MULTIPLE CYTOKINE RESPONSES TO *MYCOBACTERIUM TUBERCULOSIS* ANTIGENS IN *BACILLE CALMETTE GUERRIN* VACCINATED CHILDREN AT JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL IN KISUMU COUNTY, WESTERN KENYA.

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL IMMUNOLOGY

DEPARTMENT OF BIOMEDICAL SCIENCES AND TECHNOLOGY.

MASENO UNIVERSITY

DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for the award of a degree or any other award.

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ACKNOWLEDGEMENT

This thesis would have not been possible without the help and guidance of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. My utmost gratitude goes to Dr Kevin Cain and Dr. Kiprotich Chelimo for sponsoring my studies and for their guidance in the course of my project. Dr. Kiprotich Chelimo, has been my supervisor and academic mentor, has encouraged me to pursue this topic and spent extra time helping me to shape this research and write-up to achieve a clear structure. I would also like to acknowledge Dr Rinn Song for his support and guidance and Dr Bartholomew Ondigo for technical assistance. I thank Dr Kevin Cain and Dr Kiprotich Chelimo for their steadfast encouragement to complete this study and for the valuable insights they shared. Together they sacrificed much of their valuable time to discuss the project and thesis at various stages offering constructive criticism and helpful comments. Consequently, their support and encouragement has been priceless to the success of this thesis.

I would wish to recognize and appreciate the support of Mr. Cliff Oduor for his support that made this work a success. Last but not least I thank God the almighty, for giving me the strength to complete this study.

DEDICATION

I dedicate this thesis to my parents, Joseph Khumwa and the late Ruth Mukabane in appreciation for their encouragement and motivation.

ABSTRACT

The diagnosis and control of tuberculosis (TB) has taken various dimensions with interferon Gamma (IFN-γ) Release Assays (IGRA) currently employed for diagnosis of latent TB infection while the conventional method of vaccination is *Bacille Calmette Guerrin* (BCG). The variable efficacy and safety of BCG has led to a need of new vaccine candidates joining the development pipeline. However, the cellular mechanism of protection of BCG and the new anti-TB vaccines is not exhaustively understood while IGRA, offer limited applicability in terms of confirmation of an infection and distinction between latent and active TB. Various studies have explored the importance of various individual or configuration of cytokines in diagnosis as well as protection against TB. Understanding the cytokine profile in relation to TB infection would be important in understanding the host response to BCG as well as TB infection by identification of cytokine as biomarkers of disease and as correlates of protection. This study evaluated multiple cytokines elicited by *Mycobacterium tuberculosis* antigens in order to have an in-depth understanding of the cellular mechanism of infection, pathogenesis and protection from vaccine induced or naturally acquired immunity to TB. Twenty five-plex Luminex assay was utilized to analyze multiple cytokines in 25 plasma samples from BCG vaccinated children, aged 0-5 years, resident in Kisumu, western Kenya. Twelve children naturally exposed and 13 non-exposed to TB were selected based on IGRA in response to a cocktail of Mycobacterial antigens (ESAT-6, CFP-10 and TB-7.7). The results showed that the proportion and levels of inflammatory cytokines including IFN-γ, IP-10, IL-15, MIG, GM-CSF, IL-1ra, MIP-1a, IL-2, IL-2r, TNF-α, IL-7, IFN-α, IL-13 and IL-6 were significantly higher in IFN-γ positive (cases) as compared to IFN-γ negative (controls) children. However comparable cytokine frequencies and levels were observed in expression of MIP-1β, MCP-1, Eotaxin, IL-17, IL-12, RANTES, IL-5, IL-4, IL-8, IL-10, and IL-1β between cases and controls. This variation in individual cytokine responses between the two groups highlights the role of individual cytokines as biomarkers of infection and correlates of protection from development of active TB. The differential cytokine expression in BCG vaccinated cases and control children could be used as surrogate markers for TB diagnosis and in rational design of vaccines against TB.

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LIST OF ABBREVIATIONS

TB	Tuberculosis
HIV	Human immunodeficiency virus
CD	Cluster of differentiation
BCG	Bacille calmette guerin
IFN-γ	Interferon gamma
TNF-α	Tumor necrosis factor-1
IL	Interleukin
ESAT	Early secretory antigenic target
CFP	Culture filtrate protein
MVA	Modified vaccinia ankara virus
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
IGRA	Interferon gamma release assay
GM-CSF	Granulocyte macrophage colony stimulating factor
M-CSF	Macrophage-colony stimulating factor
MIP	Macrophage inflammatory protein
PHA	Phyto-hemaglutinin
HRP	Horse-radish-peroxidase
H_2O_2	Hydrogen peroxide
FDA	Food and drug administration
CCL	Chemokine receptor
NK	Natural killer

TLR	Toll like receptors
PPD	Purified protein derivative
TGF	Tumor growth factor
TST	Tuberculin skin test
RANTES	Regulated on activation, normal T-cell expressed and secreted
GSK	Glaxosmithkline
MPT64	Immunogenic protein from *Mycobacterium tuberculosis* H37Rv

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CHAPTER ONE: INTRODUCTION

1.1: Background

Tuberculosis (TB) is the second leading cause of death due to a single infectious agent worldwide, with the highest prevalence in sub-Saharan Africa (W.H.O, 2012a). In 2013, over 9.0 million people developed TB, a quarter of these in Africa, with 1.5 million dying from the disease (W.H.O, 2014a). About 80,000 children died of TB only, while 360,000 deaths were due to HIV associated TB (W.H.O, 2014a). In Kenya, TB prevalence is about 283/100000 and 6% of the new cases are aged less than 15 years (W.H.O, 2014b). In Kisumu county, where the current study was conducted, TB prevalence was 379/100000 (M.O.H, 2013). Hence the current global burden of TB especially in areas of high HIV transmission calls for measures beyond the existing tools.

While to date Bacille Calmette Guerin (BCG) is the only licensed vaccine which has been used for decades and proved to confer protection against TB (Aldovini and Young, 1991), the mechanism of protection is not clearly understood, but is thought to involve strong cell mediated response (Robinson HL, 2005). However, the recent setback of this vaccine is its association with the waning protection, conferring only short term protection and sometimes development of active TB or BCG-like disease among the BCG vaccinated (W.H.O, 2007). This has prompted development of novel vaccines, targeting to boost or replace BCG. One of the new vaccines is an adenovirus vectored recombinant BCG, the Aeras-402, which is in a Phase-IIb trial among infants in western Kenya. Both BCG and Aeras -402 vaccines has been shown to induce high levels of IFN-γ and TNF-α, IFN-γ and IL-2 respectively (Abel *et al*., 2010).

Interferon gamma has been associated with TB infection and is currently employed in IGRA TB diagnostic assay as a standard biomarker of TB exposure and or protection in BCG vaccinated children (Diel, 2008) . Using IFN-γ as the only gold standard in TB diagnosis has led to many potential TB vaccine candidates evaluated against their ability to induce this cytokine to be dropped early in vaccine trial pipelines. The ability of phase-IIb trial Aeras -402 vaccine to induce two other cytokines suggest that other cytokines could be important as biomarkers of diagnosis or vaccine induced and naturally exposed protection against TB. The current study was designed to further identify other potential cytokines that could be important either in diagnosis of TB infection, progression to latency or protection from development of active disease. To achieve this objective, this study assessed multiple cytokine expression among TB exposed and non-exposed BCG vaccinated children in western Kenya following whole blood stimulation with a cocktail of *Mycobacterial* antigens (ESAT-6, CFP-10 and TB-7.7) using 25-plex Luminex assay.

1.2: Statement of the problem

Tuberculosis is a major cause of death with a global estimated mortality of about 2 million people per year. To curb this problem, preventive measures including development of effective diagnostic tools, and vaccines are required. Bacille Calmette Guerrin (BCG) the only licensed vaccine in prevention of TB faces a number of challenges including short-term protection, and reversion to pathogenicity, as well as its mechanism of protection not being clearly understood. Newer vaccines candidates have to this end been developed but none so far has demonstrated efficacy in a large population of people to warrant it to offer a booster or replacement. This could be due to the likely inadequate immunologic benchmarks used to evaluate the immune responses to BCG or natural TB exposure. Iterferon gamma and recently TNF-α and IL2 have been the primary cytokines used in the conventional method of TB diagnosis in BCG vaccinated as well as naturally exposed individuals. Limited use of these cytokines as benchmarks of TB exposure could be posing a bigger challenge especially in diagnosis and development of newer vaccines that could be dropped early in development pipeline due to their inability to induce any of the three cytokine. This problem is compounded by infants and children who are not able to expectorate sputum leading to invasive methods of specimen collection to make microbiological diagnosis. Hence there is need to expand on the range of immunological markers for disease or TB infection for ease of diagnosis and development of new vaccines that circumvent the challenges experienced with the current BCG based vaccines. This study looked at the multiple cytokine responses expressed after BCG vaccination and TB infection in order to identify potential biomarkers for TB diagnosis and exposure.

1.3: Objectives

1.3.1: General objective

To investigate profiles of multiple cytokines associated with host cellular immune response against a cocktail of *M. tuberculosis* antigens among IFN-γ positive and IFN-γ negative children in Kisumu, western Kenya.

1.3.2: Specific objectives

1. To compare the proportion of cytokine responders to a cocktail of *Mycobacterium* antigens (ESAT-6, CFP-10 and TB-7.7) among TB exposed and non-exposed children.
2. To compare the levels of production of multiple cytokines secreted in response to cocktail of *Mycobacterium* antigens (ESAT-6, CFP-10 and TB-7.7) among TB exposed and non-exposed children.

1.4: Null Hypotheses

1. The proportion of cytokine responders to a cocktail of *Mycobacterium* antigens (ESAT-6, CFP-10 and TB-7.7) among TB exposed and non-exposed children is similar.

2. The levels of secreted cytokines against a cocktail of *Mycobacterium* antigens (ESAT-6, CFP-10 and TB-7.7) among TB exposed and non-exposed children is similar.

Significance

Findings from this study will contribute to understanding of the host cellular mechanism of cytokine protection and importance of individual cytokines as biomarkers during *M. tuberculosis* infection and progression to diseases. These findings will also have implications in designing better diagnostic tools and vaccines for prevention and management of tuberculosis.

