi:10.1371/journal.pone.0166954 PONE-D-16-16270 [pii]

Millington, K. A., Innes, J. A., Hackforth, S., Hinks, T. S., Deeks, J. J., Dosanjh, D. P., . . . Lalvani, A. (2007). Dynamic relationship between IFN-gamma and IL-2 profile of Mycobacterium tuberculosis-specific T cells and antigen load. *J Immunol, 178*(8), 5217-5226. doi:178/8/5217 [pii]

MOH. (2005). *Tuberculosis, Leprosy and TB/HIV prevention and control programmanual*, (3rd edition ed.), Addis Ababa

Morris, L., Martin, D. J., Bredell, H., Nyoka, S. N., Sacks, L., Pendle, S., . . . Chaisson, R. E. (2003). Human immunodeficiency virus-1 RNA levels and CD4 lymphocyte counts, during treatment for active tuberculosis, in South African patients. *J Infect Dis, 187*(12), 1967-1971. doi:JID30054 [pii]10.1086/375346

Munoz, S., Hernandez-Pando, R., Abraham, S. N., & Enciso, J. A. (2003). Mast cell activation by Mycobacterium tuberculosis: mediator release and role of CD48. *J Immunol, 170*(11), 5590-5596. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12759438

Nemeth, J., Winkler, H. M., Zwick, R. H., Muller, C., Rumetshofer, R., Boeck, L., . . . Winkler, S. (2012). Peripheral T cell cytokine responses for diagnosis of active tuberculosis. *PLoS One, 7*(4), e35290. doi:10.1371/journal.pone.0035290 PONE-D-11-23207 [pii]

Nicod, L. P. (2007). Immunology of tuberculosis. *Swiss Med Wkly, 137*(25-26), 357-362. doi:smw-11499 [pii] 2007/25/smw-11499

North, R. J. (1998). Mice incapable of making IL-4 or IL-10 display normal resistance to infection with Mycobacterium tuberculosis. *Clin Exp Immunol, 113*(1), 55-58. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9697983

Oddo, M., Renno, T., Attinger, A., Bakker, T., MacDonald, H. R., & Meylan, P. R. (1998). Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. *J Immunol, 160*(11), 5448-5454. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9605147

Oswald, I. P., Gazzinelli, R. T., Sher, A., & James, S. L. (1992). IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. *J Immunol, 148*(11), 3578-3582. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1588047

Ottenhoff, T. H., & Kaufmann, S. H. (2012). Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog, 8*(5), e1002607. doi:10.1371/journal.ppat.1002607, PPATHOGENS-D-11-02662 [pii]

Ottenhoff, T. H., Kumararatne, D., & Casanova, J. L. (1998). Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria. *Immunol Today, 19*(11), 491-494. doi:S0167569998013218 [pii]

Padmapriyadarsini, C., Narendran, G., & Swaminathan, S. (2011). Diagnosis & treatment of tuberculosis in HIV co-infected patients. *Indian J Med Res, 134*(6), 850-865. doi:10.4103/0971-5916.92630, IndianJMedRes_2011_134_6_850_92630 [pii]

Pai, M., Zwerling, A., & Menzies, D. (2008). Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann Intern Med, 149*(3), 177-184. doi:0000605-200808050-00241 [pii],10.7326/0003-4819-149-3-200808050-00241

Pai, R. K., Convery, M., Hamilton, T. A., Boom, W. H., & Harding, C. V. (2003). Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from Mycobacterium tuberculosis: a potential mechanism for immune evasion. *J Immunol, 171*(1), 175-184. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12816996

Perkins, M. D., & Cunningham, J. (2007). Facing the crisis: improving the diagnosis of tuberculosis in the HIV era. *J Infect Dis, 196 Suppl 1*, S15-27. doi:JID36838 [pii] 10.1086/518656

Pieters, J. (2001). Entry and survival of pathogenic mycobacteria in macrophages. *Microbes Infect, 3*(3), 249-255. doi:S1286-4579(01)01376-4 [pii]

Prabhavathi, M., Kabeer, B. S., Deenadayalan, A., & Raja, A. (2015). Role of QuantiFERON-TB Gold antigen-specific IL-1beta in diagnosis of active tuberculosis. *Med Microbiol Immunol, 204*(5), 567-574. doi:10.1007/s00430-014-0382-x

Qiao, D., Yang, B. Y., Li, L., Ma, J. J., Zhang, X. L., Lao, S. H., & Wu, C. Y. (2011). ESAT-6- and CFP-10-specific Th1, Th22 and Th17 cells in tuberculous pleurisy may contribute to the local immune response against Mycobacterium tuberculosis infection. *Scand J Immunol, 73*(4), 330-337. doi:10.1111/j.1365-3083.2011.02512.x

Raja, A. (2004). Immunology of tuberculosis. *Indian J Med Res, 120*(4), 213-232. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15520479

Raja, A., Uma Devi, K. R., Ramalingam, B., & Brennan, P. J. (2002). Immunoglobulin G, A, and M responses in serum and circulating immune complexes elicited by the 16-kilodalton antigen of Mycobacterium tuberculosis. *Clin Diagn Lab Immunol, 9*(2), 308-312. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11874868

