T-HELPER 17 CELLS RESPONSES AMONG TB/HIV COINFECTED SUBJECTS IN NAIROBI, KENYA

A RESEARCH THESIS PRESENTED TO THE UNIVERSITY OF NAIROBI IN PARTIAL FULFILMENT OF A MASTER OF SCIENCE DEGREE IN BIOTECHNOLOGY

BY KAMAKIA RODAH SELEYIAN

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DECLARATION

I, Kamakia Rodah Seleyian, hereby declare that this thesis is my original work and has not been presented for a degree in any other university.

Sign..................................

Date………………………………..

Supervisors

Dr. Julius O. Oyugi

Senior lecturer,

Department of Medical Microbiology,

University of Nairobi

Sign………………………… Date………………………

Dr. Adrienne F.A. Meyers

Adjunct Professor

Department of Medical Microbiology and Infectious Diseases
University of Manitoba

Sign………………………… Date……………………………

Dr. Sandra Kiazyk

Adjunct Professor

Department of Medical Microbiology and Infectious Diseases
University of Manitoba

Sign………………………… Date……………………………

Prof. James O. Ochanda

Center for Biotechnology and Bioinformatics (CEBIB),

University of Nairobi

Sign………………. Date………………………....

DEDICATION

I dedicate this work to all the students at the University of Nairobi, Center for Bioinformatics and Biotechnology (CEBIB). A brighter future lies ahead of you so unleash all the potential in you!

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LIST OF ABBREVIATIONS

AIDS- Acquired Immuno Deficiency Syndrome

ATB-Active tuberculosis

CCL-chemo tactic chemokine ligand

CD 4-Cluster of differentiation 4

CD8-Cluster of differentiation 8

CFP-10-culture filtrate protein-10

CXCL-CXC chemokine ligand

ELISA-Enzyme Linked Immunosorbent Assay

ESAT-6-Early secreted antigen target-6

HIV-Human immunodeficiency virus

IL-Interleukin

LTBI-Latent Tuberculosis infection

MIP-Macrophage inflammatory protein

QFTGT-Quantiferon ®TB Gold test

TB-Tuberculosis

T H- T helper

WHO-World Health Organization

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ABSTRACT

Background: Tuberculosis disease (TB) is caused by *Mycobacterium tuberculosis (Mtb)*. TB leads to about 2 million deaths annually and remains a major public health burden globally. According to the WHO report, in 2013 Kenya was ranked 15 out of the 22 countries with high TB burden globally. Mtb infection clinically exist in two forms, latent TB infection (LTBI) and active infection (ATB). 5 to 10% of people with LTBI develop active TB during their lifetime, while in the case of HIV co-infection, the rate is 5-15% annually. Th1-cells subset of CD4 plays a great role of host defense against Mtb though is not adequate on its own. T helper 17 cells, a newly identified subset of CD4+T cells, are associated with inflammatory disorders and clearance of extracellular pathogens, however, their role in the pathogenesis of pulmonary TB in TB/HIV co-infection and their frequencies is not clearly understood. This study aimed at determining the levels of Th17 cell responses in HIV/TB co-infected subjects.

Method: A cross-sectional study was conducted among sixty adults visiting TB clinic at Mbagathi District Hospital and the city council clinics in Nairobi, Kenya. These participants were grouped as ATB/HIV, ATB, LTBI and controls. After obtaining informed consent, an interferon gamma release assay was performed and from IGRA supernatants, Th17 milliplex assay was performed to simultaneously quantify 25 Th17 associated cytokines and chemokines. Responses from the Antigen minus Nil tubes were analyzed responses with a p value of <0.05 were considered statistically significant.

Results: Overall antigen specific responses among ATB/HIV co-infected versus ATB alone were not significant. , IL-17A (p=0.0390), IFN-γ (p=0.0024), MIP3-α (p=0.0362), IL-9(p=0.032), IL-6 (p=0.0459), IL-13(p=0.0079) and IL-5, (p=0.0002) were significantly reduced in ATB/HIV co-infected compared to LTBI while IFN-γ (p=<0.0001), IL-13(p=0.0029), IL-5(p=0.0013), IL-2 (p=<0.0001), and IL-9(p=0.0069) are significantly reduced in controls as compared to LTBI while there was no significant difference when the controls were compared to ATB.

Conclusion: This study has indicated that there could be more novel cytokines other than IFN-γ that can be used to differentiate stages of TB infection. It has also identified Th 17 associated cytokines that may be incorporated into the current ineffective TB vaccines that have been utilizing Th1 cells responses to potentiate immunity against TB. Finally, it has shown that HIV does not seem to further alter these immune responses in advance TB.

1.0 INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death globally and remains a major public health burden (WHO 2013). TB is caused by *Mycobacterium tuberculosis* (Mtb) which belongs to the *Mycobacterium* complex consisting of six closely related species; *M bovis, M.caprae, M.pinnipedii, M.africanum, M.microti and M.canettii* (Ahmad 2010). More than one-third of the world population is affected by Mtb , with approximately 8.6 million new active TB cases, and 1.3 million death cases occurring annually(WHO 2013). In Kenya, TB is still a great challenge and in 2012, Kenya was ranked position 15 of 22 countries with high TB burden in the world (The East African 2014). Five to 10% of latently-infected individuals develop active TB during their lifetime while in case of TB/HIV co-infection the risk of TB progression is approximately 50% over lifetime (Ahmad 2010; Philips & Ernst 2012).

The control of TB epidemic has been slowed due to lack of an effective vaccine, lack of a sensitive, accurate and rapid diagnostic test and emergence of drug resistant forms of *M. tuberculosis* (Dorman 2010; Kaufmann & Parida 2008). The currently available and the only licensed TB vaccine so far is Bacilli Calmette-Guerin (BCG) (Claden et al. 2013). Though this vaccine has been on the market for more than 90 years, its protection efficacy against pulmonary TB which accounts for most of the TB burden worldwide varies (Beresford & Sadoff 2010; Evans et al. 2013). It provides protection against pulmonary TB in infants and young children but not in adults. In addition, it does not prevent the spread of Mtb infection (Philips & Ernst 2012) and it also causes BCG disease among HIV infected children (Hesseling et al. 2009). Fourteen new TB vaccines candidates are in clinical trials, whether these vaccines are superior to BCG is yet to be known (Claden et al. 2013).

TB is an airborne infection and upon exposure to infected aerosols, Mtb is inhaled and deposited into the lungs (Lin & Flynn 2010). Depending on the strength of the host immune system, the pathogen is either cleared out or it multiplies causing damage to the lungs and leading to development of active disease (Cavalcanti et al. 2012). In immunocompromised individuals, Mtb infection leads to development of active TB (Piana et al. 2007).

Th17 cells are a recently identified subset of the CD4+ cells. Their effector cytokines are involve in host defense through clearance of extracellular pathogens such as *Mycobacterium tuberculosis*

though if they are not regulated they can cause autoimmune disorders (Waite & Skokos 2012). These cytokines have also been linked to disease progression in HIV/AIDS (ElHed & Unutmaz 2011). However, their levels and role in HIV/TB co-infection remains unclear.

Therefore, studying Th17 immune responses in latently infected and in active TB and how HIV infection further affects these parameters is very important. This knowledge will be useful in improving current vaccines and therapies and in developing newer ones.

2.0 LITERATURE REVIEW

2.1 Tuberculosis Infection

TB infection occurs when droplet nuclei (1-5µm in diameter) of particles containing Mtb is inhaled. This pathogen is transmitted upon exposure to expectorants from persons with active pulmonary TB , or when conducting laboratory procedures which involve handling or processing infected tissues or secretion(American Thoracic Society 1999) Its transmission is facilitated and sustained by exogenous factors such as poor case detection, poor treatment of cases, poor respiratory hygiene, overcrowding in public places and migration of individuals from highly TB endemic countries to low TB endemic countries as they pose a great risk of TB exposure. Factors such as co-infection with HIV, malnutrition and smoking alter the immune system hence posing a risk of TB progression (Narasimhan et al. 2013).

TB infection manifests clinically as either pulmonary or extra-pulmonary TB. Pulmonary TB primarily infects lungs while extra-pulmonary TB infects other organs such as the gastro intestinal tract, lymphoid organs, the central nervous system, kidneys, joints and the bones. The extra-pulmonary form of TB is mostly seen in TB/HIV co-infected cases and it is often accompanied by pulmonary TB(Golden & Vikram 2005)

2.2 Symptoms and signs of Tuberculosis

Symptoms of active TB infection include; persistent fatigue, weight loss, loss of appetite, fever, night sweats, constant chronic cough and coughing up blood (CDC 2012).

2.3 Epidemiology of Tuberculosis Infection

TB affects more than a third of the world population with about 8.6 million new cases and 1.3 million deaths occurring globally (WHO 2013). Upon exposure to Mtb, 95 % persons develop latent TB infection (LTBI) while 5% develop active TB. LTBI is the non-symptomatic and non-infectious form of TB while active TB is the infectious and symptomatic form. Out of the 95% LTBI cases only 5% develop active TB during their lifetime this is because the pathogen is cleared by the host immune system while in presence of HIV, those that reactivate are 50%. Ninety five percent of the active TB infection can be cured upon anti-TB therapy while 50% of it can lead to death if not treated. Five percent relapse can also occur even after treatment (Koul et al. 2011; WHO 2013).

The infection affects mainly young adults and this burden is higher among men compared to women. In 2012, approximately 2.9 million cases and 410,000 deaths occurring among women with 160,000 deaths being among HIV positive women worldwide. In addition, 320,000 TB death cases were reported worldwide as a result of TB/HIV co-infection (WHO 2013). Seventy five percent of the disease burden lies in the developing countries with Africa having an incidence rate of 27% and this is mainly due to high prevalence of HIV infection (WHO 2013). In Kenya, TB is still a great challenge and according to the 2013 WHO report, Kenya is still among the top 22 high TB burden countries globally. As a result, 15,000 deaths occur annually and approximately 45,000 (39%) people are infected with HIV and are living with TB were reported in 2012 (WHO 2013).