CHAPTER TWO: LITERATURE REVIEW

2.1: Tuberculosis Epidemiology

Tuberculosis is the second major cause of death worldwide. In 2012 alone 8.6 million people fell ill with TB (W.H.O, 2012b). Ninety-five percent of these deaths occurred in the developing countries especially Africa, South East Asia and Western Pacific (W.H.O, 2011). Global TB incidence is at 128 cases per 100,000. Kenya is ranked 13th among the 22 high burden countries. In 2011 alone, 1.4 million died of TB including 430,000 co-infected with HIV. Five point eight million newly diagnosed cases were notified to the national TB control programs (W.H.O, 2011, , 2012b). A TB notification rate of 338/100,000, and mortality rate of 72/100,000 people has been reported in Kenya (Sitienei, 2010). In Kenya, Nyanza province is leading in TB prevalence with a TB notification rate of 440/100,000. Siaya district, a neighboring district of Kisumu, in Nyanza province has a TB notification rate of 400/100000 (Ayisi *et al.,* 2011). The TB prevalence in Asembo and Gem areas of Siaya district was found to be 6.0/1000 (van't Hoog *et al*., 2011). All these points to a huge burden of TB infection and disease in regions of Kenya with high HIV transmission further complicating the health situation. Hence there is an urgent need to develop new effective diagnostic tools and vaccines to control the disease or improve on the current BCG based vaccines especially with the current reduced efficacy of BCG vaccines.

2.2: Pathogenesis and Host Immune Responses to Tuberculosis

On coming in contact with *M. tuberculosis,* the host alveolar macrophages (AM) via phagocytosis try to kill the *bacilli* by various mechanisms, including the generation of reactive nitrogen and oxygen intermediates. They secrete various cytokines including IL-12, IL-18, IFN-γ and TNF-α. Interferon gamma induces *Mycobacteria* killing while TNF-α controls *bacilli*

growth and granuloma formation by inducing chronic cytokine stimulation and stopping *mycobacteria* proliferation in the granuloma. Success depends on the microbicidal capacity of the macrophages and the survival stability of the *M. tuberculosis* strain (Mauel, 1982b). *Mycobacterium tuberculosis* may arrest phagosome maturation and inhibit phagolysosome formation, which is a hostile location where many *bacilli* are killed (Pelosi *et al*., 2012). *Bacilli* that evade killing by macrophages multiply within alveolar macrophages and dentritic cells and induce the production of cytokines such as TNF-*α*, IL-6, IL-12p80, IL-1*α*, and IL-1*β* that activate macrophages (Pelosi *et al*., 2012).

Different cell populations produce IFN-γ which is an important cytokine in macrophage activation and works in synergy with TNF-α (Kaufmann, 2002). Interferon gamma is produced by T cells and activated Natural Killer (NK) cells in response to IL-12 and IL-18 produced by macrophages in the alveoli and dentritic cells (Kaufmann, 2002). Monocytes whose derivatives alter cytoplasmic activity to make the cell environment suitable for *M. tuberculosis* (Cunningham, 1925) and neutrophils, which exacerbate pathology rather than host protection (Eruslanov *et al*., 2005), are also recruited to the lungs. Dendritic cells are activated through Toll-like receptors' (TLRs) signaling, and monocytes differentiate to effector macrophages leading to production of microbicidal substances such as TNF-*α*, which regulates growth and granuloma formation of *M. tuberculosis*. This comprises aggregates of T cells and infected macrophages, which prevent spread of the *bacilli*.

Pro-inflammatory cytokines including IFN-*γ*, TNF-*α*, IL-6, IL-12, IL-17, and IL-23 stabilize the granuloma and various chemokines including CCL2, CCL3, CCL5, CXCL8, and CXCL10 help in recruitment of inflammatory cells. These lead to development of a localized infection, which

may become latent. In the majority of such infections the bacterium is contained in the granuloma which prevents spreading of *M. tuberculosis*, (Aldovini and Young, 1991), (Zuniga *et al*., 2012). Macrophages therefore produce immuno-modulatory cytokines including IL-1β, prostaglandin E_2 (PGE_2), TGF-β, TNF-α and GM-CSF which in turn inhibit growth of *M. tuberculosis* and *M. avium* whereas IL-1α and IL-6, lead to macrophage inactivation and enhanced mycobacterial survival (Wallis and Ellner, 1994) .

These cytokines therefore have been demonstrated to play a major role in the host immune response against TB (Cowan *et al*., 2012), (Dimakou *et al*., 2004). Other studies have identified release of IL-10 and IL-5 in addition following stimulation with single secreted (Ag85B, ESAT6, MPT64, PstS and MPT70) and single cytosolic (DnaK, GroES and GroEL) antigens of *M. tuberculosis* (Al-Attiyah, 2006). When an individual is exposed to TB, stimulation of immune cells using a cocktail of antigens (ESAT-6, CFP-10, and TB-7.7), leads to production of IFN-γ which is diagnostic on IGRA (Al-Attiyah and Mustafa, 2010). But few studies have documented other cytokines produced in response to these cocktail antigens. A recent study in Uganda, using a 10-plex assay and ESAT-6+CFP-10+TB7.7 as stimulatory antigens among adults , noted elevation of IFN-γ, IL-2, IL-6, IL-8, IP-10, MCP-1, MIP-1β, and TNF-α responses among culture confirmed patients compared with controls (Kellar *et al*., 2011).

The current study evaluated more than 10 cytokines by employing 25-plex Luminex system to look at any additional cytokines to identify other cytokines that may play a role in diagnosis and or protection in TB.

2.3: Natural Host Response to BCG Vaccine and Natural Infection

Once an immunocompetent individual is infected by *M. tuberculosis*, the immune system mounts cellular and humoral response to control the infection (Zuniga *et al.*, 2012). Humoral immune responses utilize antibodies and complement molecules that recognize and bind to the antigenic epitopes expressed by the infectious pathogen (Greenaway *et al*., 2005). On the other hand cellular immunity involves the direct role of phagocytes, T cells and production of effector molecules such as cytokines by various immune cells (van Crevel *et al.*, 2002). Cytokines are a group of small proteins that act as key mediators of immune response to tuberculosis (van Crevel *et al.*, 2002). They are involved in cell activation leading to production of various immuno-regulatory products that lead to killing or containment of *M. tuberculosis* in the human mononuclear phagocytes (van Crevel *et al.*, 2002). Interferon gamma, one of the cytokines, which may be produced by T-cells, macrophages or dentritic cells has been shown to be a key mediator in the host immunity during BCG vaccine induced as well as natural TB infection (Herbst *et al.*, 2011). Other cytokines include IL-6, IL-10, TNF-α (J.W.Fang, 2011). However, the complete array of cytokines associated with diagnosis and protection during vaccine induced and natural TB infection has not been fully established. Recently infant and adult vaccine trials using Aeras-402 and GSK M72/ASO_1 respectively, investigated the role of these candidates in context of only three cytokines (IFN-γ, TNF-α and IL-2). The present study investigated other cytokines in addition to IFN-γ, TNF-α and IL-2 that are produced in response to TB antigens in TB IFN- γ positive and IFN- γ negative BCG vaccinated children

2.4: Association of TB and HIV

Human immunodeficiency virus (HIV) has been associated with TB due to its ability to weaken the host cell mediated immunity and HIV/TB co-infection has been associated with doubling of TB cases (Wallis and Ellner, 1994) and increasing the risk of latent TB by 20-fold (Pawlowski, 2012). HIV infects host CD4 T-cells hence destroying them. Since these cells play a crucial role both in innate and adaptive immunity, the host is left immuno-compromised leading to myriads of opportunistic infections, TB being no exception. According to the 2007 Kenya Aids Indicator survey statistics, 7.9% of the population had HIV of which TB patients with HIV were 48%. From the 8.7 million TB cases in 2011, 1.1 million included people with HIV (Sitienei, 2010; W.H.O, 2012b). Among the 1.4 million deaths from TB, 430, 000 included people with HIV, equivalent to 3900 deaths per day. The Kenya HIV prevalence among the TB patients was 39% (W.H.O, 2012a). In Asembo and Gem, TB prevalence was 6.0/1000, for all pulmonary tuberculosis, while for the 101 prevalent cases, 51% were HIV infected. Forty eight percent of prevalent and 65% of notified pulmonary tuberculosis cases were attributable to HIV (Ayisi *et al*., 2011)

It has been postulated that in HIV associated TB, cytokines may mediate enhanced susceptibility to tuberculosis or pathogen survival as HIV leads to induction of cytokines, some of which lead to proliferation of the *Mycobacterium* (Chetty *et al.,* 2014) Infection with HIV has been associated with various cytokines that modulate the disease. T-helper-1 (Th1) cytokines including IFN-γ, IL-2 are decreased while T-helper -2 (Th2) cytokines including IL-8, IL-6, IL-1, IL-10, IL4 and TNF-α are increased. Other cytokines including IL-7, IL-15, IL-16, IL-13, GM-CSF, M-CSF, IFN- α, β-chemokines, MIP-1α, MIP1β and RANTES are also expressed at varied levels due to the different roles they play in HIV pathogenesis (Kedzierska, 2001; Roberts

et al., 2010). Although the current study did not evaluate participants based on their HIV status, it was done in an area of very high HIV transmission hence cytokine profiles established could have been modulated by HIV status of individual participants.

2.5: Vaccines in Prevention and Control of TB

Bacille Calmette Guerrin (BCG) the only licensed TB vaccine, since 1921 is usually administered at birth in many countries to vaccinate against TB. It has however not always conferred the desired protection. This vaccine stimulates T-cells in a delayed type hypersensitivity type of reaction. The subcutaneous injection leads to an induration and immune cells infiltration leading to formation of an immunological memory (J. Wang *et al.*, 2002; J. Y. Wang *et al.,* 2012). BCG has been demonstrated to be ineffective in conferring long term protection and also found to cause BCG-like disease in immuno-compromised individuals (Al-Salem *et al*., 2012). For this reason, other vaccines are currently at various stages of clinical trials to bridge the setbacks currently observed when using BCG. Some of the vaccines under development include *M. tuberculosis* recombinant vaccine M72/ASO1 (Day *et al*., 2013) and the Aeras-402 *(*Abel *et al*., 2010; Kagina *et al*., 2014), targeting adults and infants respectively (McShane *et al*., 2004). M72 antigen comprises MTB32A and MTB39A immunogenic proteins with AS01 Adjuvant, and induce proliferation and production of IFN-γ (Hanekom *et al.*, 2012). In pre-clinical studies, M72/AS01 has been tested in PPD-negative, PPD-positive, and HIV-positive adults (McShane, 2011). Aeras 402, another candidate farthest in the clinical trial pipeline, is an adenovirus vectored vaccine that targets to boost expression of TB antigen 85a which is a potent stimulator of IFN-γ following priming by BCG. Both vaccines are at phase IIb of the clinical trial. The focus on IFN-γ cytokine as the gold standard in vaccine development may have blocked potential anti-TB vaccines from advancing along the development pipeline

hence the need to evaluate the potential of other cytokines in diagnosis and protection against TB.