Rangaka, M. X., Gideon, H. P., Wilkinson, K. A., Pai, M., Mwansa-Kambafwile, J., Maartens, G., . . . Wilkinson, R. J. (2012). Interferon release does not add discriminatory value to smear-negative HIV-tuberculosis algorithms. *Eur Respir J, 39*(1), 163-171. doi:10.1183/09031936.00058911, 09031936.00058911 [pii]

Raviglione, M. C., Gupta, R., Dye, C. M., & Espinal, M. A. (2001). The burden of drug-resistant tuberculosis and mechanisms for its control. *Ann N Y Acad Sci, 953*, 88-97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11795426

Redford, P. S., Murray, P. J., & O'Garra, A. (2011). The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunol, 4*(3), 261-270. doi:10.1038/mi.2011.7mi20117 [pii]

Redpath, S., Ghazal, P., & Gascoigne, N. R. (2001). Hijacking and exploitation of IL-10 by intracellular pathogens. *Trends Microbiol, 9*(2), 86-92. doi:S0966-842X(00)01919-3 [pii]

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., . . . Barry, C. E., 3rd. (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature, 431*(7004), 84-87. doi:10.1038/nature02837nature02837 [pii]

Rosenkrands, I., Slayden, R. A., Crawford, J., Aagaard, C., Barry, C. E., 3rd, & Andersen, P. (2002). Hypoxic response of Mycobacterium tuberculosis studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. *J Bacteriol, 184*(13), 3485-3491. doi:10.1128/jb.184.13.3485-3491.2002

Russell, M. S., Dudani, R., Krishnan, L., & Sad, S. (2009). IFN-gamma expressed by T cells regulates the persistence of antigen presentation by limiting the survival of dendritic cells. *J Immunol, 183*(12), 7710-7718. doi:10.4049/jimmunol.0901274 jimmunol.0901274 [pii]

Saunders, B. M., Frank, A. A., Orme, I. M., & Cooper, A. M. (2000). Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection. *Infect Immun, 68*(6), 3322-3326. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10816480

Saunders, B. M., Tran, S., Ruuls, S., Sedgwick, J. D., Briscoe, H., & Britton, W. J. (2005). Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of Mycobacterium tuberculosis infection. *J Immunol, 174*(8), 4852-4859. doi:174/8/4852 [pii]

Scanga, C. A., Mohan, V. P., Tanaka, K., Alland, D., Flynn, J. L., & Chan, J. (2001). The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice. *Infect Immun, 69*(12), 7711-7717. doi:10.1128/IAI.69.12.7711-7717.2001

Scanga, C. A., Mohan, V. P., Yu, K., Joseph, H., Tanaka, K., Chan, J., & Flynn, J. L. (2000). Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. *J Exp Med, 192*(3), 347-358. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10934223

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S. C., & Dinarello, C. A. (1990). Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood, 75*(1), 40-47. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2294996

Seah, G. T., & Rook, G. A. (2001). Il-4 influences apoptosis of mycobacterium-reactive lymphocytes in the presence of TNF-alpha. *J Immunol, 167*(3), 1230-1237. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11466338

Serbina, N. V., Lazarevic, V., & Flynn, J. L. (2001). CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection. *J Immunol, 167*(12), 6991-7000. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11739519

Sester, U., Fousse, M., Dirks, J., Mack, U., Prasse, A., Singh, M., . . . Sester, M. (2011). Whole-blood flow-cytometric analysis of antigen-specific CD4 T-cell cytokine profiles distinguishes active tuberculosis from non-active states. *PLoS One, 6*(3), e17813. doi:10.1371/journal.pone.0017813

Sharma, S., & Bose, M. (2001). Role of cytokines in immune response to pulmonary tuberculosis. *Asian Pac J Allergy Immunol, 19*(3), 213-219. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11826917

Siika, A. M., Ayuo, P. O., Sidle, M. J., Wools-Kaloustian, K., Kimaiyo, S. N., & Tierney, W. M. (2008). Admission characteristics, diagnoses and outcomes of HIV-infected patients registered in an ambulatory HIV-care programme in western Kenya.

East Afr Med J, 85(11), 523-528. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19413204

Singer, B. H., & Kirschner, D. E. (2004). Influence of backward bifurcation on interpretation of r(0) in a model of epidemic tuberculosis with reinfection. *Math Biosci Eng, 1*(1), 81-93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20369961

Sugawara, I., Yamada, H., Kaneko, H., Mizuno, S., Takeda, K., & Akira, S. (1999). Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. *Infect Immun, 67*(5), 2585-2589. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10225924

Syed Ahamed Kabeer, B., Sikhamani, R., Swaminathan, S., Perumal, V., Paramasivam, P., & Raja, A. (2009). Role of interferon gamma release assay in active TB diagnosis among HIV infected individuals. *PLoS One, 4*(5), e5718. doi:10.1371/journal.pone.0005718

Teitelbaum, R., Schubert, W., Gunther, L., Kress, Y., Macaluso, F., Pollard, J. W., . . . Bloom, B. R. (1999). The M cell as a portal of entry to the lung for the bacterial pathogen Mycobacterium tuberculosis. *Immunity, 10*(6), 641-650. doi:S1074-7613(00)80063-1 [pii]10.1016/s1074-7613(00)80063-1UNAIDS/WHO. AIDS epidemic update.