2.4 *Mycobacterium tuberculosis*

Mtb is a slowing growing, gram positive microorganism that does not form spores. It is also aerobic and facultatively intracellular (North & Jung 2004). It belongs to the *Mtb* complex consisting of six other members that are closely related; *M. bovis, M. africanum*, *M. microtti, M. pinnipedii, M. caprae and M. canetti* (Ahmad 2010). Though these members have a 99.9% similarity at their DNA level, they differ in their pathogenicity, host range and some phenotypic characteristics; *M. canetti* is very rare and its phenotypic characteristics differ with those of *Mtb*, whereby it has unusual smooth gloss colony morphology while *Mtb* forms typically dry, rough and cauliflower like colonies. *M. bovis* is mainly a bovine pathogen while *M.caprae* infects goats. *M. africanaum* and *Mtb* mainly infect humans. *M. microtti* causes disease in rodents and in immunocompromised humans while *M. pinnipedii* infects sea lions and seals (Gordon et al. 2009). Two other major human mycobacterial pathogens related to Mtb are *M. leprae* that causes leprosy, and *M. ulcerans*, the causative agent of Buruli ulcer (Gordon et al. 2009).

2.5 Tuberculosis prevention

For many years now, the only available vaccine on the market is BCG, an attenuated strain of *Mycobacterium bovis*. This vaccine has been in use since 1921 to prevent infants and young children from getting TB infection (Matsuo & Yasutomi 2011). BCG induces T helper 1 immune responses and it also triggers CD8+ T-cell responses. However, The Th1 mediated immunity play great role in clearance of Mtb though it does not completely clear it on its own (Korn et al. 2009) It is effective against pediatrics pulmonary TB as it induces mucosal immunity upon oral

administration to the newborns, it has also proven to be very safe on humans. However, its efficacy in protecting against adult pulmonary TB varies between 0-80% (Nuttall & Eley 2011; Philips & Ernst 2012) and more serious BCG disease effects have been reported in HIV infection (Arbelaez et al. 2000; Hesseling et al. 2009).

The spread of the TB epidemic shows that the BCG vaccine is not very effective. This limitation together with the emergence of drug resistance strains of *Mtb* and HIV co-infection (Ottenhoff & Kaufmann 2012) raised the need for development of better vaccines either through recombinant DNA technology or by boosting BCG immunity with a protein or viral vaccine. So far, vaccines vectors such as MVA85A, BCG- prime and others have been developed and are still undergoing clinical trial but it is not yet known whether they will be able to protect against pulmonary TB (Hanekom et al. 2008; Martin et al. 2003; Matsuo & Yasutomi 2011).

2.6 TB diagnosis

Diagnostic tests for the latent form of TB include tuberculin skin test (TST) and interferon gamma release assay (IGRA). These tests are designed to measure cell immune responses upon exposure to mycobacterium tuberculosis after a period of 6-8 weeks. TST measures adaptive immunity to purified protein derivative (PPD) when these antigens are injected intradermal while IGRA measures levels of interferon gamma cytokine produced by T-cells when exposed to *Mtb* specific antigens; the early secreted antigen target-6 (ESAT-6) and culture filtrate protein 10(CFP-10) (Schluger & Burzynski 2010).There are three commercially available IGRA tests; these include the whole blood ELISA based Quantiferon TB Gold test that utilizes ESAT-6 and CFP-10 antigens, the peripheral blood mononuclear cells enzyme-linked immunospot (ELISPOT) and Quantiferon Gold in-tube test that has an additional TB 7.7 antigen (Pai et al. 2006). Though IGRA is more sensitive and more specific than the TST test both of them have several limitations, they cannot differentiate active TB from latent TB and their sensitivity is low in immunocompromised persons (Vesenbeckh et al. 2012; Kobashi et al. 2010) The TST also has low specificity because of the cross reactivity of the PPD cocktail antigens with those present in the BCG vaccine strain and other environmental mycobacterium(Jasmer et al. 2002)

Active TB is diagnosed using sputum smear microscopy, chest X ray radiation and culture technique. Though culture technique is the gold standard method it takes almost eight weeks for results to be obtained while sputum smear microscopy which has been the primary method for

detecting and monitoring TB treatment responses for so many years in resource-limited countries has low sensitivity, low specificity in immunocompromised individuals and is subject to inter reader variability. The chest X ray on its own is inconclusive, it is subject to inter reader variability and low sensitivity in immunocompromised persons (Krasnow & Wayne 1969; WHO 2014)

2.7 Immune response to Tuberculosis infection

Upon exposure to infected aerosols, *Mtb* present in the inhaled droplet nuclei penetrate into the terminal alveoli of the lungs in which they are engulfed mainly by the alveolar macrophages(Glickman & Jacobs 2001; North & Jung 2004). These macrophages and other phagocytic cells including dendritic cells have receptors on their surfaces such as toll-like receptors, c-type lectins, dendritic cell-associated c-type lectin-1 and nucleotide binding oligomerization domain that recognize pathogen-associated molecular patterns on surface of the pathogen(Ahmad, 2010; Philips & Ernst, 2012). The *Mtb* surface ligands recognized by these receptors are 19kDa and 38kDa glycolipoproteins and glycolipids such as phoshositlylinositol mannoside (Philips & Ernst, 2012).

The presence of this pathogen in the alveoli induces an inflammatory response. The infected macrophages produce chemokines and pro-inflammatory cytokines such as IFN-γ that recruit other phagocytic cells such as neutrophils, lymphocytes and monocytes to the site of infection (Cooper et al. 1993) However, these cells are not able to eradicate the bacilli completely, the bacterium resists destruction by preventing phagosome-lysosome fusion and replicates within the macrophage, leading to macrophage necrosis. It overwhelms the phagocytic cells leading to their destruction and this occurs before cell mediated immunity is developed (Grosset 2003).

When cell mediated immunity develops (2-8 weeks after infection), a more effective immune response is mounted. Dendritic cells with engulfed pathogens migrate to the regional lymph nodes and prime T cells (both CD4 + and CD8+) against *Mtb.* The primed T cells migrate back to the infection site, attracted by chemokines produced by the infected cells (Marino et al. 2004). These T cells interact with macrophages to produce nitric oxide, a major antimicrobial mechanism (Bauer et al. 2008).CD4 +T cells produce cytokines such as IL-10, IL-12, IL-15 and TNF alpha. They help in activation and maintenance of CD8+ T cell effector and memory functions as well as the lysis of infected cells. Activation of *Mtb* specific CD8+ cytolytic lymphocytes occurs in response to both infected cells and antigen presenting cells. The accumulation of neutrophils, macrophages

and T cells at the site of infection leads to the formation of a granuloma. The granuloma is the hallmark of LTBI, and contains the *Mtb* and prevents it from spreading to other parts of the body. It also enables interaction among other immune cells and their cytokines thus mounting a sufficient immune response that eradicates the pathogen. This state of TB infection, referred to as "latent" is asymptomatic and non-infectious (Ahmad 2011).

In HIV co-infected persons, the risk of disease progression is even higher (Manabe & Bishai 2000). HIV causes depletion of CD4+ T cells that are central for the maintenance of the granuloma and protection against active TB (Collins et al. 2002).During co-infection with HIV, the immune balance maintaining the granuloma is disrupted leading to the dissemination of the bacilli and consequent development of active TB disease(Bauer et al. 2008). The exact nature of the imbalance or disruption in the immunological factors maintaining TB latency are unknown.

2.8 T-helper 17 cells

T-helper 17(Th17) cells is a subset of CD4+ T-helper cells that has been recently identified(Korn et al. 2009). TH17 cells are widely found in both non-lymphoid tissues and secondary lymphoid tissues such as peyers patches and spleen (Campillo-Gimenez et al. 2010). They were initially identified as mucosally associated. This subset is different from the other subsets of T helper cells because of the specific cytokines that define its lineage and those that carry out its effector functions. These cells are characterized by the production of IL-22, IL-21, IL-26, IL-17 cytokines (Li et al. 2012) and the expression of CCR4 and CCR6 chemokine receptors. These cytokines except IL-26 are produced by both humans and mice (Blaschitz & Raffatellu 2010). IL-26 is only secreted by humans therefore very little is known about its role (Blaschitz & Raffatellu 2010).The receptors on Th17 cells also have a unique structural features that mediate a signaling pathway through NF-κB activator 1 (ACT1) that is different from those involved in Th1 and Th2 responses (Campillo-Gimenez et al. 2010).

2.8.1 Development of T-helper 17 cells

CD4+T-helper cells consists of the Th1,Th2,Th17, Th 22, Th9,Th follicular cells and inducible T regulatory(iTregs) cells (Luckheeram et al. 2012). The Th17 subset is newly identified and various cytokines and transcription factors are involved in its development. This includes IL-23, TGF-β, IL-1β,IL-6, IL-21, retinoic-acid-related orphan receptor-γt (ROR-γt) signal transducer and activator of transcription 3 (STAT3), and aryl hydrocarbon receptor (Blaschitz & Raffatellu 2010).

TGF- β, pro-inflammatory cytokine IL-6,ROR-γt, STAT3, and aryl hydrocarbon receptor are involved in differentiation of these cells (Korn et al. 2009; Luckheeram et al. 2012).

The IL-21 cytokine is essential for amplification of T-helper 17 cells. It upregulates the IL-23R expression which in turn increases responsiveness to IL-23 cytokine(a member of 1l-12 cytokine family) that is responsible for both expansion and stabilization of T-helper 17 cell population (Bettelli et al. 2007).

Th17 cells population are up regulated by the above cytokines are but down regulated by inducible T regs,Th1 and Th2 cytokines (Blaschitz & Raffatellu 2010; Vanaudenaerde et al. 2011).

2.8.2 Roles of effector cytokines for T-helper 17 cells

2.8.2.1 IL-17 cytokine

IL-17 is a cytokine family consisting of 6 members these are IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F(Jin & Dong 2013). Major studies have focused on IL-17A, IL-17F and IL-17E but little is known about the other members of this family. IL-17E is also referred to as IL-25 and it is mainly produced by Th-2 cells. This cytokine has been reported to play a role in Th2 allergic responses and also in induction of expression of Th2 cytokines and chemokines such as Eotaxin1 and RANTES. IL-17A and IL-17F are genetically linked and they have been found to perform similar functions. They are co-expressed by neutrophils, natural killer cells, T cells, and invariant natural killer T cells, CD8 T cells, and γδT cells (Li et al. 2012; Vanaudenaerde et al. 2011). Although IL-17 is produced by other different cell types, T-helper 17 cells are the major producers with IL-17A being its hallmark cytokine (de Cassan et al. 2010; Maddur et al. 2012).