2.6: TB Diagnosis

Immunological methods of diagnosing TB is important due to the shortcomings of the current conventional microbiological and molecular methods e.g. smear microcopy, culture and gene-expert (Denkinger *et al*., 2015; Rahman *et al*., 2012). Collection of sputum specimen for these tests is invasive and or impossible especially in immuno-compromised and pediatric populations. Tuberculin skin test (Hock *et al*.) is a good immunological method but is less useful in high burden countries where majority of population are exposed to TB or BCG vaccinated individuals (Hock *et al.*, 1993). The test is invasive in terms of introducing foreign proteins, the purified protein derivative (PPD), into an individual which could have other immunological effects as well as affecting other subsequent tests. Interferon gamma release assays (IGRAs) have offered solution to the challenges experienced by TST as well as other methods by being specific, utilizing only small volumes of blood and offering results in 24 hours as opposed to TST which is nonspecific and gives results after 48-72 hours. *Mycobacterium* peptides, ESAT-6,CFP-10 and TB 7.7 are incorporated in the current FDA approved IGRAs due to their absence in BCG sub strains and most non-tuberculous mycobacteria hence minimizing confounding effects with BCG vaccination in diagnosis (Ariga and Harada, 2008). However, IGRAs, which rely on IFN-γ, cannot fully differentiate latent from active infection and few cases could exist where clinical TB diagnosis is confirmed even in cases of an IGRA negative test. Therefore there is need to search for other potential cytokines other than IFN-γ that may act as biomarkers of TB infection, progression to disease as well as correlates of protection. The current study was designed to evaluate an array of 25 cytokines in order to identify other

cytokines that could be useful in making up for the inadequacies of IFN-γ as markers of TB diagnosis, prognosis and protection.

CHAPTER THREE: MATERIALS AND METHODS

3.1: Study Area

Kisumu is the former administrative headquarters of former Nyanza province, in southwest Kenya, and the third largest city in Kenya. It's located at the shores of Lake Victoria, a fresh water lake, making fishing the major economic activity among the residents. The greater Nyanza, region latitude 00°03'S, Longitude 34°45'E, has the highest HIV prevalence, double the national average of 6.7% at 19.3%.(K.A.I.S, 2007; Nascop, 2014).This may have contributed to an increase in TB cases in Nyanza. Nyanza province has the highest TB and HIV prevalence at 48% compared to the national 7% and the poverty index at 68% compared to national 47% (Nascop, 2014).There are two major health facilities in Kisumu, the Jaramogi Oginga Odinga teaching and referral hospital (JOOTRH) and the Kisumu east district hospital (KEDH). Study participants were enrolled from the JOOTRH. The community suffers most from food insecurity, lack of water and HIV/AIDS (Leenstra *et al*., 2004). Kisumu has a population of 968,909 in an area of 2085.9 km² (KNBS, 2009; Statoids, 2005), factors accelerating spread of TB. The study samples were collected from children suspected of TB visiting the outpatient and inpatient departments of JOOTRH in this area and met the eligibility criteria of the pediatrics study, which was being carried out within the hospital.

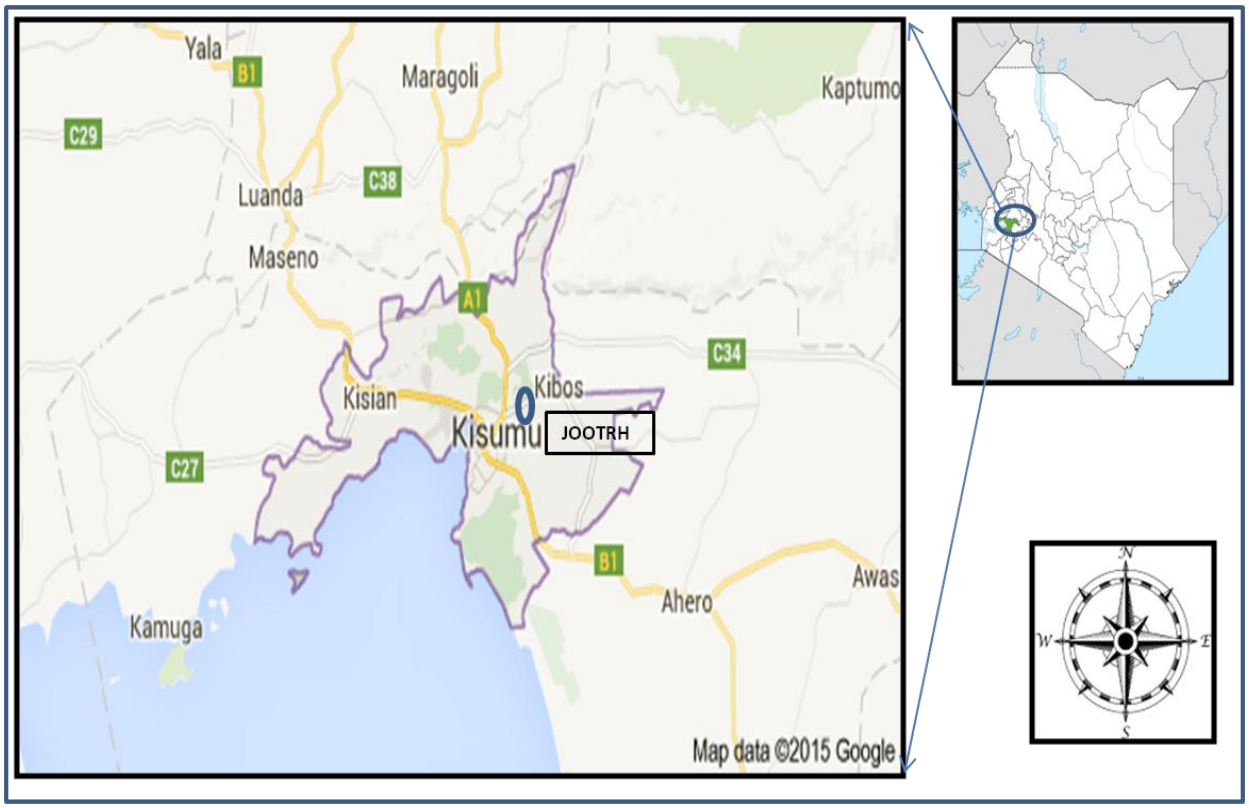

Figure 1: Map showing the study area of Kisumu. Map courtesy of http://mci.ei.columbia.edu/millennium-cities/kisumu-kenya/kisumu-maps-and-population-data.

3.2: Study Design

This was a cross-sectional study forming part of the larger ongoing prospective pediatrics TB study in Kisumu. Immune response to mycobacterial antigens (ESAT-6, CFP-10 and TB-7.7) was analyzed from frozen plasma, collected at one time-point during screening of TB exposed and non-exposed infants to determine their natural cytokine responses to TB antigens.

3.3 Study Population

This study was part of an on-going pediatrics study that seeks to determine the best combination of TB diagnostic methods among infants. Twenty five children, mean age 2.5 years, BCG vaccinated and either exposed or not exposed to TB were selected from the population. The test group comprised of 12 IFN-γ release assay confirmed positive children while the control group comprised 13 children that were negative based on IFN- γ release assay and free from any acute or chronic conditions based on clinical evaluation. *Mycobacterium* culture and genexpert was also performed.

3.4: Inclusion and Exclusion Criteria

Inclusion

Children testing positive on IGRA, aged less than five years, symptoms or history suggestive of TB exposure and whose parents or guardian consented to participate in the study were randomly selected and included in the study irrespective of the HIV status. Children testing negative on IGRA were randomly selected for inclusion in this study as controls.

Exclusion

Children with indeterminate IGRA results, aged 5 years and above, undergoing or had undergone TB treatment within 1year, and weigh less than 2.5kg were excluded from this study.

3.5: Sample Size Determination

The proportions method (NIHR, 2009) as indicated below was used to calculate sample size.

$N=K\{P1(1-P1)+P2(1-P2/P1-P2)^2\}$

Where;

N=Desired sample size

K=Value of α=0.05 and β=0.2 from the table:

	Power 50% β=0.5	80%β=0.2	90%β=0.1	95%β=0.05
α: 0.10	2.7	6.2	8.6	10.8
0.05	3.8	7.9	10.5	13
0.02	5.4	10	13	15.8
0.01	6.6	17.8	14.9	17.8

P1=Proportion 1

P2=Proportion2

In this study, the proportions of IGRA positive and IGRA negative children were investigated and these formed proportion 1 and 2 respectively. A similar study in Uganda was inferred since it also looked at cytokine responses to purified protein derivative (PPD) and found the percentages of TST positive/IFN-γ positive to TST positive/IFN-γ Negative (construed as true positive to false positive) to be 49.3% and 15.9% giving proportions of 0.493 to 0.159 as P1 and P2 respectively (Lewinsohn, 2008). The study was similar to the current study since BCG

vaccination was one of the eligibility criteria for the study. Hence this prevalence values was selected for inference. Therefore at K value of 7.9 (i.e. 80% confidence interval and α of 0.05), sample size for this study was calculated as follows:

$$N = \frac{K\,(P1\,(1-P1) + P2\,(1-P2))}{(P1-P2)^2}$$

$$= 7.9\left(\frac{(0.493(1-0.493) + 0.159(1-0.159))}{(0.493-0.159)^2}\right)$$

$$= 7.9\left(\frac{(0.493 \times 0.507) + (0.159 \times 0.8411))}{(0.334)^2}\right)$$

$$= 7.9\left(\frac{0.249951 + 0.1337349}{0.111556}\right)$$

$$= \frac{3.03107}{0.1116}$$

$$= 27.1$$

Twenty seven samples were selected to get an alpha (α) value of 0.05 at a power of 80.