van Crevel, R., Ottenhoff, T. H., & van der Meer, J. W. (2002). Innate immunity to Mycobacterium tuberculosis. *Clin Microbiol Rev, 15*(2), 294-309. doi:10.1128/cmr.15.2.294-309.2002

van Pinxteren, L. A., Cassidy, J. P., Smedegaard, B. H., Agger, E. M., & Andersen, P. (2000). Control of latent Mycobacterium tuberculosis infection is dependent on CD8 T cells. *Eur J Immunol, 30*(12), 3689-3698. doi:10.1002/1521-4141(200012)30:12<3689::AID-IMMU3689>3.0.CO;2-4 [pii] 10.1002/1521-4141(200012)30:12<3689::AID-IMMU3689>3.0.CO;2-4

Wallis, R. S., Pai, M., Menzies, D., Doherty, T. M., Walzl, G., Perkins, M. D., & Zumla, A. (2010). Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. *Lancet, 375*(9729), 1920-1937. doi:10.1016/S0140-6736(10)60359-5S0140-6736(10)60359-5 [pii]

Walzl, G., Ronacher, K., Hanekom, W., Scriba, T. J., & Zumla, A. (2011). Immunological biomarkers of tuberculosis. *Nat Rev Immunol, 11*(5), 343-354. doi:10.1038/nri2960nri2960 [pii]

Wang, C. H., Lin, H. C., Liu, C. Y., Huang, K. H., Huang, T. T., Yu, C. T., & Kuo, H. P. (2001). Upregulation of inducible nitric oxide synthase and cytokine secretion in peripheral blood monocytes from pulmonary tuberculosis patients. *Int J Tuberc Lung Dis, 5*(3), 283-291. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11326829

Wang, X., Zhang, J., Liang, J., Zhang, Y., Teng, X., Yuan, X., & Fan, X. (2015). Protection against Mycobacterium tuberculosis infection offered by a new multistage subunit vaccine correlates with increased number of IFN-gamma+ IL-2+ CD4+ and IFN-gamma+ CD8+ T cells. *PLoS One, 10*(3), e0122560. doi:10.1371/journal.pone.0122560, PONE-D-14-49407 [pii]WHO. (2011). Country TB profile.

WHO. (2015). Global tuberculosis control e surveillance,planning, financing. Geneva: WHO Report; 2015 (WHO/HTM/TB/2015.08).

WHO. (2018). Global Tuberculosis Report Retrieved from www.who.int/tb/publications/global_report/en/.

Winkler, S., Necek, M., Winkler, H., Adegnika, A. A., Perkmann, T., Ramharter, M., & Kremsner, P. G. (2005). Increased specific T cell cytokine responses in patients

with active pulmonary tuberculosis from Central Africa. *Microbes Infect, 7*(9-10), 1161-1169. doi:S1286-4579(05)00110-3 [pii] 10.1016/j.micinf.2005.03.020

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol, 28*, 445-489. doi:10.1146/annurev-immunol-030409-101212

APPENDICES

Appendix I

1.0 DEMOGRAPHICS QUESTIONNAIRE

COMPARISON OF IMMUNE PROFILES WITH SPUTUM SMEARS STATUS IN TUBERCULOSIS PATIENTS CO-INFECTED WITH HUMAN IMMUNODEFIENCY VIRUS

Demographics

Name …… Form No. ……..

Gender …….

Age ……..

AMPATH No. …….

Inpatient No. ……

Clinical data

1. Site of TB infection: ☐ Pulmonary ☐ Extrapulmonary (Please specify) _____
2. Infection type: ☐ New ☐ Retreatment ☐ Re-infection
3. Has the patient ever been on prophylaxis for TB? ☐ *Yes* ☐ *No*

If yes, when was it done (month and year) _____

Section 4: Results of tests performed

1. Sputum AFB staining: ☐ *Negative* ☐ *Positive*
2. Sputum culture: ☐ *Negative* ☐ *Positive*
3. CD4 count _____ CD8 count_____

   CD19 count _____ CD16/56 count_____

   IFN-γ, _____ TNF-α, _____

   IL-2, _____ IL-4, _____

   IL-6, _____ and IL-8 _____

   IL-10 _____ IL-12p70 _____

   Interviewed by _____

Appendix II

2.0 FLOW CYTOMETRY TO DETERMINE CD3/CD4/CD8/CD16/CD56 /CD 45/CD19

MultiTEST IMK Kit Reagent

1. INTENDED USE

BD MultiTEST IMK kit is a four-color direct immunofluoresence reagent kit for use with a suitably equipped flow cytometer to identify and determine the percentage and absolute counts of the following mature human lymphocyte ($CD3^+$), B lymphocyte ($CD19^+$), helper/inducer T lymphocytes($CD3^+CD4^+$), suppressor/cytotoxic T lymphocytes ($CD3^+CD8^+$) and natural killer lymphocytes ($CD3^-CD16^+$ and/or $CD56^+$). TruCOUNT Tubes are used for determining absolute counts.

2. SUMMARY AND EXPLANATION

Human lymphocyte can be divided into three major populations based on their biologic function and cell-surface antigen expression: T lymphocyte, B lymphocytes, and natural killer (NK) lymphocytes.