IL-17A and IL-17F produced by Th17 cells act on different cell types to induce production of cytokines such as IL-8, IL-6, GM-CSF, chemokines (CXCL1, CXCL10), and metalloproteinase (Liang et al. 2006; Lienhardt et al. 2012). These cytokines have pro-inflammatory roles and are also key in the recruitment, activation and migration of neutrophils during an infection,(Campillo-Gimenez et al. 2010; Bettelli et al. 2007).IL-17 is important in protection against infections and extra cellular pathogens such as Mtb and *Klebsiella pneumonia* that are not efficiently cleared by Th1 and Th2 responses.

In presence of Mtb pathogen, Th17 cells induce expression of CXCL9, CXCL10 and CXCL11 that attract IFN-γ-producing CD4+ Th1 cells to the site of infection to aid in the elimination of the pathogen. However, if IL-17 is persistently and uncontrollably expressed due to the dysfunction

of T-helper 17 cells, it leads to inflammatory and many autoimmune diseases such as allergy, asthma, rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus (Maddur et al. 2012; Waite & Skokos 2012).

A protective role for IL-17 can be dependent or independent on interferon gamma(IFN-γ).The interferon gamma dependent way involves induction of IL-12 by IL-17 that allows generation of Th-1 responses (Li et al. 2012).

2.8.2.2 T helper 17 cells receptors

Th17 cells receptors are expressed on epithelial cells, lymphocytes (both B cells and T cells), dendritic cells, macrophages, fibroblasts and marrow stromal cells. These receptors include the lectin receptor CD 161 which is expressed specifically by human Th17 cells, IL-17RA and IL-17RC (utilized by IL-17 cytokine), IL-21R, IL-26R and IL-22R (Blaschitz & Raffatellu 2010)

Th17 cell population also expresses $CCR6^+$,$CCR4^+$ or $CCR2^+$ and $CCR5^-$ memory $CD4^+$T cells on their surfaces (Lim et al. 2008; Blaschitz & Raffatellu 2010).

2.8.2.3 Th17 cell responses in TB

Th17 effector cytokines play a protective role against mycobacterial infection. They act on different types of cells to induce expression of pro-inflammatory cytokines that are key in granuloma formation that contains and kills mycobacterium present in the lung (Bettelli et al. 2007; Campillo-Gimenez et al. 2010; Saunders & Britton 2007).Various studies have been conducted on Th17 responses in the different forms of pulmonary TB and has been reported that Th17 immune response suppression is associated with TB severity (Chen et al. 2010; Scriba et al. 2008). Some studies have also reported that Th17 responses are reduced in active TB individuals compared to those with latent TB (Chen et al. 2010; Jurado et al. 2012).

2.8.2.4 Th17 cell responses in HIV

Th17 cells express significantly higher levels of CCR5 co-receptors than other subsets and the core receptors are the preferred targets for HIV-1 replication (Gosselin et al. 2010; Brenchley et al. 2008).This leads to depletion of these cells and contribute to the pathogenesis of HIV. Several studies conducted on Th17 responses in HIV found out that these responses are significantly reduced in subjects with chronic HIV infection compared to healthy ones(Clark et al. 2011; Gosselin et al. 2010) Yue et al. also found that Th17 responses were detectable during early stages of HIV infection but were reduced to undetectable levels in chronic infection (Yue et al. 2008).

2.8.3 Milliplex assay

Milliplex assay is based on simultaneous quantification of cytokines. This technology combines both flowcytometry and ELISA where color coded magnetic beads are conjugated with a specific capture antibody while a fluorochrome is conjugated to a secondary antibody then the antigen and antibody reaction is quantified by measuring fluorescence intensity.

2.8.3.1 Milliplex Human Th17 magnetic bead

The milliplex Human Th17 magnetic bead panel is the most flexible system in the CD4 T helper research in that it allows simultaneous screening of cytokines produced by all the CD4+ T subsets. This kit enables simultaneous quantification of the following cytokines;IL-33,IL-2,1L-6,IL-5,IL-22,IL-23,IL-31,IL-27,IL-28A,IL-4,IL-9,IL-13,IL-15,GM-CSF,IL-10,IL-22,IL17E/IL-25,IL-1β,TNF-α,TNF-β,MIP-3α,IL-17A,IL-17F,IL-12p70 and IFN-γ. These cytokines are expressed by major CD4 + T cells subsets i.e. Th1, Th2, Th17 and Tregs.Th1 cells express IFN-γ upon induction by IL-12p70, whereas Th2 express IL-4,IL-5,IL-13 and IL17-E/IL-25.When Th17 cells are induced by IL-β,IL-6,IL-21,TGF β and IL-23 they produce,IL-17A,IL-17F,IL-22 and TNF- α (Jin & Dong 2013; Qu et al. 2013; Kleinschek et al. 2007; van de Veerdonk et al. 2010).

2.9 Hypothesis of the study

Th17 cells and their effector cytokines are present at different levels in active and latent TB infection and further altered by HIV infection. In addition, Th17 responses levels correlate with immunodeficiency to due HIV.

2.9.1 General objective To determine the levels of Th17 cell responses in pulmonary TB and the effect of HIV infection among patients with TB/HIV co-infection.

2.9.2 Specific objectives

- To determine Th17 responses to *Mtb* in ATB among participants co-infected with HIV and study participants who are HIV negative.
- To compare Th17 cell responses among ATB study participants versus responses among LTBI study participants.
- To correlate immunodeficiency due to HIV with Th17 responses among HIV/ATB co-infected study participants.

2.9.3 Significance of the study

Tuberculosis is the leading cause of death among HIV infected subjects (WHO, 2013). In 2012, WHO reported 320,000 death cases of HIV that were related to TB (WHO, 2013).The persistence of this epidemic is a clear indication that the current preventive and therapeutic strategies for TB are not very effective.

Th1 cells pro-inflammatory cytokines do not completely clear the pathogen from the host immune system. The BCG vaccine that utilizes the Th1 immunity does not protect adults against pulmonary TB and its protective mechanism is lowered too in TB/HIV co-infection. Therefore, there is a great need for understanding of more immune responses of the host to the TB pathogen so as to obtain knowledge that can be used to improve the theraupetic and preventive strategies.

Th17 is a newly identified subset of CD4+T cells and its role on host defense has been researched on. However, the frequencies of Th 17cell responses in TB/HIV co-infection and the correlation of HIV immunodeficiency levels with these cell responses is not well understood.

3.0 MATERIALS AND METHODS

3.1 Study site

Study participants' recruitment and sample collection were done at the TB clinic in Mbagathi District Hospital after obtaining informed consent. This TB clinic receives approximately 40 TB cases per day from its surroundings and other regions within Nairobi. Thereafter, Immunological assays were performed at the University of Nairobi Institute of Tropical and Infectious Diseases laboratories (UNITID) and Dr. John Charles (JC) Wilt Infectious laboratory in Winnipeg, Canada. UNITID laboratories are located at the College of Health Science, faculty of medicine with a mandate of harnessing research and initiating training opportunities to control and manage infectious diseases while JC Wilt infectious laboratories have been established by the Public Health Agency of Canada as an additional space to the national microbiology for extensive research of infectious diseases including HIV and AIDS and is located in Winnipeg city, Manitoba Canada.

3.2 Study population

A cross-sectional study was conducted to enroll active TB (ATB) patients (both HIV+ and HIV – participants), visiting TB clinics in Mbagathi District Hospital, and a subset of household contacts (HHC) of ATB patients.

3.3 Sample size

This study was a proof of concept immunological study and therefore only 60 participants were recruited and distributed as shown in Table 1 below. This sample size is an approximation from other similar published studies with sample sizes ranging from 40-100.An example is a study conducted by **Nancy et al.2012 entitled" Reduced Frequency of memory T cells and increased Th17 responses in patients with active Tuberculosis.**

Table 1: study participants' distribution

Gender	ATB	ATB/HIV	Controls	LTBI	Total
Female n (%)	4(21.05%)	9(52.94%)	6(75%)	8(50%)	27(45%)
Male n (%)	15(78.95%)	8(47.06%)	2(25%)	8(50%)	33(55%)
Total	19(31.7%)	17(28.3%)	8(13.3%)	26.67%	60(100%)

4. 4 Sampling

The legible and willing study participants for the ATB/ HIV co-infected and ATB study groups were recruited as they came to seek medical services at the clinics. For the HHC those who accompanied the ATB/ HIV co-infected and the ATB patients were requested to participate in the study. For the participants who were not accompanied, the HHC were traced to their homes. Patient index was sought for the house hold contacts to be contacted. The HHCs were then tested for latent TB infection and grouped as either latent TB infected (LTBI) participants or controls. All study participants were screened for HIV using HIV rapid kits. The HIV negative HHC participants who were infected with latent TB were recruited to the LTBI group while those without latent TB were group as controls. The participants who had active TB but were HIV negative were enrolled into the ATB group while those who had both HIV and active TB were recruited into the ATB/HIV co-infected group.

3.4.1 Inclusion criteria

In all the study groups, subjects who were 18 years of age and older and were willing to participate were recruited into the study.

In the **ATB group**, HIV negative participants with TB clinical symptoms, positive sputum smear microscopy and/ or CXR results suggestive of TB and were not under any anti-TB treatment were recruited.

HHCs who had been living in the same household with an adult pulmonary TB case for more than 2 months and had no indicators of active TB were recruited into the study. This HHCs group was later on subdivided into LTBI and controls groups following their IGRA and HIV results as follows; the **LTBI group**, these were latent TB positive according to IGRA and HIV negative.

The **Control group** who were also HIV negative according to HIV rapid tests but were IGRA negative according to IGRA test .In the **ATB/HIV co-infected group**, study participants' positive for both HIV and active pulmonary TB according to HIV rapid tests and sputum smear microscopy and/ or CXR suggestive of TB respectively and were not under anti-TB treatment were also recruited

3.5 Exclusion criteria

In all the study groups, the following patients were excluded; those under 18 years of age, those unwilling to give consent and those without HIV results.

In the **HIV/ATB co-infected study group**, subjects who were negative for both TB and HIV and those with extra pulmonary TB infections were excluded. In the **ATB group**, patients who have extra pulmonary TB, TB/ HIV co-infection were also excluded. In the **LTBI group**, HHCs who had not been living in the same house with an adult pulmonary TB case for more than 2 months and those without HIV results were left out. **In the control group**, HHCs who were positive for latent TB alone according to IGRA test were excluded.

3.6 Ethical considerations

Ethical approval for this study (ref no.P380/07/2013) was obtained from the Kenyatta National Hospital and the University of Nairobi ethics review committee. See an attached copy of the approval letter at the appendices.