3.6: Preparation of Samples

This cross-sectional study utilized plasma samples from children recruited into an ongoing pediatrics TB diagnosis study. The samples were collected between September 2013 and June 2014. For standardization of the assay, 2.4 milliliters of venous blood was collected from each child and 0.8mL distributed into each of 3 tubes as described below: A mitogen tube, coated with phytohemaglutinin (PHA) acting as positive control, a TB test antigen tube, coated with TB

antigens (ESAT-6, CFP-10 and TB 7.7(4) and an uncoated Nil Tube, acting as negative control, for each participant. Tubes were shaken vigorously to ensure coating of the blood collection tube surfaces with the blood to ensure adequate contact of blood cells with the coated antigens and incubated at 37°C for 20 hours *ex-vivo*. Following the *in-vitro* stimulation, tubes were spun, plasma harvested, an aliquot used to run IGRA according to the manufacturer's instructions (Appendix 1) and the remaining aliquot frozen at -80°C. Plasma was frozen at -80°C in order to batch to the required numbers for the subsequent cytokine luminex assay. Twelve IGRA positive and 13 IGRA negative samples were selected from the frozen samples to form the test and control arms

3.6.1: Interferon gamma release assay

Fifty microliters of test plasma was added into wells of a 96-well plate to which anti-human IFN-γ HRP working conjugate had been added. Serially diluted recombinant IFN- γ standards, S1, S2, S3, and S4, were added to respective standard wells. The plate was incubated for two hours. Following six washes, tetramethylbenzidine (TMB) substrate was added and reaction stopped using 0.5M sulphuric acid (H_2SO_4) following a thirty-minute incubation. Optical densities (OD) were captured on a Tecan® ELISA reader and using the Magellan® software supplied by the manufacturer and analyzed using Quantiferon TB Version 2.62 (QuantiFERON-TB Gold In-Tube, Cellestis, Carnegie, Australia). Results were interpreted as positive, negative or indeterminate for IFN-γ. (Detailed procedure in appendix 1)

3.6.2 : The Luminex assay

Procedure

Multiplexed microsphere-based immunoassay developed by Luminex (Luminex Corp., Austin TX) was used. Briefly, following reagents equilibration and thawing of plasma, standards were reconstituted in assay diluent and an 8 point standard curve made by serially diluting the reconstituted standard in assay diluent and blanks prepared. Wells were pre-wet, beads, added, washed and plasma samples, standards and incubation buffer added to assay plate and following an incubation and wash steps, detection antibody was added followed by streptavidin-PE. After incubation and a wash step, beads were re-suspended in assay buffer, and plate read at low PMT (Bio-Plex Manager 6.0 - low RP1 target). (Detailed procedure is outlined in appendix 2). A total of 25 cytokines namely IFN-γ, IP-10, IL-15, MIG, GM-CSF, IL-1ra, MIP-1a, IL-2, IL-2r, TNF-a, IL-7, IFN-α, IL-13, IL-6, MIP-1β, MCP-1, Eotaxin, IL-17, IL-12, RANTES, IL-5, IL-4, IL-8, IL-10, and IL-1β were analyzed in this assay. Two samples failed during the Luminex assay and were excluded from subsequent analysis.

3.7: Data Analysis

Cytokine responses were determined among the IFN-γ positive and negative samples. Data collected from this study was entered into excel spreadsheets and analyzed using Graphpad prism® version 6. The frequency of responders was compared between IFN-γ positive and negative using a Chi-square test. Each cytokine response was determined by subtracting the background cytokine concentration in plasma from blood incubated with buffered saline (Nil) from the cytokine concentration in plasma from blood stimulated with mitogen (phyto-hemagglutinin) or *M. tuberculosis* cocktail antigens. The levels of cytokine produced were

evaluated using Mann-Whitney U test. All the analyses were done at 95% confidence level and values of $P \leq 0.05$ were considered statistically significant.

3.8: Ethical Considerations

Approval to carry out this study was provided by the School of Graduate Studies (SGS), Maseno University (Appendix 4). Ethical approval was obtained from the Kenya Medical Research Institute Ethical Review Committee (KEMRI ERC) (Appendix 3). Informed consent was obtained from the parents or guardians who assented on behalf of the children. The assent form was signed and a copy issued to the guardian and a copy retained in the study file.

CHAPTER FOUR: RESULTS

4.1. Demographic Characteristics of the Study Population

A total of 25 children were enrolled in this study. Twelve (12) of them were IFN-γ positive for TB and 13 were IFN-γ negative age matched controls. The proportions of exposed verses unexposed were comparable between the cases and controls as shown in table 1 below.

Table 4.1: Clinical, demographic and laboratory characteristics of the study participants

	IFN- γ Positive (n)	IFN- γ Negative (n)	P-value
No. of subjects, n=25	12/25	13/25	
Age	2.57	2.58	
Gender			
Male	4/12	6/13	0.688[a]
Female	8/12	7/13	
HIV Status			
Positive	2 /12	3/13	1.00[a]
Negative	10/12	10/13	
TB			
Culture positive	3/12	0/13	0.25[a]
Culture negative	9/12	13/13	

[a] *Statistical significance determined by χ 2 . Values in bold were statistically significant at P≤0.05.*

4.2. Association of Th1 Cytokines and TB

Th1 cytokines are critical in the cellular immune response and they play an important role in host defense systems for intracellular microbial agents and viruses including mycobacterium. The frequency of Th1 cytokine responders and non-responders were calculated by mean plus two standard deviations of the non-responders for each individual cytokine as the cut off concentrations. Any value above this output in both groups was regarded as a responder. Table 2 below summarizes the frequencies of each cytokine.

Table 4.2: Frequency of Th1 cytokine responders in TB exposed and non-exposed

	Frequency of cytokine responders		
Cytokine	**IFN-γ positive**	**IFN-γ negative**	***p*-value(χ^2)**
IFN-γ	10/12	1/13	**0.0001**
IL-2	9/12	1/13	**0.0006**
IL-12	2/12	1/13	0.4903
TNF-α	7/12	1/13	**0.0067**
IFN-α	4/12	1/13	0.1093
IL-1ra	5/12	1/13	**0.0469**
RANTES	2/12	1/13	0.4903
Eotaxin	1/12	0/13	0.2881
MCP-1	4/12	1/13	0.1093
MIP-1β	0/12	1/13	0.3268
IL-15	10/12	2/13	**0.0011**
IP-10	8/12	1/13	**0.0021**
IL-2r	6/12	1/13	**0.0186**
MIG	10/12	0/13	**0.0001**
IL-8	3/12	1/13	0.2383

Th1 cytokines depicting proportions of individuals expressing IFN- γ, IL-2,TNF- α, IL-1ra, IL-15, IP-10, IL-2r and MIG showing significant difference in number between IFN- γ positive and IFN- γ negative arm with IL-12, IFN-α, RANTES, *Eotaxin, MCP-1 ,MIP1 β and IL-8 showing no significant difference between the two study arms. Values in bold were statistically significant at $p \leq 0.05$.*

Table 4.3: Concentration levels of Th1 cytokines and TB

Cytokine	Median cytokine responses(pg/ml)		*p*-value(*Mann Whitney U test*
	IFN-γ positive	IFN-γ negative	
IFN-γ	145.5	0.0	**0.0001**
IL-2	65.8	0.0	**0.0002**
IL-12	15.4	11.7	0.1480
TNF-α	2.6	0.0	**0.0280**
IFN-α	4.5	0.0	**0.0198**
IL-1ra	706.6	0.0	**0.0005**
RANTES	1827.0	663.9	0.5807
Eotaxin	0.0	0.0	0.4800
MCP-1	0.0	0.0	0.0662
MIP-1β	673.6	296.1	0.1081
IL-15	96.9	0.0	**0.0001**
IP-10	3419.0	21.2	**0.0001**
IL-2r	122.6	0.0	**0.0420**
MIG	429.5	3.6	**0.0001**
IL-8	0.0	0.0	0.0680

Th1 cytokines depicting IFN- γ, IL-2, TNF- α, IL-1ra, IL-15, IP-10, IL-2r and MIG cytokines showing significant difference in levels between IFN-γ positive and IFN-γ negative arm with IL-12, RANTES, Eotaxin, MCP-1, MIP-1β and IL-8 showing no significant difference between the two study arms. Values in bold were statistically significant at $p \leq 0.05$.

4.3: Association of Th2 Cytokines and TB

Th2 cells mediate the activation and maintenance of the humoral immune response against extracellular parasites, bacteria, allergens, and toxins by producing various cytokines such as IL-4, IL-5, IL-6, IL-9, IL-13, and IL-17E (IL-25) that are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses. Most of the Th2 cytokines evaluated were comparable between the two groups except for IL-6 and IL-13 which were significantly higher in IFN-γ positive as compared to IFN-γ negative children as shown in table 3 below.

Table 4.4: Frequency of Th2 cytokine responders in TB exposed and non-exposed

Cytokine	Frequency of Th2 cytokine responders		*p*-value(χ^2)
	IFN-γ positive	IFN-γ negative	
IL-4	1/12	1/13	0.9529
IL-5	3/12	1/13	0.2383
IL-6	0/12	0/13	0.9999
IL-10	1/12	1/13	0.9529
IL-13	4/12	0/13	**0.0231**
IL-7	5/12	0/13	**0.0093**

Th2 cytokines depicting proportions of individuals expressing IL-13 and IL-7 showing significant difference in numbers between IFN-γ positive and IFN-γ negative arm with IL-4, IL-5, IL-6 and IL-10 showing no significant difference between the two study arms. Values in bold were statistically significant at $p \leq 0.05$.

Table 4.5: Concentration levels of Th2 cytokines and TB

Cytokine	Median Cytokine values(pg/ml)		*p*-value*(Mann Whitney U Test)*
	IFN-γ +	IFN-γ -	
IL-4	2.7	1.7	0.3831
IL-5	0.0	0.0	0.6802
IL-6	77.5	0.0	**0.0360**
IL-10	0.0	0.0	0.7943
IL-13	2.1	0.0	**0.0225**
IL-7	7.7	0.0	**0.0196**

Th2 cytokines depicting IL-6, IL-13, and IL-7 showing significant difference in levels between IFN- γ positive and IFN- γ negative arm with IL-4, IL-5 and IL-10 showing no significant difference between the two study arms. Values in bold were statistically significant at $p \leq 0.05$

4.4: Association of Th17 Cytokines and TB

IL-17 cytokines play a role in pathology of TB as some studies have suggested. Many studies however found no major role in tuberculosis. The current study similarly did not find any significant difference between the study and control groups (p=0.22) with the exception of MIP-1α, GM-CSF and IL-6.