3. PRINCIPLES OF THE PROCEDURE

When whole blood is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leucocyte surface antigens. The stained samples are treated with MultiTEST IMK Kit lysing solution to lyse erthrocytes. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter and fluorescence signals, detected by the instrument provide information about the cell's size, internal complexity, and relative fluorescence intensity.

MultiTEST reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population to reduce contamination of unlysed or nucleated red blood cells in the gate.

When TruCOUNT Tubes are used, a known volume of sample is stained directly in a TruCOUNT Tube. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells/µl) of positive cells in the sample can be determined by comparing cellular events to bead events. If appropriate software such as MultiSET is used, absolute counts are determined by the software. If manually performing data analysis using software such as CellQuest, simply divide the number of positive cellular events by the number of bead events, then multiply by the TRuCOUNT bead concentration.

4. REAGENTS

BD MultiTEST IMK kit, sufficient for 50 tests when used as directed, is supplied as one vial each of the following reagents:

- MultiTEST CD3/CD8/CD45/CD4 reagent provided in 1ml of buffered saline with 0.1% sodium azide. It contains CD3 fluorescence isothiocynate (FITC), clone SK7, CD8 phycoerthrin (PE), clone SKI, CD45 peridinin chlorophyll protein (PerCP), clone 2D1 (HLe-1), and CD4 allophycocyanin (APC), CLONE SK3.
- MultiTEST CD3/CD16+CD56/CD45/CD19 reagent provided in 1ml of buffered saline with 0.1% sodium azide. It contains FITC-labeled CD3, clone SK7, PE-labeled CD16, clone B73.1, and CD56, clone NCAM 16.2, PerCP labeled CD45, clone 2D1 (HLe-1), and APC-labeled CD19, clone SJ25C1.
- MultiTEST IMK kit Lysing Solution, 10X concentrate, a proprietary buffered solution containing <15% formaldehyde and <50% diethylene glycol.

5. INSTRUMENT

MultiTEST IMK kit and TruCOUNT Tubes are designed for use on a flow cytometer equipped with appropriate computer hardware and software. BD recommends the FACScalibur or FACSort flow cytometer; however results can be achieved using other platforms. The cytometer must be equipped with 635nm and 488nm lasers and must be capable of detecting in four ranges: 515-545nm, 562-607nm, >650nm and 652-668nm. It must be able to threshold or discriminate using the >650nm channel. The FACS Loader can also be used with product.

BD recommends using CaliBRITE beads and FACSComp software, version 4.0 or later, for setting the fluorescence compensation, and checking instrument sensitivity before use. For users of flow cytometers manufactured by companies other than BD, refer to the manufacturer's instructions for setting up four-color immunophenotyping.

BD has developed software applications such as MultiSET that automatically calculate absolute counts when TruCOUNT Tubes are used. However, other software packages can be used for data acquisition and analysis and the absolute counts can be calculated manually.

6. SPECIMEN COLLECTION AND PREPARATION

Collect blood aspectically by venipuncture into a sterile K_3 EDTA (ethylenediamine-tetraacetic acid) VACUNTAINER blood collection tube (lavender top). MultiSET IMK Kit reagents and TruCOUNT Tubes have been validated with both liquid and dry formulation of K_3 EDTA.

A minimum of 200µL of whole blood is required for this procedure. Follow the collection tube manufacturer's guidelines for the minimum volume of blood to be collected to ensure proper specimen dilution, especially when determining absolute counts with TruCOUNT beads.

Obtain a white blood cell (WBC) count and a differential white cell count from the same whole blood sample before staining to ensure that the WBC count is within the linear range or to calculate absolute counts from percentages.

Anticoagulated blood stored at room temperature (20-25 ^{0}C) must be stained within 48 hours of draw and then analyzed within 24 hours of staining.

Interfering conditions

Do not use previously fixed and stored patient specimens. Whole blood samples refrigerated before staining may give aberrant results. Samples obtained from patients taking immunosuppressive drugs can yield poor resolution. Blast cells can interfere with test result. Hemelolyzed samples should be rejected.

Procedure

Reagents provided

- MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
- MultiTEST CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC
- TruCOUNT TuBES (IMK Kit Catalog No. 340504)
- MultiTEST IMK Kit lysing Solution, 10X concentrate.

Reagents and materials required but not provided

1. CaliBRITE 3 and APC beads (BD Catalog Nos. 340486 and 340487, respectively)
2. IX MultiTEST IMK Kit Lysing Solution, diluted as indicated below.
3. Reagent-grade (distilled or deionized) water
4. K_3 EDTA VACUTAINER blood collection tubes (BD Catalog No. 366457), or equivalent (if not using TruCOUNT Tubes)

5. Disposable 12 x 75-mm Falcon capped polystyrene test tube (BD Catalog No. 352058), or equivalent (if not using TruCOUNT Tubes)
6. Vortex mixer
7. Micropippettor with tips (BD Electronic pipette, Catalog No. 343246 [US], or 343208 [Europe]; Pipetman, Rainin Instrument Co. Inc; or equivalent)
8. Bulk dispenser or pipettor (450µL) for dispensing 1X MultiTEST Lysing Solution.
9. Sheath fluid (FACSFlow, BD Catalog No. 340398 [US and Latin America] or 342003 or equivalent)
10. TruCOUNT controls (BD Catalog 340335), necessary if using TruCOUNT Tubes
11. Lysable whole blood control (available commercially)

Dilution instructions for MultiTEST IMK Kit Lysing Solution

Dilute the 10X concentrate 1:10 with room temperature (20-25), deionized water. The prepared solution is stable for 1 Month when store in a glass container at room temperature.