3.6.1 Personnel

Two nurses with many years of experience from the Mbagathi TB clinic were requested for this project. Each of the nurses was required to have a minimum qualification of a diploma in Nursing. They assisted in the recruitment of the study participants, obtaining consent, administering questionnaire and collecting venous blood from the participants. There were also responsible for follow-up of patients, contact tracing, packaging and transporting of samples to the laboratory for processing.

3.6.2 Consenting process

Following ethical approval of this study, patients who were seeking medical services at the clinic during the study period were informed on the purpose of the study and were requested to participate. They were allowed to any ask questions regarding the study. Those willing to participate in the study were given written informed consent formed witnessed by a member of our research study team. The study participants were allowed to take a copy of the informed consent form while the other copy was filed in our records.

3.6.3 Data collection

Upon obtaining consent, the nurses interviewed the study participants using questionnaires and obtained information on age, marital status, HIV status ,occupation, living conditions, health status, However the information on the CD4 counts ,on whether or not the patient was on HAART or TB drugs naïve or not and the HIV status of the patients were obtained from their records.

The questionnaire and the informed consent document formats are attached in the appendices.

3.7 Sample collection

After obtaining informed consent from each participant, one of the nurses collected 3ml of venous blood (1ml of blood per tube) into 3 specialized Quantiferon tubes that were well labeled (labeled with unique patient indices and date of collection only). The three tubes included one containing no antigen, another containing phytohemaglutinin (PHA) as a positive control (mitogen), and the third one containing *Mtb* specific antigens (Early secreted antigen target-6, Culture filtrate protein-10 and TB 7.7). After filling, the tubes were shaken thoroughly to ensure the entire surface of the tube is coated with blood to dissolve antigen on the tube walls and then transported in a cool box to the UNITID laboratories for further processing within 2 hours of collection.

3.8 Sample preparation.

Upon arrival at the UNITID laboratories, the tubes were incubated uprightly at 37C$^{\circ}$ and zero CO_2 for 22 hours. Following incubation, the samples were centrifuged for 15 minutes at 2000 to 3000 RCF (g).Equal volumes of plasma from each Quantiferon tube was harvested into 2 cryovials; one for IGRA and another for human TH17 kit milliplex assay. The cryovials were then frozen at -70 C$^{\circ}$ until the respective assays were performed.

3.8.1 Interferon gamma release assay (IGRA)

3.8.1.1 Reagents preparation

Prior to use, all reagents except conjugate 100X concentrate were brought to room temperature for at least 60 minutes. Then the freeze dried Kit standard was reconstituted with distilled water and a solution of 8.0 IU/ml was produced. The solution was mixed gently to avoid frothing and to ensure complete solubilisation then it was used to produce a 1 in 4 dilution series of interferon gamma. Freeze dried conjugate 100X concentrate was also reconstituted with 0.3ml of distilled water then mixed gently. Working strength conjugate was prepared by diluting the required amount of the reconstituted concentrate in green diluent as per the instruction in the kit.

3.8.1.2 IGRA immunoassay procedure

Quantiferon TB Gold In-Tube test was done to detect latent TB infection according to the manufacturer's instructions (Cellestis, Australia).A sandwich Enzyme linked immunosorbent assay (ELISA) was performed using the QFT ELISA kit (Cellestis, Australia) to quantify the amount of secreted interferon gamma cytokine. Briefly, after all reagents and samples were brought to room temperature,50µl of plasma samples/standards were added to appropriate wells on a 96 well plates and mixed using a shaker. The plates were then incubated for 2 hours at room temperature. Following incubation, the wells were washed at least 6 times with 400µl of wash buffer then 100µl of enzyme substrate was added and mixed properly then incubated for 30 minutes. Finally, 50µl of stop solution was added to all wells, mixed using a shaker and results read immediately at 450nm with 620 to 650nm reference filter.

The raw data generated from the ELISA experiment was analyzed and interpreted using QFT software (version 2.5.0, Cellestis, Australia). As recommended by the manufacturer, a positive result for latent TB infection was considered if the TB Antigen minus Nil was >0.35 IU/ml .The negative results were considered if the TB Antigen minus Nil was <0.35 IU/ml and if the Mitogen minus Nil was ≥0.5 IU/ml. The indeterminate results from test were accepted if the TB Antigen minus Nil was≤ 0.35 IU/ml and if the Mitogen minus Nil <0.5 I U/ml.

3.8.2 Human TH17 kit Multiplex assay

Th17 responses were evaluated using Milliplex MAP Human TH17 Magnetic Bead Kit on xMAP platform following manufacturer's instructions (Millipore, MO, USA)

3.8.2.1 Preparation of plasma samples

Frozen plasma samples from the quantiferon nil and antigen tubes were thawed completely, mixed well by vortexing and centrifuged prior to use. All samples were ran in duplicates and those with insufficient volume were diluted with serum matrix and the dilution factor multiplied with absorbed concentration of each of the samples.

3.8.2.2 Preparation of reagents

Before use all reagents were brought to room temperature. Premixed beads were sonicated for 30 seconds then vortexed for 1 minute before they were used. Quality control 1 and 2 were reconstituted with 250µl deionized water. The vials were inverted several times to mix well and vortexed. Then they were allowed to sit for 5-10 minutes before they were transferred to appropriately labeled polypropylene microfuge tubes. Sixty millitres of 10X wash buffer were diluted with 540ml deionized water before it was used. Serum matrix was also prepared by adding 0.5ml deionized water to the bottle containing lyophilized serum matrix and was mixed well and allowed 10 minutes for complete reconstitution. Human TH17 standard was also reconstituted with 250µl deionized water and the vial inverted severally to mix. Then it was vortexed for 10 seconds and allowed to sit for 5-10 minutes. This was used as the stock standard (standard 7). Finally, 6 working standards were prepared in microfuge tubes labeled 1 to 6.One fifty microliters of assay buffer was added into each of the tubes then 1:4 serial dilutions were prepared by adding 50 µl of standard 7 to standard 6 tube then was mixed well and 50 µl of standard 6 was transferred to tube 5, it was mixed well then 50 µl of tube 5 was transferred to tube 4 and mixed well. Fifty microliters of tube 4 were transferred to tube 3 and mixed then 50 µl of tube 3 were transferred to tube 2 and mixed well. Fifty microliters of tube 2 were transferred to tube one and mixed well. The 0 standard (background) was the assay buffer.

3.8.2.3 Immunoassay procedure

Briefly, 96-well plates were prewet with 200 µl assay buffer for 10 minutes with shaking at room temperature. The assay buffer was then decanted and residual amount removed from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Twenty five microliters of each standard, control or samples were added with appropriate and assay buffer. Premixed beads corresponding to the 25 analytes were added and incubated at 4°C overnight with shaking. Following incubation, the plates were washed, 25µl of detection antibodies added into each well and incubated for 1 hour with shaking at room temperature. Twenty five microliters of

streptavidin-phycoerythrin was then added to each well containing detection antibody and incubated with agitation for 30 minutes at room temperature. After incubation, 150 μl of sheath fluid was added into all the wells, mixed for 5 minutes. Finally, the plates were read using a luminex machine with Bioplex manager software version 5.0 and absorbed concentration of the diluted samples were multiplied by the dilution factor.

3.9 Data analysis

Statistical analysis for the data generated from IGRA and human TH17 kit milliplex assay was done using Graph Pad Prism 6 software. Antigen-Nil responses among the study groups were compared using Mann-Whitney U test and one way ANOVA among multiple groups while Th17 cell responses and CD4 counts correlation was determined using the Spearman rank correlation test. A p-value of 0.05 was considered statistically significant. The level of significance was determined as per the p-value scheme provided in graphPad prism statistical guide(Motulsky 2007). The p-value scheme is as shown below;

P-value	**Wording**	**Summary**
< 0.0001	Extremely significant	****
0.0001 to 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥ 0.05	Not significant	ns

4.0 RESULTS

4.1 Study participants clinical characteristics

Out of the sixty study participants, 17(28.3%) were HIV positive. All the 19(31.7%) active pulmonary TB and HIV negative patients had their Quantiferon (QFT) results and they all tested positive. Out of the 17(28.3%) ATB/HIV co-infected, 11(65.1%) had positive QFT results while 3(17.7%) had negative QFT results and 3(17.7%) had indeterminate results.CD 4 counts for only 14 study participants were available and all these participants belonged to the ATB/HIV co-infected group. The mean of the ages was also determined and were as follows; ATB-34.4, LTBI-34.4, ATB/HIV co-infected-37.1 and controls-40.3. The clinical characteristics of the study participants are illustrated in Table 2 below.

Table 2: Clinical characteristics of study participants

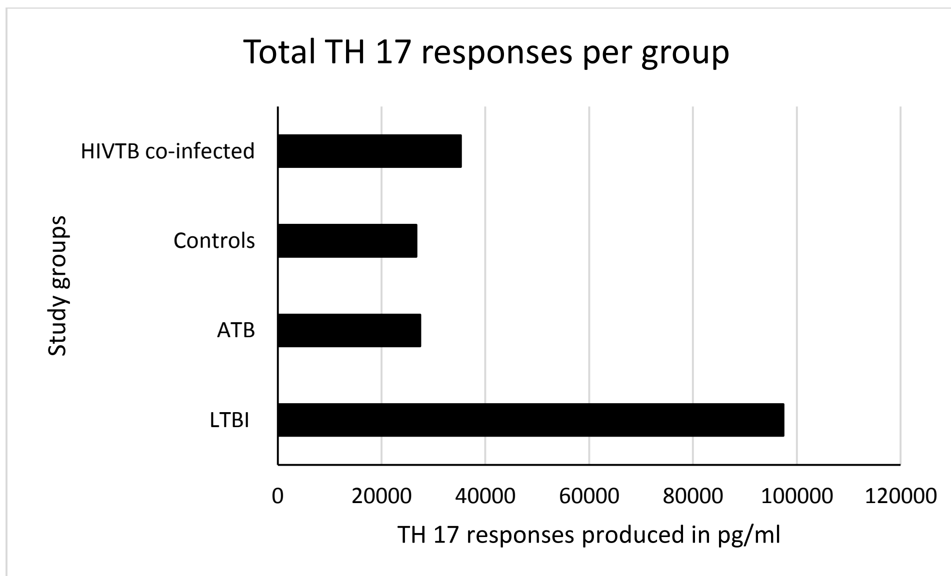
Clinical characteristic	**ATB(n=19)**	**LTBI(n=16)**	**ATB/HIV co-infected**	**Controls**
Gender				
Females	4(21.052%)	8(50%)	9(52.9)	6(75%)
Males	15(78.95%)	8(50%)	8(47.1%)	2(25%)
Mean of their age	34.4	34.4	37.1	40.3
Quantiferon®				
Negative	0	0	3(17.7%)	8(100%)
Positive	19(100%)	16(100%)	11(65.1%)	0
Indeterminate	0	0	31(7.7%)	0
HIV status(+)	0	0	17(100%)	0
CD4 counts(available)	0	0	14(77.8%)	0

4.2 Human Th17 milliplex Assay

Th17 responses among all the participants were determined using Th17 milliplex assay, which involved analyzing a kit of 1 chemokine, the MIP3α and 24 cytokines. These cytokines included IL-17F, IFN-γ, GM-CSF, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-22, IL-9, IL-β, IL-33, IL-2 , IL-4, IL-21, IL-23, IL-5, IL-6, IL-17E/IL-25, IL-27, IL-31, TNF-α, TNF-β and IL-28A.Responses from the nil tubes were subtracted from the antigen tubes and their differences were used for data analysis.