Table 4.6: Frequency of Th17 cytokine responders in TB exposed and non-exposed

Cytokine	Frequency of Th17 cytokine responders		*p*-value(χ^2)
	IFN-γ positive	IFN-γ negative	
IL-1β	1/12	1/13	0.9529
IL-6	0/12	0/13	0.9999
GM-CSF	9/12	0/13	**0.0002**
IL-17	2/12	0/13	0.1249
MIP-1α	7/12	1/13	**0.0067**

Th17 cytokines depicting proportions of individuals expressing GM-CSF and MIP-1α showing significant difference in levels between IFN-γ positive arm and IFN-γ negative arm with IL-1β, IL-6 and MIP-1α showing no significant difference between the two study arms. Values in bold were statistically significant at $p \leq 0.05$

Table 4.7: Concentration levels of Th17 cytokines and TB

Cytokine	Median Cytokine values(pg/ml)		*p*-value *(Mann Whitney U test)*
	IFN-γ positive	IFN-γ negative	
IL-1β	175.1	28.8	0.0551
IL-6	77.5	0.0	**0.0360**
GM-CSF	1.3	0.0	**0.0001**
IL-17	0.0	0.0	0.2200
MIP-1α	158.9	0.0	**0.0001**

Th17 cytokines depicting IL-6, GM-CSF, and MIP-1α showing significant difference in levels between IFN- γ positive arm and IFN- γ negative arm with IL-1β and IL-17 showing no significant difference between the two study arms. Values in bold were statistically significant at $p \leq 0.05$

CHAPTER FIVE:DISCUSSION

Tuberculosis is one of the leading causes of mortality and morbidity especially in sub-Saharan Africa where there is emergence of HIV/AIDS pandemic. Historically BCG vaccine has been used during childhood to prevent TB infection and progression to an active case. However, since the advent of the HIV/AIDS, TB has been a big opportunistic infection that complicates the progression of HIV infection to full blown AIDS condition due to reduced immunity in the later case. Although BCG has been effective in control and prevention of TB, the actual mechanism underlying the protection is not completely understood. Furthermore, there is an increasing trend in detectable active TB cases especially in regions with high prevalence of HIV/AIDS. These two factors have necessitated new investigation to establish correlates of protection and diagnostic biomarkers which would help in enhancing the TB diagnosis and anti-TB vaccine efficacy. To this end the current study was designed to evaluate different cytokines that are elicited in response to a cocktail of specific antigens expressed by *M. tuberculosis*. Of the 25 different cytokines evaluated in the plasma, majority of the Th1 and a few Th2 and Th17 cytokines were found to be significantly higher in IFN- γ positive as compared to IFN- γ negative group.

Several studies have demonstrated the cellular and humoral immune responses with the major cells being alveolar macrophages (AM) that phagocytose the bacilli and kill them by various mechanisms, including the generation of reactive nitrogen and oxygen intermediates. Macrophages secrete IL-12, IL-18, IFN-γ and TNF-α. IFN-γ induces mycobacteria killing while TNF-α controls bacilli growth and granuloma formation by inducing chronic cytokine stimulation and stopping mycobacteria proliferation in the granuloma. These lead to development of a localized infection, which may become latent. In the majority of such

infections the bacterium is contained in the granuloma which prevents spreading of the *M. tuberculosis* (Aldovini and Young, 1991), (Zuniga *et al.*, 2012) and (Germann *et al.*, 1996). Success depends on the microbicidal capacity of the macrophages and the survival stability of the *M. tuberculosis* strain (Mauel, 1982a, , 1982b).

A few studies have looked at multiple cytokines during the course of natural TB infection by activating host cells with TB antigens. A study in Uganda, using a 10-plex assay and ESAT-6+CFP-10+TB7.7 as stimulatory TB antigens among adults , noted elevation of IFN-γ, IL-2, IL-6, IL-8, IP-10, MCP-1, MIP-1β, and TNF-α responses among culture confirmed TB patients compared with controls (Kellar *et al*., 2011). Furthermore three cytokines, IL4, IL-6 and Il-10 have been shown to be elevated during active disease (Verbon *et al.*, 1999)

Findings from this study indicate additional cytokines that may not have been identified by previous studies as potential biomarkers of TB diagnosis and/or protection since they were found to be secreted at higher levels in response to BCG antigen in children within IFN-γ positive group. The proportion of cytokine responders as well as levels of following cytokines were found to be elevated in the IFN-γ positive as compared to the IFN-γ negative children: IFN-γ, IP-10, IL-15, MIG, GM-CSF, IL-1ra, MIP-1α, IL-2, IL-2r, TNF-α, IL-7, IFN-α, IL-6, and IL-13. This study was consistent and lends support to a previous study looking at multiple cytokines in a similar group using two different assays (Kellar *et al*., 2011). Kellar noted elevation of IFN-γ, IL-2, IL-6, IL-8, IP-10, MCP-1, MIP-1β, and TNF-α. These findings were consistent with the findings on IFN-γ, IP-10, IL2, TNF-α, and IL-6 but in contrast lower levels of MIP-1β, MCP-1, and IL-8 were observed in the current study. Kellar's study also did not evaluate IL-15, MIG, GM-CSF, IL-1ra, MIP-1α, IL-7, IFN- α, EOTAXIN, IL-17, IL12, RANTES, IL-5, and IL-1β nor report on proportion of individuals responding to individual cytokines as was done in this study.

In a study that evaluated 14 different cytokines responses to differentiate latent from active infections using ESAT-6 and CFP-10 antigens to stimulate whole blood, Borgstrom and others found IFN-γ, IP-10, MIP-1β, IL-2, TNF-α, IL-6, IL-10, IL-13 and GM-CSF to be different between the two groups (Borgstrom *et al.,* 2012) which corroborates with the current study except for lower observation in MIP-1 β and IL-10.

Th1 Cytokine Responses to TB

Th1 cell promoting factors include IFN-γ, IL-12 (p70), and the activation of the transcription factors STAT1 and STAT4 (ebioscience, 2015). The expression of the Interleukin-12 receptor β2-chain (IL-12Rβ2) is required for Th1 cellular differentiation through induction of IL-12 and IFN-γ expression through STAT1 signals. Th1 cells are the primary source for the inflammatory cytokines IFN-γ, IL-2, and TNF-β that stimulate macrophages, lymphocytes, and PMNs in the destruction of bacterial pathogens including *M. tuberculosis* (ebioscience, 2015).

The primary cytokine that has so far been documented by several studies to directly suppress *M. tuberculosis* is IFN-γ. It has been demonstrated to induce resistance to mycobacteria (Flynn *et al* 1993,Flynn and bloom,1996), latent TB diagnosis and determining progression from latent to active disease (Diel *et al.*, 2011). It's an autocrine factor in the establishment of Th1 cells and its action is enhanced by the presence of IL-12. It activates macrophages and stimulates B cells to produce receptors that enhance the attachment of microbes to phagocytes facilitating clearance (Xing *et al.*, 2000). It is one of the primary markers and key protective cytokine induced by current tuberculosis vaccines. It has also been demonstrated to be useful in diagnosis of tuberculosis in special groups for instance the HIV infected (Brock *et al*., 2006). In support of previous studies on the role of IFN-γ as a biomarker of TB diagnosis and protection, the current

study observed higher IFN-γ responses in TB exposed as compared to the non-exposed control group.

Interleukin 2 (IL-2) is secreted by activated T cells and mainly stimulate growth, differentiation, and survival of antigen-specific effector and memory T cells (ebioscience, 2015). During TB infection IL-2 plays a critical role in pathogenic clearance as demonstrated by its inverse proportional increase with decreasing bacterial load (Millington *et al*., 2007). This cytokine has been focused as one of the critical cytokines required for effective control of TB infection in BCG and new vaccines trials (Abel *et al.*, 2010). In the present study, the proportion and levels of IL-2 responses were higher in TB exposed as compared to the non-exposed control group. The high levels of IL-2 elicited during TB infection may be important in the activation and differentiation of TB specific T cells to produce IFN-γ that would enhance the anti-microbial activity of macrophages. This activation leads to eventual intracellular killing of the mycobacterium within the infected macrophages. Interleukin 12 (IL-12) is secreted by activated macrophages and promotes survival and growth of Th1 cells (ebioscience, 2015). Although the role of this cytokine in the present and earlier studies seems to be unclear due to similar levels in cases and controls, it could be possible that the sustained survival of Th1 cells producing IFN-γ suggest that this cytokine may indirectly influence the course of TB progression through IFN-γ production.

TNF-α is a general potent and pleiotropic immune activator and regulator of immune cell function. It induces the specific production of class I MHC antigen, GM-CSF, and IL-1 which are key in pathogen elimination hence disease control. Apart from IFN-γ and IL-2, this cytokine has been shown in previous studies to be of greater significance in TB infection and control. It has been one of the target cytokines in most of the TB vaccine candidates in the advanced phases

of clinical trials including Aeras-402 (Abel *et al*., 2010) and Glaxosmithklines M72/ASO1 (Day *et al*., 2013; Idoko *et al*., 2014; Montoya *et al*., 2013; Thacher *et al*., 2014). The present study lends support to the potential role of this cytokine in TB infection and resolution since it was demonstrated to be higher in cases compared to controls.

Th2 Cytokine Responses to TB

Th2 cells mediate the activation and maintenance of the humoral immune response against extracellular parasites, bacteria, allergens, and toxins by producing various cytokines such as IL-4, IL-5, IL-6, IL-9, IL-13, and IL-17E (IL-25) that are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses. These cytokines also counteract the Th1 responses that allow for the Th2 responsiveness to IL-4. IL-4 signals through STAT6 to up-regulate GATA3 expression, the master regulator of Th2 cell differentiation. Repression of this activity results in the development failure of IL-4 producing cells. IL-4 also suppresses Th1 and Th17 cell responses through the up-regulation of transcriptional repressor(s) of IFN-γ and IL-17 production. Functionally, Th2 cytokines have effects on many cell types in the body as the cytokine receptors are widely expressed on numerous cell types. Th2 cells stimulate and recruit specialized subsets of immune cells, such as eosinophils and basophils, to the site of infection or in response to allergens or toxin leading to tissue eosinophilia and mast cell hyperplasia. They also control the regulation of B cell class-switching to IgE.

IL-10_is an auto-regulator of Th1 cell activation. It's an anti-inflammatory cytokine. A recent study identified that TB distorts its inhibitory impact in an HIV co-infection (Chetty *et al.*, 2014). This cytokine has not however been demonstrated to play a major role in TB. The current

study did not find any significant difference in the levels of this cytokine between the IFN- γ positive and IFN- γ negative groups.

Interleukin 4 (IL-4) and IL-6 are required for Th2 priming and maturation. They mainly function by inhibiting proliferation and differentiation of Th1 cells and stimulation of B cell proliferation and maturation into plasma cells. Although IL-4 has been associated with reduced risk of extra-pulmonary and severe tuberculosis in certain human populations (Qi *et al.*, 2014), the exact mechanism is not well understood since it opposes the function of Th1 cytokines that are known to be the primary regulators of immunity to TB. Although the current study showed no difference in response between cases and controls suggesting limited role of IL-4 to TB infection, it is possible that this cytokine may contribute towards the humoral responses to TB. IL6 has unique ability to carry out both pro and anti-inflammatory effects (Scheller *et al.*, 2010). This complexity could explain the observed higher responses in cases as compared to controls. Previous studies have also shown similar trend of higher IL-6 responses in active disease (Cowan *et al*., 2012).

IL-13 stimulates B-cell production of IgE attracts basophils and mediates the release of granules and triggers mast cells to release granules. It has been shown to be involved in tissue pathology in tuberculosis (Heitmann *et al*., 2014). The higher levels observed in cases compared to controls in this study suggest its role in degranulation and releases of cellular contents that would potentially act on mycobacterium.