Staining the cells

Lyse red blood cells following staining using diluted (IX) MultiTEST Lysing Solution. Use care to protect the tube from direct light. Perform the procedure at room temperature (20-25^{0}C).

Staining

1. For each patient sample, label two 12 x 75-mm tubes with the sample identification number. Use letters such as A and B to differentiate the two tubes.

For absolute counts, label two TruCOUNT Tubes in place of the 12 x 75-mm tubes.

NOTE: Before use, verify that the TruCOUNT bead is intact and within the metal retainer at the bottom of tube. If this not the case, discard the TruCOUNT Tube and replace it with another.

2. Pipette 20µL of MultiTEST CD3/CD8/CD45/CD4 reagent into the bottom of each tube labeled A. pipette 20µLof MultiTEST CD3/CD16 + CD56/CD19 reagent into the bottom of each tube labeled B.

If using TruCOUNT Tubes, pipette just above the stainless retainer. Do not touch the pellet.

3. Pipette 50 µL of well-mixed, anticoagulated whole into the bottom of each tube.

NOTE: Avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with reagent.

When using TruCOUNT Tubes, accuracy is critical. Use the reverse pipetting technique to pipette samples onto the side of the tube just above the retainer.

4. Cap the tubes and vortex gently to mix. Incubate for 15 minutes in the dark room at room temperature (20-25^{0}C).
5. Add 450 µL IX MultiTEST Lysing Solution to each tube.
6. Cap the tubes and vortex gently to mix. Incubate for 15 minutes in the dark room temperature (20-25^{0}C). The samples are now ready to be analyzed on the flow cytometer.

Flow cytometry

If samples are not to be analyzed immediately after preparation, store them in the dark at room temperature (20-25^{0}C).

Vortex the cells thoroughly (at low speed) to reduce aggregation before running them on the flow cytometer

Quality control

Run a control sample daily from a normal adult subject or a commercially available whole blood control to optimize instrument settings and as a quality control check of the system.

Use commercial controls providing established values for percent positive and absolute counts with each run to assess system performance.

Visually inspect the CD45 vs SSC dot plot. The lymphocyte population should appear as a bright, compact cluster with low SSC. Monocyte and granulocyte should appear as distinct clusters. Do not proceed with analysis if populations are diffuse and there is little or no separation between clusters.

Results

Results are reported as the percentage of positive lymphocyte population or as the number of positive cells per microliter of blood (absolute count).

Appendix III

3.0 ZIEL NELSEEN FOR AFB STAINING

REQUIREMENTS

-0.5% Carbol fuchsin

-0.3 methyleen blue

-decoulourizer, either 3% acid alcohol or 25% sulphuric acid

- Bunsen burner or spirit lamp

-Tap water

Principle

Some microorganism especially of the family *mycobactericea*, on their cell wall have mycolic acid which when it come into contact with some dyes forms a complex, which resist decolourization,with strong acid/alcohol.

Method:

- On a clean, grease- free, slide prepare a smear, 1cm by 2cm. leave it to air-dry
- Heat fix by passing I over the flame for 3-4 seconds
- Flood with 0.5% carbol fuchsin, heat to steam, don't boil. Leave it for 5min
- Wash with tap water
- Decolorize with any decolorizing agent. Leave it for 3min
- Wash with tap water
- Flood with methyleen blue
- Leave it for 2min

- Wash with tap water, leave it to dry.
- Observe under X100 using oil immersion. Observe for red pink rods over a blue background

Appendix IV

4.0 LOWENSTEIN - JENSEN MEDIUM

Principles of the Procedure

L-Asparagine and Potato Flour are sources of nitrogen and vitamins in Lowenstein-Jensen Medium. Monopotassium Phosphate and Magnesium Sulfate enhance organism growth and act as buffers. Glycerol and the Egg Suspension provide fatty acids and protein required for the metabolism of mycobacteria. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. Sodium Citrate and Malachite Green are selective agents to prevent growth of most contaminants and allow early growth of mycobacteria.

Lowenstein-Jensen Medium Slants and Bottles

1. Inoculate.
    a. Using sterile disposable 0.01 mL calibrated loops, inoculate the slants or bottles using mycobacterial cultures prepared as described above.
    b. Incubate containers with loosened caps at 35 ± 2°C in an aerobic atmosphere supplemented with carbon dioxide.
2. Examine tubes or bottles after 7, 14 and 21 days for growth, selectivity and pigmentation.

Quality Control

1. Visually examine representative tubes or bottles to assure that any existing physical defects will not interfere with use.
2. Determine the pH potentiometrically at room temperature for adherence to the specification of 7.0 ± 0.2.
3. Incubate uninoculated representative tubes or bottles at 20–25°C and 30–35°C and examine after 7 and 14 days for microbial contamination.