The GM-CSF, IL-4 and TNF-β responses were excluded from analysis as they were all below detectable levels. IL-15 responses was undetectable in the control group therefore was excluded from data analysis. Using Microsoft Excel, total cytokines and chemokine responses in all groups, which is a sum of all individual responses, was determined in order to get a sense of overall total TH17 responses produced. Proportions of these responses obtained are illustrated in the figure 1 below.

Figure 1: Total Th17 responses produced per study group



Total Th17 responses per study group were evaluated upon stimulation of whole blood using Mtb-specific antigens. Antigen minus Nil responses of each participant were calculated then their total was determined and compared per group

In the above figure, the highest total Th17 responses were from participants in the LTBI group with a production of almost 100,000 pg/ml. Responses from the HIV/TB co-infected group followed with a production of close to 40,000 pg/ml. Responses from the controls were slightly higher than from the ATB group though both were approximately 30,000 pg/ml.

In addition, proportions of individual chemokine and cytokines in each group were determined in order to find out their different levels of expression. Proportions obtained are illustrated in Table 3.

Table 3: Proportions of Th17 responses in each particular study group

Analyte	**Controls(pg/ml)**	**ATB(pg/ml)**	**LTBI(pg/ml)**	**ATB/HIV(pg/ml**
IL-17F	211.8(1%)	307.8 (1%)	97402.7(2%)	257.4 (1%)
IFN-γ	38.3 (0%)	2385 (9%)	19746.3(20%)	10168.4(29%)
IL10	27.9(0%)	101.1(0%)	148.1(0%)	36.1(0%)
MIP-3α	3005.0 (11%)	1631.0(6%)	11724.6(12%)	1532.9(4%)
IL12p70	69.2(0%)	60.2(0%)	120.0(0%)	60.1(0%)
IL13	83.9(0%)	355.2(1%)	3933.3(4%)	668.3(2%)
IL15	6.1(0%)	25.6(0%)	20.6(0%)	23.8(0%)
IL17A	44.2(0%)	34.2(0%)	139.1(0%)	54.3(0%)
IL-22	1079.4(4%)	1555(6%)	2085.3(2%)	1087.1(3%)
IL-9	3.4(0%)	20.2(0%)	59.4(0%)	15.5(15%)
IL-1β	2211.4(1%)	2144.2(8%)	3563.0 (4%)	615.8(2%)
IL-33	152.7(1%)	141.0(1%)	313.6(0%)	157.9(0%)
IL-2	9.8(0%)	765.6(3%)	10605.5(11%)	1399.9(4%)
IL-21	162.3(1%)	138.5(1%)	310.7(0%)	152.7(0%)
IL-23	5488.8(21%)	4915.4(18%)	8429.9(9%)	5157.6(15%)
IL-5	5.1(0%)	35.5(0%)	293.6(0%)	13.5(0%)
IL-6	6722.0(25%)	5598.0(20%)	1776.8(18%)	3708.9(11%)
IL-17E/IL-25	1391.4(5%)	929.3(3%)	2454.1(3%)	1147.7(3%)
IL-27	1905.5(7%)	1836.8(7%)	2729.9(3%)	2417.5(7%)
IL-31	1064.7(4%)	971.1(4%)	1873.1(2%)	1091.2(3%)
TNF-α	923.6(3%)	1028.9(4%)	5491.5(6%)	2896.6(8%)
IL-28A	2135.2(8%)	2487.1(9%)	3592.1(4%)	2646.5(7%)
TOTAL	**26741.6(100%)**	**27467.3(100%)**	**97402.7(100%)**	**3509.5(100%)**

This analysis was performed using Ms Excel in which the nil tube minus antigen tubes responses from each study participant was calculated in pg/ml and their totals obtained in each group. The percentages of each analyte produce per group was also determined by dividing the total amount of each analyte by the sum of all the responses in that particular group.

As shown in Table 2, the analyte in the control group with the highest production compared to the rest upon stimulation with *Mtb* specific antigens was IL-6 (25%) followed closely by IL-23 (21%) and MIP-3α (11%).The other 19 analytes were below 10% with IFN-γ, IL-13, IL-9 and IL-5 all being at 0%.

In the ATB group, only two analytes had high production above 10%; the IL-6 (20%) and IL-23 (18%), the rest of the responses were below 10%.

In the LTBI group, IFN- γ response was the highest at 20% compared to the other analytes.IL-6 .followed at 18%, MIP-3α (12%) and IL-2 at 11%.The rest of the responses were below 10%.

In the ATB/HIV co-infected group, IFN- γ response still was the highest (29%) when compared to the other responses in this group.IL-23 and IL-9 followed both at 15% then IL-6 response at 11%. The rest of the responses were below 10%.

Across the four study groups and as shown in the table 2 above, total MIP-3α production and IL-6 were higher in controls compared to the rest. IL-12p70 (69.2 pg/ml), IL-23 (5488.8pg/ml) and IL-17E/IL-25 (1391.4 pg/ml) were slightly higher in controls compared to the ATB and ATB/HIV co-infected participants while IL-17A, IL-31, IL1-β, IL-27, IL-33 were slightly higher in controls than in ATB. IL-2 was very high in both LTBI and TB/HIV co-infected participants but very low in controls and ATB.IL-33 and IL-17A is slightly lower in controls than in ATB/HIV co-infected. In addition, IL-10 IL-9, IL5, IL-17A, IL-33, IL-21, IL-15 and IL-12p70 were generally reduced in all groups.

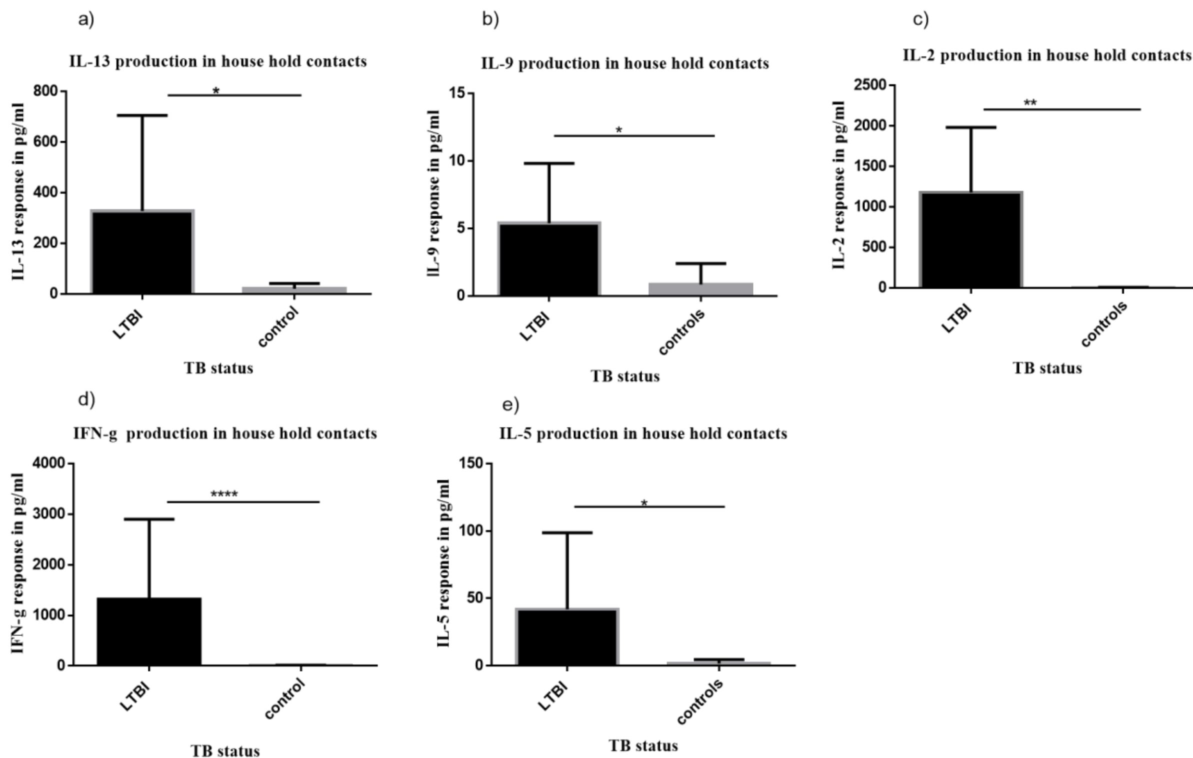
4.3 Comparison of Th17 responses among ATB/HIV co-infected and ATB study participants

The antigen-nil responses from the ATB/HIV co-infected and ATB were compared using Mann Whitney U test to determine the effect of HIV on immunity to active TB and from the analysis done, none of the responses was significantly different in both groups.

4.4 Comparison of Th17 responses among household contacts

Statistically analysis was carried out on all the household contacts responses. Household contacts included participants in the LTBI and control groups. These were HIV negative individuals who were exposed to active TB and were either IGRA positive for the LTBI or IGRA negative for the controls.Th17 responses(antigen-nil) were isolated and compared with Mann Whitney U test and a p value <0.05 was considered significant. From the analysis performed, only IFN-γ (p=<0.0001), IL-13(p=0.0198), IL-9 (p= 0.0168), IL-2 (p=0.0091) and IL-5(p=0.0333) were significantly higher in LTBI compared to controls as illustrated in the figure 2 below.