Th17 Cytokine Responses to TB

Th17 cytokine responses to TB Th17 cells play a key role in host defense against extracellular microbes such as bacteria and fungi and play a significant role in autoimmune disease and its inflammatory response. They are localized in tissues that separate the host from the environment, mainly the skin and mucosa. Through their activation and subsequent cytokine production, they trigger pro-inflammatory signaling that promotes neutrophil mobilization and the expression of antimicrobial peptides (Torrado and Cooper, 2010).

Up-regulation of Th17 cytokines in collaboration with Th1 has been reported in protection against TB through elevation of Interleukin 1Beta (IL-1β) (Wareham *et al*., 2014), a cytokine involved in early Th17 differentiation and maintenance of post-polarization. It mediates inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The direct role in TB infection has not been documented. Although IL-17 regulates local tissue inflammation through coordinated expression of pro-inflammatory and neutrophil-mobilizing cytokines and chemokines, its levels were found to be comparable in cases and controls in the present study. Some studies have found IL-17 to accelerate apoptosis of polymorphonuclear cells (PMNs) in TB (Jiang L, 2014) ,while other studies have found no role in osteoarticular TB (Tiwari *et al.*, 2014). A recent study has implicated this cytokine in immune pathology of TB and sought for a balance between Th1 and Th17 (Fan L, 2015).

GM-CSF is critical for the pro-inflammatory functions of Th17 cells. This cytokine has recently been demonstrated to mediate invariant natural killer T-cells (iNKT) activation, anti-microbial activity and *Mycobacterium* growth control demonstrating its antimicrobial effector function and

important role in T cell immunity against *M. tuberculosis* contrary to earlier knowledge that indicated requirement for IL-12 and IL-18 (Rothchild *et al.*, 2014). The present study demonstrated significance response levels between the cases and controls supporting other studies on the role of this cytokine in TB. The lack of major trend in Th17 family of cytokines is possible due to the fact that little is unknown on Th17 and association with diseases as is one family of recently defined cytokines. More studies are required to definitely pinpoint their contribution to disease pathogenesis and protection.

CHAPTER SIX: CONCLUSION

This study identified several cytokines in addition to the conventional IFN-γ that would be useful in protection and/or diagnosis of TB. These include IFN-γ, IP-10, IL-15, MIG, GM-CSF, IL-1ra, MIP-1a, IL-2, IL-2r, TNF-a, IL-7, IFN-α, IL-6, and IL-13.These cytokines would potentially aid in TB diagnosis as well as serve as correlates of protection against TB since their levels of expression were significantly higher in IFN-γ positive as compared to the IFN-γ negative children. Cytokines which did not show any significant difference between the cases and control groups included MIP-1β, MCP-1, Eotaxin, IL-17, IL-12, RANTES, IL-5, IL-4, IL-8, IL-10 and IL-1β and could either play a less significant role in tuberculosis or are under negative regulation by the other cytokines.

6.1. RECOMMENDATIONS FROM THE PRESENT STUDY

1. Detection of differential cytokine levels between IFN-γ positive as compared to the IFN-γ negative children could be useful in making clinical decisions for patient management as well as determining disease prognosis.

2. Vaccines targeted to induction or regulation of these cytokines would equally be useful in combating and managing tuberculosis.

6.2. RECOMMENDATIONS FOR FUTURE STUDIES

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1. The role of all the cytokines which demonstrated significant difference between the cases and controls should be evaluated in future studies for potential role in diagnosis, predisposition and protection against TB infection and progression to active disease.

2. Combination of multiple cytokines would be important in accurate diagnosis of TB and in overcoming the challenge with differentiating latent from active TB infection.

3. Furthermore, the role of common co-infection such as HIV, malaria and schistosomiasis in influencing the course of multiple cytokines in TB infection and progression to disease should be considered future studies.

REFERENCES

Abel, B.Tameris, M.Mansoor, N.Gelderbloem, S.Hughes, J.Abrahams, D.Makhethe, L.Erasmus, M.de Kock, M.van der Merwe, L.Hawkridge, A.Veldsman, A.Hatherill, M.Schirru, G.Pau, M. G.Hendriks, J.Weverling, G. J.Goudsmit, J.Sizemore, D.McClain, J. B.Goetz, M.Gearhart, J.Mahomed, H.Hussey, G. D.Sadoff, J. C., and Hanekom, W. A. (2010). The novel tuberculosis vaccine, AERAS-402, induces robust and polyfunctional CD4+ and CD8+ T cells in adults. *Am J Respir Crit Care Med, 181*(12), 1407-1417.

Al-Attiyah, R. (2006). Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of Mycobacterium tuberculosis. *FEMS Immunol Med Microbiol, 47*(2), 254-261.

Al-Attiyah, R., and Mustafa, A. S. (2010). Characterization of human cellular immune responses to Mycobacterium tuberculosis proteins encoded by genes predicted in RD15 genomic region that is absent in Mycobacterium bovis BCG. *FEMS Immunol Med Microbiol, 59*(2), 177-187.

Al-Salem, A. H.Kothari, M. R.AlHani, H. M.Oquaish, M. M.Khogeer, S. S., and Desouky, M. S. (2012). Safety of intradermal Bacillus Calmette-Guerin vaccine for neonates in Eastern Saudi Arabia. *Saudi Med J, 33*(2), 172-176.

Aldovini, A., and Young, R. A. (1991). Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. *Nature, 351*(6326), 479-482.

Ariga, H., and Harada, N. (2008). [Evolution of IGRA researches]. *Kekkaku, 83*(9), 641-652.

Ayisi, J. G.van't Hoog, A. H.Agaya, J. A.McHembere, W.Nyamthimba, P. O.Muhenje, O., and Marston, B. J. (2011). Care seeking and attitudes towards treatment compliance by newly

enrolled tuberculosis patients in the district treatment programme in rural western Kenya: a qualitative study. *BMC Public Health, 11*, 515.

Brock, I.Ruhwald, M.Lundgren, B.Westh, H.Mathiesen, L. R., and Ravn, P. (2006). Latent tuberculosis in HIV positive, diagnosed by the M. tuberculosis specific interferon-gamma test. *Respir Res, 7*, 56.

Chetty, S.Porichis, F.Govender, P.Zupkosky, J.Ghebremichael, M.Pillay, M.Walker, B. D.Ndung'u, T.Kaufmann, D. E., and Kasprowicz, V. O. (2014). Tuberculosis distorts the inhibitory impact of interleukin-10 in HIV infection. *Aids, 28*(18), 2671-2676.

Cowan, J.Pandey, S.Filion, L. G.Angel, J. B.Kumar, A., and Cameron, D. W. (2012). Comparison of interferon-gamma-, interleukin (IL)-17- and IL-22-expressing CD4 T cells, IL-22-expressing granulocytes and proinflammatory cytokines during latent and active tuberculosis infection. *Clin Exp Immunol, 167*(2), 317-329.

Cunningham, B. (1925). North Carolina Academy of Science. *Science, 62*(1593), 37-38.

Day, C. L.Tameris, M.Mansoor, N.van Rooyen, M.de Kock, M.Geldenhuys, H.Erasmus, M.Makhethe, L.Hughes, E. J.Gelderbloem, S.Bollaerts, A.Bourguignon, P.Cohen, J.Demoitie, M. A.Mettens, P.Moris, P.Sadoff, J. C.Hawkridge, A.Hussey, G. D.Mahomed, H.Ofori-Anyinam, O., and Hanekom, W. A. (2013). Induction and regulation of T-cell immunity by the novel tuberculosis vaccine M72/AS01 in South African adults. *Am J Respir Crit Care Med, 188*(4), 492-502.

Denkinger, C. M.Kik, S. V.Cirillo, D. M.Casenghi, M.Shinnick, T.Weyer, K.Gilpin, C.Boehme, C. C.Schito, M.Kimerling, M., and Pai, M. (2015). Defining the needs for next generation assays for tuberculosis. *J Infect Dis, 211 Suppl 2*, S29-38.

Diel, R. (2008). Predictive value of a whole blood IFN-gamma assay for the development of active tuberculosis disease after recent infection with Mycobacterium tuberculosis. *Am J Respir Crit Care Med, 177*(10), 1164-1170.

Diel, R.Goletti, D.Ferrara, G.Bothamley, G.Cirillo, D.Kampmann, B.Lange, C.Losi, M.Markova, R.Migliori, G. B.Nienhaus, A.Ruhwald, M.Wagner, D.Zellweger, J. P.Huitric, E.Sandgren, A., and Manissero, D. (2011). Interferon-gamma release assays for the diagnosis of latent Mycobacterium tuberculosis infection: a systematic review and meta-analysis. *Eur Respir J, 37*(1), 88-99.

Dimakou, K.Papaioannides, D.Latsi, P.Katsimboula, S.Korantzopoulos, P., and Orphanidou, D. (2004). Disseminated tuberculosis complicating anti-TNF-alpha treatment. *Int J Clin Pract, 58*(11), 1052-1055.

ebioscience. (2015). T Helper 1 (Th1) Cells & Cytokines. from http://www.ebioscience.com/knowledge-center/antigen/cytokines/th1-cells.htm

Eruslanov, E. B.Lyadova, I. V.Kondratieva, T. K.Majorov, K. B.Scheglov, I. V.Orlova, M. O., and Apt, A. S. (2005). Neutrophil responses to Mycobacterium tuberculosis infection in genetically susceptible and resistant mice. *Infect Immun, 73*(3), 1744-1753.

Fan L, X. H., Mai G, Su B, Ernst J, and Hu Z. (2015). Impaired M. tuberculosis Antigen-Specific IFN-γ Response without IL-17 Enhancement in Patients with Severe Cavitary Pulmonary Tuberculosis. *PLoS One, 10*(5).

Germann, T.Hess, H.Szeliga, J., and Rude, E. (1996). Characterization of the adjuvant effect of IL-12 and efficacy of IL-12 inhibitors in type II collagen-induced arthritis. *Ann N Y Acad Sci, 795*, 227-240.

Greenaway, C.Lienhardt, C.Adegbola, R.Brusasca, P.McAdam, K., and Menzies, D. (2005). Humoral response to Mycobacterium tuberculosis antigens in patients with tuberculosis in the Gambia. *Int J Tuberc Lung Dis, 9*(10), 1112-1119.

Hanekom, W. A.Hawkridge, A.Mahomed, H.Scriba, T. J.Tameris, M.Hughes, J.Hatherill, M.Day, C. L., and Hussey, G. D. (2012). SATVI - after 10 years closing in on a new and better vaccine to prevent tuberculosis. *S Afr Med J, 102*(6), 438-441.

Heitmann, L.Abad Dar, M.Schreiber, T.Erdmann, H.Behrends, J.McKenzie, A. N.Brombacher, F.Ehlers, S., and Holscher, C. (2014). The IL-13/IL-4Ralpha axis is involved in tuberculosis-associated pathology. *J Pathol, 234*(3), 338-350.