Appendix V

5.0 Institutional Research and Ethics Committee (IREC) approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET

Reference: IREC/2013/239
Approval Number: 0001163

22nd April, 2014

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
22 APR 2014
APPROVED
P.O. Box 4606-30100 ELDORET

Job Kisuya,
Moi University,
School of Public Health,
P.O. Box 4606-30100,
ELDORET-KENYA.

Dear Mr. Kisuya,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:-

"The Role of Cytokine in Immune Response to Pulmonary Tuberculosis Smear Status in Patients Co-Infected with Human Immunodeficiency".

Your proposal has been granted a Formal Approval Number: ***FAN: IREC 1163*** on 22nd April, 2014. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 21st April, 2015. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc Director - MTRH
Principal - CHS
Dean - SOM
Dean - SOP
Dean - SON
Dean - SOD

Appendix VI

6.0 Proposal Approval

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PG/PHD/00028/2012

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 26th February, 2014

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR WAPANG'ANA JOB KISUYA—PG/PHD/00028/2012

The above named is registered in the Doctor of Philosophy in Immunology Programme of the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "The Role of Cytokine in Immune Response to Pulmonary Tuberculosis Smear Status in Patients Co-infected with Human Immunodeficiency Virus" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Dr. Pauline Andang'o
ASSOCIATE DEAN, SCHOOL OF GRADUATE STUDIES

Appendix VII

7.0 INFORMED CONSENT

THE ROLE OF CYTOKINES IN IMMUNE RESPONSE TO PULMONARY TUBERCULOSIS CO-INFECTED WITH HUMAN IMMUNODEFIENCY VIRUS

You must read this preview to the respondent and proceed with interview only after she/he gives consent.

Good morning, my name is Job Kisuya and I am a student at Maseno University, School of Public Health and Community Development; Department of Biomedical Science and Technology carrying out a research on the role of cytokines in immune response to pulmonary tuberculosis co-infected with Human Immunodeficiency Virus. The research has been approved by Moi Teaching and Referral Hospital, Institutional Research and Ethics Committee and Maseno University. (Show IREC letter if Respondent wishes to see). It will involve obtaining of two sputum samples (*spot and early morning samples*), and 4ml blood sample from each of the suspected pulmonary tuberculosis patient who accepts to participate in the study. The sputum sample will be used to determine your smear status so as know whether you have TB or not while the blood samples will be used to determine a distinct cytokine profile (diagnostic markers) that can be useful for diagnosis of TB. All information collected will be handled confidentially and kept under lock and key.

Benefits

This research and findings will be used to improve diagnosis of tuberculosis with use of immune profiles. We intend to develop diagnostic markers that will be used to confirm or rule out active TB at point of diagnosis and patient receives treatment promptly.

Risks

I understand that matters pertaining heath are sensitive and most people would rather not talk about, but I guarantee that all information got from research will be confidential and that your name will not at any time be linked to findings when they are released. If you choose not to participant there will no harm in any way. You will experience a mild pain and may be bruises when blood will be drawn other than there will no other risk.

If you will participate in the process will last 30 minutes. In case of any questions in future, you are free to contact the research team members and their contacts are given below. Can we continue?

Participant: Signature: **Date**

Interviewer: Signature: **Date**

Contacts for Research Team:

1. Job Kisuya – Moi University (0721-884-347)
2. Prof. Collins Ouma – Maseno University (0722-381-214)
3. Prof. Alex Chemtai – Moi University (0722-903-078)

Appendix VIII

8.0 Abstract presented at JKUAT-3RD Africa International Biotechnology and Biomedical Conference (AIBBC), September 13th and 14th 2017

The diagnostic accuracy of Th1 (IFN-γ, TNF-α, and IL-2) and Th2 (IL-4, IL6 and IL-10) cytokine response in AFB microscopy smear negative PTB- HIV co-infected patients

Job Kisuya[1, 2], Alex Chemtai[3], Evans Raballah[4], Alfred Keter[2], Collins Ouma[1, 5, 6]

1. Department of Biomedical Science and Technology, Maseno University, Private Bag, Maseno, Kenya, **2.** Academic Model for Providing Access to Healthcare (AMPATH), P.O Box 4606-30100 Eldoret, Kenya, **3.** Department of Immunology, Moi University, P.O. Box 4606-30100, Eldoret, Kenya, **4.** Department of Medical Laboratory Sciences, Masinde Muliro University of Science and Technology, P.O. Box 190-50100, Kakamega, Kenya, **5,.** Centre for Global Health Research/Kenya Medical Research Institute, P.O. Box 1578-40100, Kisumu, Kenya, **6.** Ideal Research Centre, P.O. Box 7244-40123, Kisumu, Kenya

Background/Objectives: Acid Fast Bacilli (AFB) microscopy smear is the most widely used technique for Pulmonary Tuberculosis (PTB) diagnosis in low middle income countries. Although it is highly specific, the sensitivity varies between 20 and 80% in immune competent people, with only 50% case detection among HIV/TB co-infected patients, therefore the need to evaluate AFB negative smears to determine the PTB status.