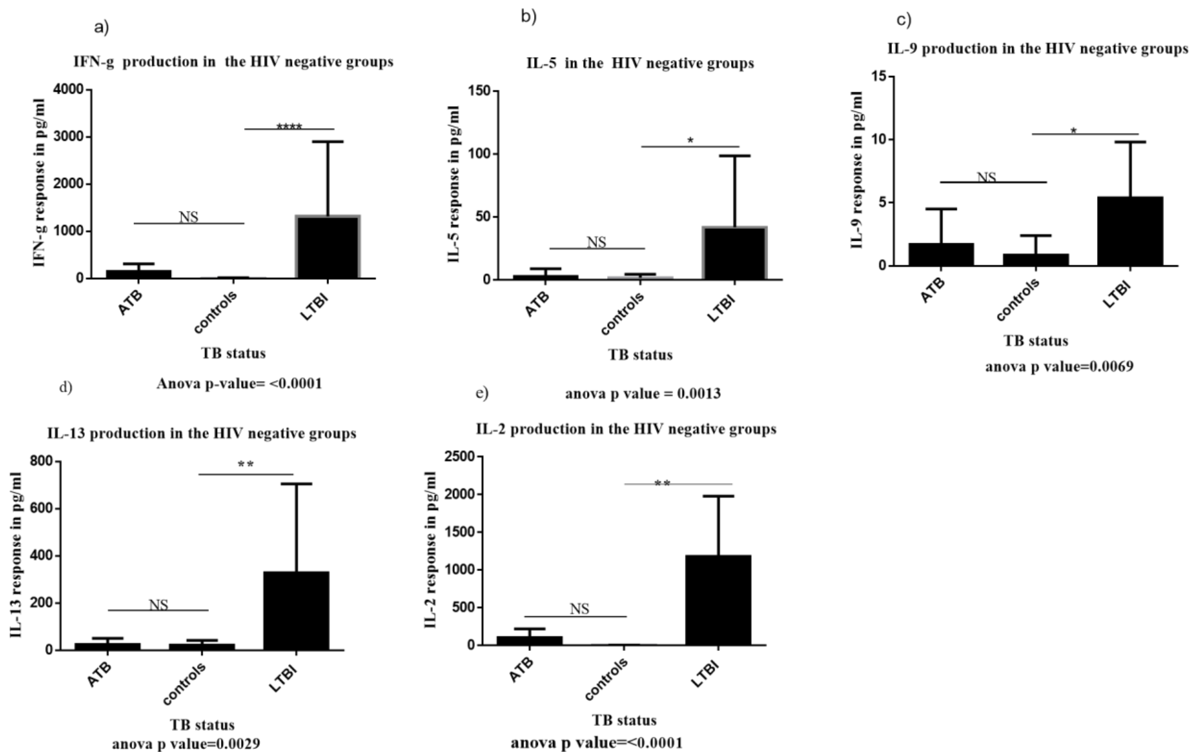
Figure 2: Responses that were significantly different among household contacts



4.5 Determination of TH17 responses among HIV negative participants with ATB and LTBI

In this study, HIV negative participants were in the ATB, LTBI and control groups. Responses from these three groups were isolated and compared using the ANOVA-Kruskal Wallis statistical with Dunn multiple comparison post- test to find out the effect of pulmonary TB on TH17 immune responses. From the analysis done, IFN-γ (p=<0.0001), IL-13(p=0.0029), IL-5(p=0.0013), IL-2 (p=<0.0001), and IL-9(p=0.0069) responses were significantly higher across the groups and in LTBI compared to controls according Dunn's multiple comparison test. However, none of these responses showed any significance difference when controls were compared with ATB. MIP-3α(p=0.0083),IL-17A(p=0.0404) and IL1-β(p=0.0285) were significantly different among the three groups in ANOVA but when Dunn's multiple comparison test was done, no significance difference was observed between any two groups. Responses with significance differences between groups are illustrated in 3 figure below.

Figure 3: Responses that are significantly different among the HIV negative groups

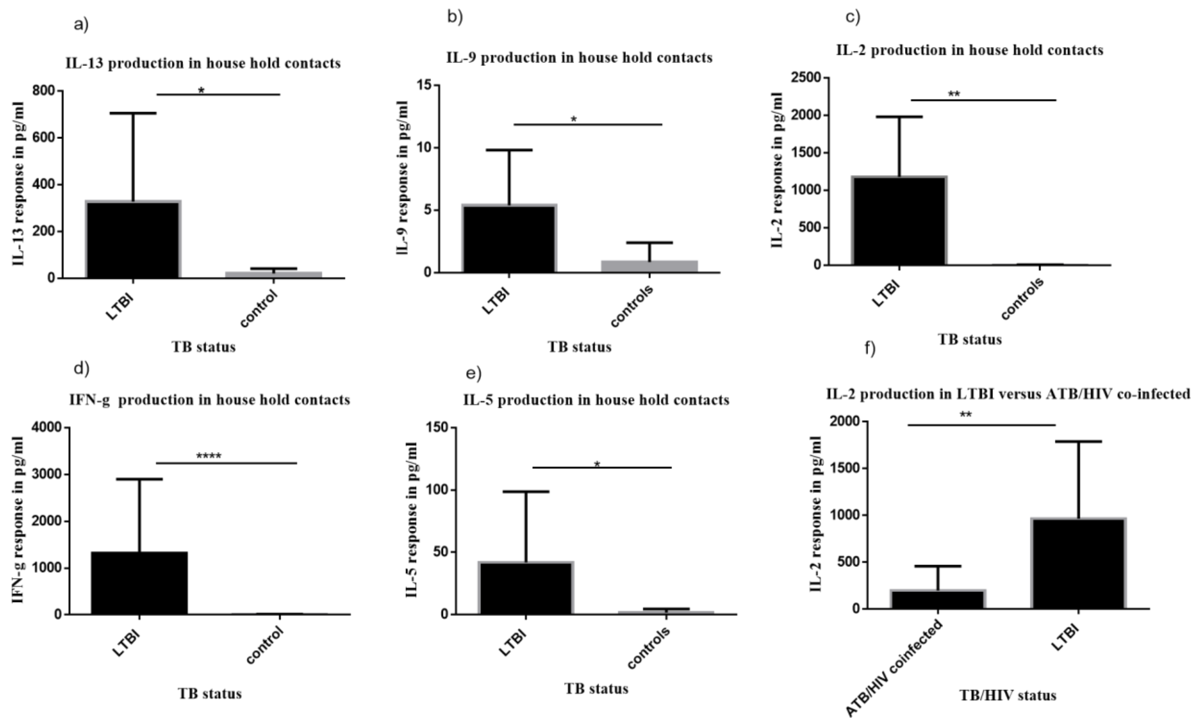


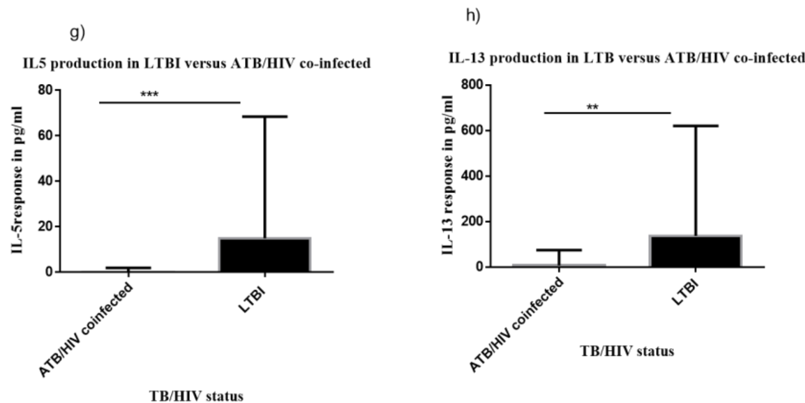
4.6 Comparison of Th17 responses among the LTBI and ATB/HIV co-infected study participants

Th17 effector cytokines levels of HIV negative household contacts who were latently infected with TB (the LTBI) and those of ATB/HIV co-infected were isolated and compared using Mann Whitney U test.

From the analysis conducted, IL-17A (p=0.0390), IFN-γ (p=0.0024), MIP3-α (p=0.0362), IL-9(p=0.032), IL-6 (p=0.0459), IL-13(p=0.0079) and IL-5, (p=0.0002) were the only cytokines with significant differences among the two groups. These cytokines were significantly reduced in ATB/HIV co-infected compared to the LTBI as illustrated below in figure 4,

Figure 4: Responses among LTBI and ATB/HIV co-infected study participants





4.7 Correlation between CD4 counts and TH17 responses of HIV positive study participants

A total of 18 HIV positive participants were recruited into this study and all these participants were in the TB/HIV co-infected group. Out the 18, only 14 (77.78%) had the CD4 counts available. These CD4 counts ranged between 14 - 455 mm^3, with an average of 191.3 mm^3. None of the participants had CD4 counts that was 500 mm^3 and above.

Correlation between these CD4 counts and TH17 immune responses from respective study participants was determined using Spearman's rank correlation test, by comparing CD4 count from each participant with each cytokine produced. IL-2 was excluded from this analysis because it had few pairs for comparison. All the P-values obtained from the test were not significant as shown in Table 4.

Table 4; Comparison of individual cytokines with CD4 counts in HIV positive study participants

Cytokine vs CD4 count in mm^3	r value	P value
IL-17F	-0.2543	0.3879
IFNγ	0.1469	0.6509
IL10	-0.2316	0.2571
MIP-3α	0.2570	0.4288
IL12p70	0.2956	0.3024
IL13	0.1581	0.6621
IL15	-0.2156	0.5012
IL17A	-0.3133	0.3425
IL-22	0.003527	0.9862
IL-9	0.4638	0.3722
IL-1β	0.2478	0.4582
IL-33	-0.1667	0.6777
IL-21	-0.3118	0.2675
IL-23	-0.01703	0.9210
IL-5	0.2052	0.7333
IL-6	-0.1747	0.2645
IL-17E/IL-25	0.02456	0.9426
IL-27	0.5011	0.1155
IL-31	0.1345	0.7132
TNF-α	0.03119	0.8157
IL-28A	-0.1681	0.6463

5.0 DISCUSSION

Pulmonary tuberculosis remains a major global public health burden. One way of eradicating this epidemic involves improving the current vaccine that is ineffective or developing new ones. However, this has been limited by incomplete understanding of the immunopathogenesis of this microorganism particularly among cases of HIV co-infection. Generating knowledge on the alteration of T helper 17 cells responses in relation to TB/HIV co-infection may provide further insight on improving current TB preventive and therapeutic strategies or even developing new ones.