Herbst, S.Schaible, U. E., and Schneider, B. E. (2011). Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. *PLoS One, 6*(5), e19105.

Hock, H.Dorsch, M.Kunzendorf, U.Uberla, K.Qin, Z.Diamantstein, T., and Blankenstein, T. (1993). Vaccinations with tumor cells genetically engineered to produce different cytokines: effectivity not superior to a classical adjuvant. *Cancer Res, 53*(4), 714-716.

Idoko, O. T.Owolabi, O. A.Owiafe, P. K.Moris, P.Odutola, A.Bollaerts, A.Ogundare, E.Jongert, E.Demoitie, M. A.Ofori-Anyinam, O., and Ota, M. O. (2014). Safety and immunogenicity of the M72/AS01 candidate tuberculosis vaccine when given as a booster to BCG in Gambian infants: an open-label randomized controlled trial. *Tuberculosis (Edinb), 94*(6), 564-578.

J.W.Fang. (2011). Interleukin-17A differentially modulates BCG induction of cytokine production in human blood macrophages. *Journal of Leukocyte Biology, 90*, 333-341.

Jiang L, Y. C., Jin Q, and Li B. (2014). Effect of interleukin-17 on neutrophil apoptosis in patients with tuberculosis. *30(8)*, 833-836.

K.A.I.S. (2007). Kenya Aids Indicator survey.

Kagina, B. M.Tameris, M. D.Geldenhuys, H.Hatherill, M.Abel, B.Hussey, G. D.Scriba, T. J.Mahomed, H.Sadoff, J. C.Hanekom, W. A.Mansoor, N.Hughes, J.de Kock, M.Whatney, W.Africa, H.Krohn, C.Veldsman, A.Kany, A. L.Douoguih, M.Pau, M. G.Hendriks, J.McClainc, B.Benko, J.Snowden, M. A., and Hokey, D. A. (2014). The novel tuberculosis vaccine, AERAS-402, is safe in healthy infants previously vaccinated with BCG, and induces dose-dependent CD4 and CD8T cell responses. *Vaccine, 32*(45), 5908-5917.

Kaufmann, S. H. E. (2002). *Protection against tuberculosis:cytokines,T cells,and macrophages*.

Kedzierska. (2001). Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother., 2001 May;12(3):*, 133-150.

Kellar, K. L.Gehrke, J.Weis, S. E.Mahmutovic-Mayhew, A.Davila, B.Zajdowicz, M. J.Scarborough, R.LoBue, P. A.Lardizabal, A. A.Daley, C. L.Reves, R. R.Bernardo, J.Campbell, B. H.Whitworth, W. C., and 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.

KNBS. (2009). http://www.scribd.com/doc/36672705/Kenya-Census-2009.

Leenstra, T.Kariuki, S. K.Kurtis, J. D.Oloo, A. J.Kager, P. A., and ter Kuile, F. O. (2004). Prevalence and severity of anemia and iron deficiency: cross-sectional studies in adolescent schoolgirls in western Kenya. *Eur J Clin Nutr, 58*(4), 681-691.

Lewinsohn, D. A. (2008). Whole Blood Interferon-Gamma Responses to Mycobacterium tuberculosis Antigens in Young Household Contacts of Persons with Tuberculosis in Uganda. *PLoS One, 3*(10).

M.O.H. (2013). Kisumu County health at a glance. Retrieved 31 Mar 2015, 2015

Mauel, J. (1982a). In vitro induction of intracellular killing of parasitic protozoa by macrophages. *Immunobiology, 161*(3-4), 392-400.

Mauel, J. (1982b). Macrophage activation and effector mechanisms against microbes. *Adv Exp Med Biol, 155*, 675-686.

McShane, H. (2011). Tuberculosis vaccines: beyond bacille Calmette-Guerin. *Philos Trans R Soc Lond B Biol Sci, 366*(1579), 2782-2789.

McShane, H.Pathan, A. A.Sander, C. R.Keating, S. M.Gilbert, S. C.Huygen, K.Fletcher, H. A., and Hill, A. V. (2004). Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med, 10*(11), 1240-1244.

Millington, K. A.Innes, J. A.Hackforth, S.Hinks, T. S.Deeks, J. J.Dosanjh, D. P.Guyot-Revol, V.Gunatheesan, R.Klenerman, P., and 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.

Montoya, J.Solon, J. A.Cunanan, S. R.Acosta, L.Bollaerts, A.Moris, P.Janssens, M.Jongert, E.Demoitie, M. A.Mettens, P.Gatchalian, S.Vinals, C.Cohen, J., and Ofori-Anyinam, O. (2013). A randomized, controlled dose-finding Phase II study of the M72/AS01 candidate tuberculosis vaccine in healthy PPD-positive adults. *J Clin Immunol, 33*(8), 1360-1375.

Nascop. (2014). *Kenya HIV estimates*.

NIHR. (2009). Sampling and Sample Size Calculation.

Pawlowski, A. (2012). Tuberculosis and HIV Co-infection. *PLOS PATHOGENS, 8*(2).

Pelosi, A.Smith, D.Brammananth, R.Topolska, A.Billman-Jacobe, H.Nagley, P.Crellin, P. K., andCoppel, R. L. (2012). Identification of a novel gene product that promotes survival of Mycobacterium smegmatis in macrophages. *PLoS One, 7*(2), e31788.

Qi, H.Sun, L.Jin, Y. Q.Shen, C.Chu, P.Wang, S. F.Yin, Q. Q.Qi, Z.Xu, F.Jiao, W. W.Wu, X. R.Tian, J. L.Xiao, J., andShen, A. D. (2014). rs2243268 and rs2243274 of Interleukin-4 (IL-4) gene are associated with reduced risk for extrapulmonary and severe tuberculosis in Chinese Han children. *Infect Genet Evol, 23*, 121-128.

Rahman, S.Magalhaes, I.Rahman, J.Ahmed, R. K.Sizemore, D. R.Scanga, C. A.Weichold, F.Verreck, F.Kondova, I.Sadoff, J.Thorstensson, R.Spangberg, M.Svensson, M.Andersson, J.Maeurer, M., and Brighenti, S. (2012). Prime-boost vaccination with rBCG/rAd35 enhances CD8(+) cytolytic T-cell responses in lesions from Mycobacterium tuberculosis-infected primates. *Mol Med, 18*, 647-658.

Roberts, L.Passmore, J. A.Williamson, C.Little, F.Bebell, L. M.Mlisana, K.Burgers, W. A.van Loggerenberg, F.Walzl, G.Djoba Siawaya, J. F.Karim, Q. A., andKarim, S. S. (2010). Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression. *Aids, 24*(6), 819-831.

Robinson HL, A. R. (2005). T cell vaccines for microbial infections. *Nat Med, 11*(S25-S32).

Rothchild, A. C.Jayaraman, P.Nunes-Alves, C., and Behar, S. M. (2014). iNKT cell production of GM-CSF controls Mycobacterium tuberculosis. *PLoS Pathog, 10*(1), e1003805.

Scheller, J.Chalaris, A.Schmidt-Arras, D., and Rose-John, S. (2010). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta, 1813*(5), 878-888.

Sitienei, J. (2010). Engaging Professional Associations in TB Control in Kenya: DLTLD MOPHS - Kenya.

Statoids. (2005). http://www.statoids.com/yke.html.

Thacher, E. G.Cavassini, M.Audran, R.Thierry, A. C.Bollaerts, A.Cohen, J.Demoitie, M. A.Ejigu, D.Mettens, P.Moris, P.Ofori-Anyinam, O., and Spertini, F. (2014). Safety and immunogenicity of the M72/AS01 candidate tuberculosis vaccine in HIV-infected adults on combination antiretroviral therapy: a phase I/II, randomized trial. *Aids, 28*(12), 1769-1781.

Tiwari, U.Ramachandran, V. G.Das, S., and Kumar, S. (2014). Interleukin-3 and interleukin-17 do not play a dynamic role in the immunopathogenesis of osteoarticular tuberculosis. *Indian J Tuberc, 61*(2), 142-147.

Torrado, E., and Cooper, A. M. (2010). IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev, 21*(6), 455-462.

van't Hoog, A. H.Laserson, K. F.Githui, W. A.Meme, H. K.Agaya, J. A.Odeny, L. O.Muchiri, B. G.Marston, B. J.DeCock, K. M., and Borgdorff, M. W. (2011). High prevalence of pulmonary tuberculosis and inadequate case finding in rural western Kenya. *Am J Respir Crit Care Med, 183*(9), 1245-1253.

van Crevel, R.Ottenhoff, T. H., and van der Meer, J. W. (2002). Innate immunity to Mycobacterium tuberculosis. *Clin Microbiol Rev, 15*(2), 294-309.

Verbon, A.Juffermans, N.Van Deventer, S. J.Speelman, P.Van Deutekom, H. a., and Van Der Poll, T. (1999). Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol, 115*(1), 110-113.

W.H.O. (2007). Revised BCG vaccination guidelines for infants at risk for HIV infection. *Wkly Epidemiol Rec, 82*(21), 193-196.

W.H.O. (2011). Tuberculosis global facts 2010/2011. *Cent Eur J Public Health, 18*(4), 197.

W.H.O. (2012a). Tuberculosis factsheet No.104. *104*.

W.H.O. (2012b). W.H.O Global Tuberculosis report 2012.

W.H.O. (2014a). *Global Tuberculosis report 2014*. France: W.H.O.

W.H.O. (2014b). Tuberculosis country profiles. Retrieved 31/03/2015, 2015

Wallis, R. S., andEllner, J. J. (1994). Cytokines and tuberculosis. *J Leukoc Biol, 55*(5), 676-681.

Wang, J.Zganiacz, A., and Xing, Z. (2002). Enhanced immunogenicity of BCG vaccine by using a viral-based GM-CSF transgene adjuvant formulation. *Vaccine, 20*(23-24), 2887-2898.

Wang, J. Y.Chang, H. C.Liu, J. L.Shu, C. C.Lee, C. H.Wang, J. T., and Lee, L. N. (2012). Expression of toll-like receptor 2 and plasma level of interleukin-10 are associated with outcome in tuberculosis. *Eur J Clin Microbiol Infect Dis*.

Wareham, A. S.Tree, J. A.Marsh, P. D.Butcher, P. D.Dennis, M., and Sharpe, S. A. (2014). Evidence for a role for interleukin-17, Th17 cells and iron homeostasis in protective immunity against tuberculosis in cynomolgus macaques. *PLoS One, 9*(2), e88149.