Methods: We recruited a total of 86 participants, who were screened for PTB by AFB smear microscopy. A total of 70 (81.4%) were AFB smear negative while 16 (18.4%) were AFB smear positive (Table 1). The respective sputum AFB smear negative samples were then cultured using Lowenstein Jensen Medium with 46 being culture-positive and 24 being culture-negative. Furthermore, a blood sample was collected to evaluate cytokine profiles using Enzyme-Linked Immunosorbent Assay (ELISA). Statistical analyses were performed using R software. Independent samples t-test was used to compare means between the AFB smear negative but culture-positive and AFB smear negative but culture-negative while the medians were compared using two-sample Wilcoxon rank sum test. To determine the predictive power of Th1 and Th2 on PTB culture status among those who screened negative for AFB microscopy smears, binary logistic regression models were fitted for Th1/Th2 covariates and Receiver Operating Characteristic (ROC) curves plotted. In all analyses, P≤0.05 were considered statistically significant.

Results and Conclusion: Among the participants who had initially screened negative for AFB microscopy smears, Th1 cytokines IFN-γ (P<0.001), TNF-α (P=0.004) and IL-2 (P=0.004) levels were elevated in PTB culture-positive compared to PTB culture-negative in participants (Table 2). In addition, Th2 cytokine, IL-4 median levels were elevated in PTB culture-positive compared to PTB culture-negative among the participants who had initially screened negative for AFB microscopy smears.
Finally, when Th1 cytokines (IFN-γ, TNF-α and IL-2) and Th2 cytokines (IL-4) were separately included in the binary logistic regression models of PTB outcome, the predictive power of discriminating between those who were AFB smear negative in the diagnosis of PTB was good with area under the curves (AUC) being 69.2%, and 79.6 for Th1 and Th2, respectively (Table 1 and Table 2). This study provides evidence for the ability of Th1 and Th2 in separately determining PTB status in AFB microscopy smear negatives patients co-infected with HIV.

Tables

Table 1: Demographic characteristic of relationship AFB microscopy smear status

		AFB Smear Results		
	N	Negative (N=70) Mean (SD) or n (%)	Positive (N=16) Mean (SD) or n (%)	P-value
Age (years)	86	38.8 (12.3)	40.0(11.1)	0.721[a]
Male	42	35 (50.0%)	7 (43.8%)	0.652[b]

There was no difference in the demographic characteristics (age and gender) between those who were AFB smear negative culture-positive and those who were AFB smear negative culture-negative. Compared using [a] independent sample t-test, and [b] Pearson's Chi-square test

Table 2: Relationship between Th1 and Th2 cytokines in PTB culture-negative and -positive participants who screened negative for AFB microscopy smears

		Culture Results		
	N	Negative (N=46) Median (IQR)	Positive (N=24) Median (IQR)	P-value
IFN -γ	70	7.6 (5.6, 16.9)	70.6 (8.6, 202.7)	<0.001
TNF - α	70	16.1 (14.4, 19.2)	19.7 (15.8, 22.9)	0.004
IL-2	70	15.3 (8.4, 26.9)	53.1 (14.1, 122.1)	0.004
IL-4	70	8.6 (1.1, 26.0)	39.5 (4.5, 56.5)	0.009
IL-6	70	12.1 (3.9, 27.6)	14.5 (7.6, 44.0)	0.598
IL-10	70	12.4 (8.4, 15.7)	10.8 (7.1, 13.7)	0.092

Among those who were negative for AFB smear the participants who were confirmed to be having PTB via culture test had significantly higher IFN-γ, TNF-α, and IL-2 profiles compared to those were negative for culture. The participants who were negative for AFB smear but positive for culture had a significantly higher IL-4 profile compared to those were confirmed to be negative for PTB, (P = 0.009). Statistical significance determined by two-sample Wilcoxon rank sum test.

Figures

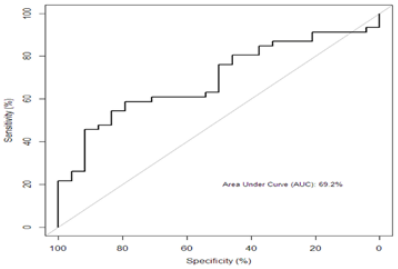

Figure 1: Predictive power of Th1 cytokines on PTB culture status among those who screened negative for AFB microscopy smears

AUC of 69.2% can be interpreted to mean that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group 69.2 percent of the times.

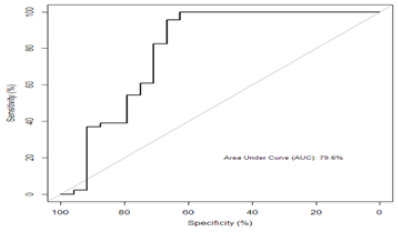

Figure 2: Predictive power of Th2 cytokines on PTB culture status among those who screened negative for AFB microscopy smears

The AUC of 79.6% demonstrates a good predictive power of the model with Th2 cytokines among those who were negative for AFB smear in the diagnosis of PTB. The curve confirms that higher values of Th2 are sensitive for the presence of PTB while lower values are highly specific to absence of PTB.