From the analysis performed to determine the overall cytokine/chemokine production in each particular group ,it was found out that Th17 responses from the LTBI group were generally higher compared to the other groups (see figure 1). This observation could be due to high inflammatory activities taking place at the site of infection in which many immune cells are being recruited which in turn express effector cytokines such as TNF and IFN- γ that fight back the invading pathogen (Cooper et al. 2011).These results are similar to those found in an unrelated study conducted by Demissie et al in which Th1 associated cytokines were highly expressed in latent TB individuals that were able to control Mtb (Demissie et al. 2004).

Th17 responses were generally reduced in both ATB/HIV co-infected, ATB and controls (see figure1).Slightly higher responses were observed among ATB/HIV co-infected as compared to ATB. However, when further statistical analysis was done to compare responses among these two groups, it was found that these responses were not significantly different. This could be due to both TB and HIV infections inducing immune activation in a similar pattern. This observation is similar to that seen by Mihret et al. when they evaluated the impact of HIV co-infection cytokines and chemokines in pulmonary tuberculosis (Mihret et al. 2014).They found out that HIV co-infection did not have impact on chemokines and cytokines of pulmonary tuberculosis patients.

From the analysis done to find out cytokines that were generally abundantly produced in each particular group. Higher levels of some cytokines such as IL-6, IL-23 and MIP-3α were detected as compared to IFN-γ. This is expected because Mtb pathogen stimulates all the other CD 4 subsets apart from the Th1 subset that secrete IFN-γ measured in IGRA test. This is an indicator that there could be other cytokines that are produced upon exposure to Mtb specific antigens that are not captured by the IGRA test as it was also shown in a study conducted by Biselli et al. to identify

other cytokines that could be used as biomarkers. These researchers reported that IL-2 could be used to differentiate controls and LTBI (Biselli et al. 2010). In the LTBI group, though IFN-γ was the most abundant though there were other cytokines that were abundantly produced during latent TB. These included IL-6, IL-2 and MIP-3α. In the ATB and ATB/HIV co-infected groups, IFN-γ was still the most abundant. However, IL-6, IL-23 and IL-9 in ATB/HIV co-infected were also detected in high levels. These findings in LTBI, ATB and ATB/HIV co-infected groups indicate that there could be other cytokines/chemokines apart from IFN-γ that could be used to detect or differentiate the different forms of TB as has been reported in different studies (Chegou et al. 2009; Biselli 2010; Sutherland et al. 2010; Mihret et al. 2013; Wang et al. 2012).

Among the household contacts and the HIV negative groups only IFN- γ, IL-2 IL-5, IL-13 and IL-9 were significantly different in that they were significantly higher in LTBI as compared to controls and to ATB (see figure 2 and 3).Reduction of these cytokines in the ATB could be due to exhaustion by *Mtb* pathogen.Th17 responses among LTBI and ATB/HIV also compared and additional cytokines/chemokine; MIP-3α, IL-6 and IL-17A were detected in significantly reduced levels in ATB/HIV co-infected as compared to LTBI. Reduction of these cytokines/chemokines in ATB/HIV could be due to exhaustion of Th1 and Th2 cytokines by both HIV and Mtb as was also observed by Bordon et al. in unrelated study on development of TB among HIV infected women (Bordon et al. 2011).They observed reduced levels of Th2 cytokines and IL-12 in peripheral mononuclear cells of TB/HIV co-infected women. It could also be due to impairment of T cells or preferential depletion of TH17 cells by the HIV as it has also reported in several studies (Clark et al. 2011; Erikstrup et al. 2010; Kanwar et al. 2010). Erikstrup et al. observed T-cell dysfunction among HIV-1 infected patients with impaired recovery of CD 4 despite suppression of viral replication. Kanwar et al. reported loss of Th cells in AIDS pathogenesis while Clark et al. found out that reduced Th1/Th17 cell numbers are associated with impaired PPD specific cytokine responses.

IFN- γ and IL-2 are expressed mostly by T helper 1 lymphocytes upon immune activation and inflammation and are the main cytokines involved in the control of *Mtb* (Zhang et al. 1995).In the present study, these two cytokines were found to significantly reduced in ATB, ATB/HIV co-infected, controls compared to LTBI. Several other unrelated studies obtained similar findings

(Rook et al. 2005; Biselli et al. 2010; Zhang et al. 1995) when they compared ATB, LTBI and controls. However, Kobashi et al. controversially reported significantly higher levels of IFN- γ in ATB compared to LTBI when they compared T-cell interferon gamma release assay for mycobacterium tuberculosis specific antigens in patients with latent and active TB (Kobashi et al. 2010).The difference in the findings they obtained could because they used QFT-G2 IGRA test that is less sensitive as compared to QFT-Gold-In-Tube or T-SPOT-TB tests (Richeldi 2006).To the best of our knowledge there are no reports comparing Th17 responses among LTBI and ATB/HIV co-infected. Hence generating knowledge on the impact of HIV on each stage of TB infection is very important. This will provide insight on the stage of TB infection to be targeted in control of Mtb in ATB/HIV co-infection.

IL-5 and IL-13 are anti-inflammatory cytokines produced by T helper 2 cytokines (Qu et al. 2013). IL-5 cytokine modulates the activity of T helper 1 cytokines enabling persistence of the *Mtb* pathogen. IL-5 in this study was found to be significantly elevated in LTBI as compared to the controls and ATB/HIV co-infected and there was no significant difference in IL-5 production in controls as compared to ATB. These results are supported by study done by Moura et al. in which they also reported no significant differences in production of IL-5 IN ATB and in healthy subjects (Moura et al. 2004). In contrast, a study conducted by Morisini et al. reported that IL-5 levels was lower in healthy subjects as compared to ATB (Morisini et al. 2005). This variation could be as a result of the antigen used in stimulation since the PPD they used is less specific as compared to the ESAT-6/CFP-10 and TB7.7 used in this study. IL-13 on the other hand directly inhibits production of IL-23, IL-1β and IL-6 by dendritic cells. These cytokines are involved in the development of the T helper 17 cells that are involved indirectly in the clearance of *Mtb* through induction of T helper 1 CD4+ T cell (Dhanasekaran et al, 2013). The significantly elevated IL-13 levels in LTBI compared controls reported in this study is similar to that obtained in a study conducted on BCG-vaccinated young children in southern India in which IL-13 was found to be significantly increased in latently infected children compared to uninfected controls (Dhanasekaran et al. 2013).IL-9 on the hand is a T helper 17 cells cytokine that is mainly induced by IL-1β only under the presence of TGF-β(Stephens et al. 2011).This cytokine promotes elimination of *Mtb* by the T helper 1 cytokines through up regulating IFN-γ (Alvarez et al. 2013) In this study IL-9 production is not significantly different among controls and ATB. However, in another study conducted by Yu et al. significantly higher levels of IL-9 were reported in ATB

subjects compared to controls (Yu et al. 2012).This contrast in results could be because of the length of stimulation of the cells using *Mtb* antigens. In the later study stimulation was done for 72 hours as compared to overnight stimulation done in the present study.

CCL20/MIP-3α is a chemokine ligand that utilizes CCR6+ as its only receptor (Lee et al. 2007). Its production is induced by TNF-α or IFN-γ and IL-17 (Kao et al. 2005; Lee et al. 2007). It plays a great role in attracting immature dendritic cells, effector/memory cells and B cells to the site of infection .In this study, CCL2O/MIP-3α production was not significantly different among controls, ATB and LTBI. However, a study conducted by Lee at el., when they stimulated peripheral blood mononuclear cells and monocytes derived macrophages with 30-kDa mycobacterium antigens. Their results showed significant production of CCL20/MIP-3α in ATB as compared to healthy individuals (Lee et al, 2007).This contrast in these findings could be due to individual variability. Slight significant difference in CCL20/MIP-3α among LTBI and ATB/HIV co-infected and this is interesting because to the best of my knowledge no studies have reported this yet.

IL-17A and IL-6 cytokines are produced by Th17 cells(Scriba et al. 2008). These cytokines are involved in early control of *Mtb* infection (Saunders et al. 2000; Okamoto Yoshida et al. 2010).In this study IL-17A is significantly reduced in ATB/HIV co-infected as compared to LTBI while in other studies found IL17A to be significantly reduced in ATB patients as compared to LTBI and healthy donors(Chen et al. 2010; Scriba et al. 2008).In this study,IL-6 and IL-17A were significantly reduced in ATB/HIV co-infected as compared to LTBI and this is also interesting because no studies have reported this yet.

Finally, a correlation test was done on Th17 responses of the ATB/HIV co-infected group and their CD4 counts. Each cytokine response for all study participants was compared to find out if the levels of CD4 counts correlate with the amount of cytokines expressed .From the analysis, there was no significant difference between the CD4 counts and levels of expression of the cytokines. Most of the cytokines levels were not altered with change in the number of CD4 counts while levels from a few of them decreased as the CD4 count though they were not significant as indicated by the r values and p values respectively in table 3. Further investigation should be done on this because this study had few number of CD4 counts from the HIV positive participants which limited further comparisons of the same parameters. This is the first study to correlated these two parameters .This finding is very important because it enables know us whether CD4 counts levels

have any effect on the Th17 immune responses and if altering CD counts of an individuals would be of any significance.

6.0 CONCLUSION AND RECOMMENDATIONS

Often many responses are sequestered to the lung in ATB infection, and measuring of Th1 responses only may lead to misdiagnosis of TB because of low specificity of IGRA test that is currently used and its inability differentiate LTBI from ATB. In addition, utilizing Th1 responses only in BCG to potentiate immunity against Mtb is not enough. This study has shown that Th17 immune responses could also be incorporated to improve the current TB vaccines or even develop new ones.

This study has also identified novel Th17 associated cytokines and chemokines that could be boosted or regulated at the latent stage of TB to prevent progression of TB even in case of a co-infection. High levels of IL-13 and IL-5 anti-inflammatory cytokines could be regulated while IL-2, IFN-γ, IL-17A, IL-9, MIP3-α and IL-6 pro-inflammatory cytokines could be boosted to prevent progression of TB infection. This study has also indicated that there could be other cytokines there could be measured in addition to IFN-γ to differentiate the different stages of TB.