Appendix IX

9.0 Manuscript published in Pan African Medical Journal

PanAfrican Medical Journal

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Research

The role of *Mycobacterium tuberculosis* antigen specific cytokines in determination of acid fast bacilli culture status in pulmonary tuberculosis patients co-infected with human immunodeficiency virus

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Key words: HIV-TB co-infection, *Mycobacterium tuberculosis*, culture, cytokines

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Abstract

Introduction: The interaction between *Mycobacterium tuberculosis* and HIV leads to rapid progression of tuberculosis (TB) and human immunodeficiency virus (HIV)-induced immunosuppression. Diagnosis of TB in these patients is more difficult due to its atypical presentations giving contradicting results. The overall aim of this study was to evaluate the ability of pro-inflammatory cytokine (Th1) and anti-inflammatory cytokine (Th2) to discriminate between culture-positive and -negative smear status in HIV-TB co-infected patients. **Methods:** In a prospective cohort, a total of 86 study participants were recruited: 46 culture-negative and 40 culture-positive. Blood and sputum samples were collected from all participants. The blood was then analyzed using FACSCalibur flow cytometer to immunophenotype the cells and ELISA performed for cytokine profiles. Sputum samples were analyzed to determine smear status using direct microscopy and Lowenstein Jensen medium. Statistical analyses were performed using R software. Independent samples t-test was used to compare means between the two groups, while the medians were compared using two-sample Wilcoxon rank sum test. Pearson's Chi-square test was used to compare the proportion of male and female participants across the culture and AFB smear status. In order to determine the predictive power of Th1 and Th2 in discriminating Pulmonary Tuberculosis status (PTB) (culture status was used as a confirmatory test), binary logistic regression models were fitted for Th1 covariates [IFN-γ, TNF-α, IL-2 and IL-12(p70)] and Receiver Operating Characteristic (ROC) curves plotted. **Results:** The overall mean age of the participants was 39 years (SD=12), 42% being male. Although, lymphocytes counts were higher in culture-positive relative to culture-negative, the CD8, CD19, and CD16/CD56 were comparable in the two groups. The CD4 counts differed between the two groups (P=0.012). The Th1 showed a better discrimination between culture-positive and -negative PTB individuals; IFN-γ (P=0.001), TNF-α (P=0.001), IL-2 (P=0.001) and IL-12(p70) (P=0.016). The Th2 cytokines (IL-4, IL-6 and IL-10) were comparable between the culture-positive and -negative groups. However, when the combination of Th1 cytokines [IFN-γ, TNF-α, IL-2 and IL-12(p70)] was fitted in binary logistic regression models, the predictive power was high with area under curve (AUC) being 89.7% in discriminating PTB. **Conclusion:** This study provides evidence for the ability of a combination of Th1 cytokines in discriminating against culture-positive and culture-negative PTB.

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Appendix X

10.0. Manuscript published in Scientific Report

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SCIENTIFIC REPORTS

OPEN

The diagnostic accuracy of Th1 (IFN-γ, TNF-α, and IL-2) and Th2 (IL-4, IL-6 and IL-10) cytokines response in AFB microscopy smear negative PTB- HIV co-infected patients

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Acid Fast Bacilli (AFB) microscopy smear remains the most widely used laboratory diagnostic technique for Pulmonary Tuberculosis (PTB) in low-and-middle income countries. Although it is highly specific, the sensitivity varies between 20–80% in immune-competent people, with only 50% case detection among HIV/TB co-infected patients, hence the need to determine the diagnostic accuracy of Th1 and Th2 cytokine response in AFB microscopy smear negative PTB-HIV co-infected patients. A total of 86 participants were recruited; 70 (81.4%) AFB microscopy smear negative and 16 (18.6%) AFB microscopy smear positive. The AFB microscopy smear negative samples were then cultured using Lowenstein Jensen Medium with 46 being culture-negative and 24 being culture-positive. Blood samples were also collected, cultured using QFT-GIT and the supernatant (plasma) harvested to evaluate cytokine profiles using Enzyme-Linked Immunosorbent Assay. IFN-γ ($P < 0.001$), TNF-α ($P = 0.004$), IL-2 ($P = 0.004$) and IL-4 ($P = 0.009$) median levels were elevated in PTB culture-positive (AFB microscopy smear negative) as compared to PTB culture-negative (AFB microscopy smear negative) participants. Finally, when Th1 cytokines (IFN-γ, TNF-α and IL-2), Th2 cytokines (IL-6 and IL-10) and T cells were included in the logistic regression fit for PTB outcome, the predictive power of discriminating between those who were AFB smear negative in the diagnosis of PTB was good with cross validated area under the curve (AUC) being 0.87 (95% CI: 0.78, 0.96). This study provides evidence for the ability of Th1 and Th2 cytokines to determine PTB status in AFB microscopy smear negative patients co-infected with HIV.

Tuberculosis (TB) is among the main HIV and AIDS defining illnesses[1]. In 2016 there were an estimated 10.4 million new cases of TB worldwide, with 1.2 million people living with HIV developing TB. TB is the leading cause of death among people living with HIV, accounting for 400,000 deaths in HIV-associated TB. Africa accounted for 75% of all deaths[2]. Kenya is among countries with the highest annual number of TB cases, with an estimated incidence of 348 per 100,000 population and 36,000 incident TB cases occurring among persons living with HIV infection[2].

Mycobacterium tuberculosis (MTB) complex is the causative agent of TB, one of the oldest diseases known to affect humans. Although all MTB complex members are obligate pathogens and cause TB, they exhibit distinct phenotypic characteristics and host range. MTB is a rod-shaped, non-spore-forming, thin aerobic bacterium

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