Immunodeficiency due to HIV has also been shown to independent of the Th17 associated cytokines/chemokines levels. However, a larger sample size is needed to confirm this finding. . In the present study, it was also observed that HIV co-infection does not further alter Th17 immune responses in advance TB disease. However, it would be interesting to further investigate the peripheral blood mononuclear cells of the ATB/HIV co-infected study participants to find out whether significantly reduced Th17 responses were due to diminishing T cells populations expressing the cytokines or dysfunctioning of the T cells caused by co-infection of HIV. A longitudinal study should also be conducted on all the study groups to determine the role of Th17 cells responses in TB/HIV co-infected cases.

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8.0 APPENDICES

8.1 Informed Consent Form

Code of the participant: ……………………………………..

Title of the study: T helper 17 cells responses among HIV/TB co infected subjects, in Nairobi Kenya.

Investigator: Rodah Seleyian Kamakia

Supervisors: Dr. Julius Oyugi

: Dr. Andrienne Meyers

: Prof. Ochanda

My name is Rodah Seleyian Kamakia. I am a postgraduate student at the University of Nairobi and I would like to invite you to participate in this study. The information on this document is meant to help you make a decision on whether or not to participate in the study. Kindly feel free to ask any questions or raise your concerns.

Introduction

Tuberculosis (TB) is an airborne infectious disease that is a major public health burden globally. TB is caused by a bacterium known as *mycobacterium tuberculosis* (Mtb) and this infection causes nearly 2 million deaths annually. Upon exposure to Mtb, a non-infectious and asymptomatic form of TB develops known as latent TB. Individuals with latent TB alone have 5 to 10 % chances of developing active TB during their lifetime. However, if the individuals are co infected with HIV, the chances of progression of latent TB to active TB are 5 to 15 % annually. Kenya is ranked position 12 out 22 of high burden TB countries worldwide. The rising TB epidemic clearly indicates that the only licensed BCG vaccine and the current TB treatment regimens are not very effective and that there is a need for further research for better vaccines. Improving TB vaccines requires better understanding how of the body fights Mtb. CD4+ T helper cells are a subgroup of the white blood cells that protect the body against infections. These cells are further divided according to their functions into smaller groups such as T helper 17(Th17) cells group. Th17 cells have two roles; they are involved either in the development of inflammatory diseases or in the control of TB and other bacterial infections such as pneumonia. However, their function in the development of TB of the lungs in case of a TB/HIV co-infection is not clearly understood.

The purpose of the study

This study seeks to investigate the role played by a subgroup of white blood cells particularly T helper 17 cells in the development of TB of the lungs in TB/HIV co infection .This information will be useful in the development of better vaccines.

Study Procedures

This study will involve one visit only. During this visit, the interviewer will inform you about the study. If you give consent to participate in this study, you will sign or mark 2 copies of the form confirming that you have been informed about the study and that you voluntarily agree to take part in it. One copy is yours and the other will be kept in our confidential study file. If you do not wish to keep your copy, you will sign or mark a form that states that you do not want to take it and we will keep it for you. You will also be asked questions about your general health and a medical examination will be performed. Forty (40) ml (about 4 table spoons or about 4 tubes) of your blood will be drawn for immunological tests.

Risks and/or Discomfort

There are no anticipated risks that will rise from participating in this study other than the pain and bruising that you may have where the needle goes during blood collection.

Voluntary participation

Your participation in this study is completely voluntary. Your decline to participate or withdrawal from the study will not deny you any services from the clinic.

Costs and Benefits

Participation in this study is free of charge. There will also be no direct benefits from the study to the participants. However, the knowledge that will be obtained from this study will be useful in vaccine development.

Confidentiality

During this study, all information collected about you and all laboratory test results will be available to no one except our study team. You will be identified only by your own unique identity number, which is known only by you and the clinic staff. Apart from the study team members that you meet, members of the Ethics Committee may check the records to make sure that the study was conducted properly.

Questions

If you have any question regarding this study now or later you can call

The principal investigator, Rodah Seleyian Kamakia, at 0723034248 or

Co-investigator, Dr. Julius Oyugi at, 0713898564.

If you have any question about your rights as a research volunteer you should contact **KNH/UoN ERC Chairperson, Prof. Guantai at phone number (254-020)276300 ext 44355 or email uonknh erc@uonbi.ac.ke**

8.2 Informed Consent Document

I (name of volunteer)………………………………………………………………………….

Of (address)……………………………………………………………………………

Agree to take part in the research project entitled: **T helper 17 cells responses among HIV/TB co infected subjects, in Nairobi Kenya.**

I have been told in detail about the study and know what is required of me. I understand and accept the requirements. I understand that my consent is entirely voluntary and that I may withdraw from the research study for any reason, and this will not affect the legal rights I may otherwise have. My questions have been answered to my satisfaction.

Participant Print Name:…………………………………………………………………….

Signature/Mark or Thumbprint:…………………………………………………………..

Date: …………………………

Person Obtaining Consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer and answered his/her questions:

Print Name:…………………………………………………………………………………

Signature:……………………………………………………………………………...

Date:………………………………………………………………………………………..

Impartial Witness: (only necessary if volunteer was not able to read and read and understand the Consent Information Sheet and Informed Consent Document):

I affirm that the Informed Consent Document has been read to the volunteer and he/she understands the study, had his/her questions answered, and I have witnessed the volunteer's consent to study participation.
Print Name:……………………………………………………………

Signature/ Mark or Thumbprint………………………………………

8.3 Questionnaire that will be used by this TB/HIV study

Date: …………………………………………………………………….

Volunteer ID: ………………………

Section I-Personal Details

Visit Date: ………………………… (DD/MM/YY)

Gender:

- ☐ Male
- ☐ Female

D.O.B:………………………. (DD/MM/YY)

Are you available (i.e. resident in Nairobi)

- ☐ Yes
- ☐ No

Where do you leave? ....

Marital status:

- ☐ Single-never married
- ☐ Married (specify if monogamist or polygamist)…………………………………………...
- ☐ Separated
- ☐ Divorced
- ☐ Windowed
- ☐ Other, please specify…………………………………………………………………....

How would you describe your current work situation?

- ☐ Unemployed
- ☐ Housewife
- ☐ CSW
- ☐ Casual worker
- ☐ Professional worker
- ☐ Self employed/Business person
- ☐ Student
- ☐ Other, please specify…………………………………………………………………

How would you describe your living/accommodation conditions?

- ☐ Single room
- ☐ Double room
- ☐ Other (please specify)……………………………………………………………….

Section 2-Health

I would like to ask a few questions about your present state of health:

1. How would you describe your present state of health
    - Excellent
    - Good
    - Average
    - Poor (please give details)……………………………………………………………………………… ………………………………………………………………………………… …………………………………………………………………………………
2. Have you recently been diagnosed with TB
    - No
    - Yes (please give details)…………………………………………………………………………… ………………………………………………………………………………… …………………………………………………………………………………
3. Have you recently been exposed to TB?
    - Yes
    - No
4. Are you currently taking any TB medicine prescribed by a doctor, or self prescribed?
    - No
    - Yes
5. If yes, what medication are you taking?
    ....

6. Are you currently taking any antiretroviral (ARV's) drugs prescribed by a doctor, or self prescribed?
    - Yes
    - No
7. Were you taken an X-ray?
    - Yes
    - No
8. Was your sputum collected?
    - Yes
    - No

Section 3- Lifestyle

Now I would like to ask you a few questions about your life-style, some of which are quite private:

Family and Contraception;

1. Do you have any children?
   - ☐ No
   - ☐ Yes
2. Are you or is your partner using contraception?
   - ☐ No
   - ☐ Yes
3. If yes are you or your partner using:
   - ☐ Hormonal contraception(oral)
   - ☐ Hormonal contraception(injection)
   - ☐ IUD
   - ☐ Implant
   - ☐ Male condoms
   - ☐ Female condoms
   - ☐ Other (specify)………………………………………………………………..

Signature of the interviewer: ………………………… Date…………………………………….

Signature of the reviewer: …………………………… Date……………………………………

Date entered by (sign):………………………………...Date…………………………………….

RESULTS:

1. HIV test results:

- ☐ Positive
- ☐ Negative

2. CD4 Counts……………………counts/ml

3. X-ray results………………………………………………………..

8.4 Reagent Recipes

8.4.1 IGRA assay components

	2-plate Kit
1. Human IFN-γ Standard, lyophilized (contains *recombinant human IFN- γ, bovine casein, 0.01% w/v Thimerosal*)	1 x vial (8 IU/mL when reconstituted)
2. Green Diluent (*contains bovine casein, normal mouse serum , 0.01% w/v Thimerosal)*	1x30 mL
3. Conjugate 100X Concentrate, lyophilized (*murine anti-human IFN- γ HRP,contains, 0.01% w/v Thimerosal)*	1x0.3 mL
4. Wash Buffer 20X Concentrate (pH 7.2, *0.01% w/v Thimerosal)*	1x100 mL
5. Enzyme Substrate Solution *(contains H_2O_2, 3, 3', 5, 5' Tetramethylbenzine)*	1x30mL
6. Enzyme Stopping Solution *(contains 0.5M H_2SO_4)*	1x15mL
7. Microplate strips coated with murine anti-human IFN-γ monoclonal antibody	2 x 96 well plates

8.4.2 Human Th17 Milliplex assay

Reagents(all are stored at 2 -8°C)	**Volume**	**Quantity**
Human Th17 Standard	Lyophilized	1 vial
Human Th17 Quality Controls 1and 2	Lyophilized	2vials
Serum Matrix(contains 0.08% Sodium Azide)	Lyophilized	2 vials
Set of one 96-well plate with 2 sealers	--------	1plate 2 sealers
Assay Buffer	30mL	1 bottle
10X Wash Buffer (contains 0.05% Porclin)	30mL	2 bottles
Human Th17 Detection Antibodies	3.2mL	1 bottle
Streptavidin-Phycoerythrin	3.2mL	1 bottle
Mixing Bottle	-------	1 bottle
Bead Diluent	3.5 mL	1 bottle
Human Th17 Antibody-immobilized Premixed Magnetic Beads		
Premixed 24-plex Beads +IFNγ	3.5mL 90uL	1bottle +1 bead vial
25 customizable antibody-immobilized magnetic beads	50X concentration, 90µl	