Xing, Z.Zganiacz, A., and Santosuosso, M. (2000). Role of IL-12 in macrophage activation during intracellular infection: IL-12 and mycobacteria synergistically release TNF-alpha and nitric oxide from macrophages via IFN-gamma induction. *J Leukoc Biol, 68*(6), 897-902.

Zuniga, J.Torres-Garcia, D.Santos-Mendoza, T.Rodriguez-Reyna, T. S.Granados, J., and Yunis, E. J. (2012). Cellular and humoral mechanisms involved in the control of tuberculosis. *Clin Dev Immunol, 2012*, 193923.

APPENDICES

Appendix 1: Quantiferon Gold in tube assay procedure

1. Collect samples in three tubes namely the Nil, Antigen and Mitogen,

2. Incubate at 37+-2 °C for 16-24 hours without CO2.

3. Centrifuge tubes at 3000rpm at room temperature and harvest plasma.

4. Prepare the IFN-γ Elisa plate layout corresponding to the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	1A	1M	*S1*	*S1*	*S1*	13N	13A	13M	21N	21A	21M
B	2N	2A	2M	*S2*	*S2*	*S2*	14N	14A	14M	22N	22A	22M
C	3N	3A	3M	*S3*	*S3*	*S3*	15N	15A	15M	23N	23A	23M
D	4N	4A	4M	*S4*	*S4*	*S4*	16N	16A	16M	24N	24A	24M
E	5N	5A	5M	9N	9A	9M	17N	17A	17M	25N	25A	25M
F	6N	6A	6M	10N	10A	10M	18N	18A	18M	26N	26A	26M
G	7N	7A	7M	11N	11A	11M	19N	19A	19M	27N	27A	27M
H	8N	8A	8M	12N	12A	12M	20N	20A	20M	28N	28A	28M

5. Reconstitute the lyophilized 100x conjugate using 300ul of distilled water. Prepare the working conjugate by diluting the reconstituted conjugate in green diluent, quantity depending on the number of strips to be used.

6. Add 50µl of working conjugate to wells of the micro plate strips of the murine anti-human IFN- γ monoclonal antibody coated plate to be used.

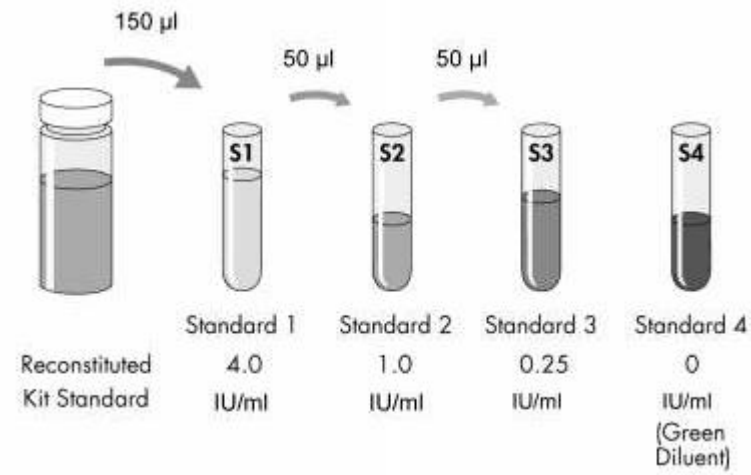
7. Add 50µl of plasma.

8. Reconstitute the human IFN-γ lyophilized standard by adding the recommended volume of distilled water as indicated on the bottle, mixing gently and preparing a serial dilution for a four point curve as follows:

a. Add 150 µl of green diluent to four tubes for a four point standard curve.

b. Add 150µl of reconstituted standard to first tube (Concentration, 4.0 IU/ml), mix and transfer 50ul to second tube (Concentration 1.0 IU/ml).

c. Mix and transfer 50ul to third tube (concentration 0.25 IU/ml) leaving the fourth tube as zero (0 IU/ml) standard.



9. Add standards S1,S 2, S3, and S4 to respective assay well plates. Shake the plate at 380rpm for 1 minute. Incubate in the dark at room temperature for 120+-5 minutes.

10. Wash plate six times using a 1x wash buffer (diluted from the 20x wash buffer concentrate.

11. Add 100ul of substrate solution (containing hydrogen peroxide and TMB), shake for 1 minute at 380rpm and incubate for 30 minutes in the dark at room temperature.

12. Add 50ul of stop solution and read plate at wavelength 450 with a reference of 650.

13. Interpret data using QFT gold in tube software version 2.62.or based on this chart:

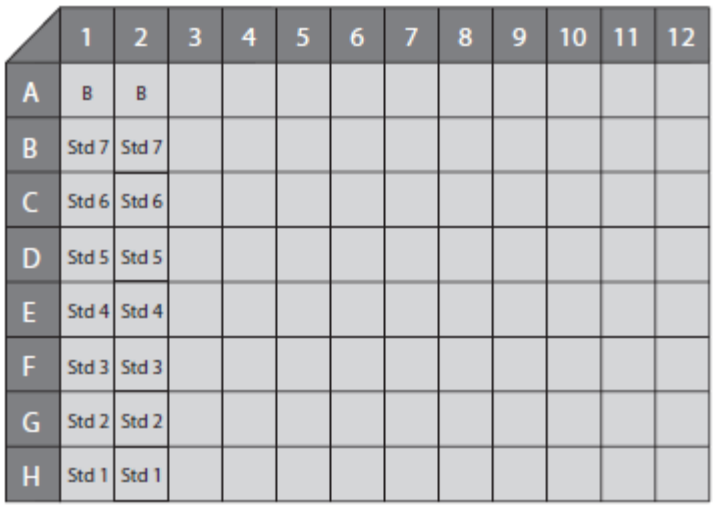
Nil (IU/ml)	TB Antigen minus Nil (IU/ml)	Mitogen minus Nil (IU/ml)*	QFT result	Report/Interpretation
≤8.0	< 0.35	≥ 0.5	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and < 25% of Nil value	≥ 0.5	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and ≥ 25% of Nil value	Any	Positive†	*M. tuberculosis* infection likely
	< 0.35	< 0.5	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness
	≥ 0.35 and < 25% of Nil value	< 0.5	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness
> 8.0§	Any	Any	Indeterminate‡	Results are indeterminate for TB-Antigen responsiveness

14. Report results as positive, negative or indeterminate.

15. For more information, refer to the package insert

Appendix 2: Luminex assay protocol (Refer to the package insert for more information)

Plate layout



	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B										
B	Std 7	Std 7										
C	Std 6	Std 6										
D	Std 5	Std 5										
E	Std 4	Std 4										
F	Std 3	Std 3										
G	Std 2	Std 2										
H	Std 1	Std 1										

Preparing Wash Solution

Prepare a 1X Working Wash Solution for use with a 96-well plate by transferring the entire contents of the Wash Solution Concentrate bottle to a 500 mL container (or equivalent) and then add 285 mL of deionized water. Mix well.

Reconstituting Lyophilized Standards

1. To the standard vial(s), add the suggested reconstitution volume of the appropriate diluent.

Donot vortex. When mixing or reconstituting protein solutions, always avoid foaming.

2. Replace the vial stopper and allow the vial to stand undisturbed for 10 minutes.

3. Gently swirl and invert the vial 2 to 3 times to ensure complete reconstitution and allow the vial to sit at room temperature for an additional 5 minutes.

Preparing Reagents,

One vial of standard: Reconstitute the standard vial in the suggested reconstitution volume, usually 1 mL, of appropriate diluent.

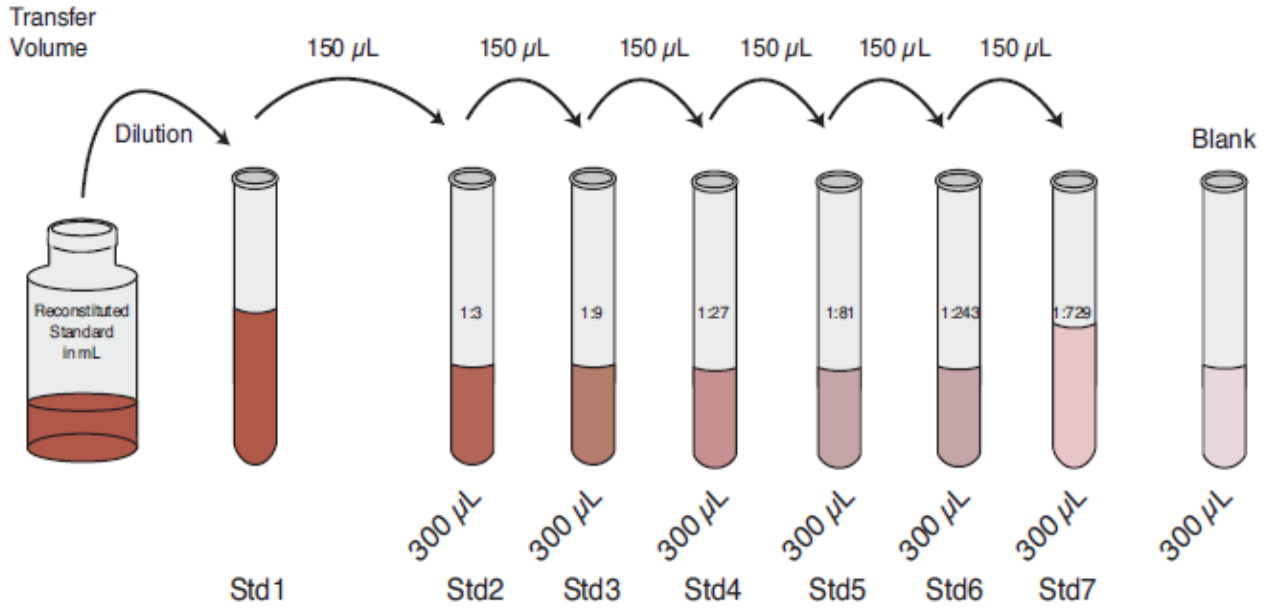
Two vials of standards: Reconstitute each vial with 0.5 mL of appropriate diluent. Combine 300 µL from each vial and mix by gently pipetting up and down 5 to 10 times.

Three vials of standards: Reconstitute each vial with 0.333 mL of appropriate diluent. Combine 200 µL from each vial and mix by gently pipetting up and down 5 to 10 times.

Four vials of standards: Reconstitute each vial with 0.250 mL of appropriate diluent. Combine 150 µL from each vial and mix by gently pipetting up and down 5 to 10 times.

Preparing Standard Curve

The standard curve is made by serially diluting the reconstituted standard in Assay Diluent for serum samples or a mixture of 50% Assay Diluent and 50% tissue culture medium for tissue culture supernatant samples. See below. **Do not vortex.** Mix by gently pipetting up and down 5 to 10 times.



Preparing 1X Antibody Beads

Determine the number of wells required for the assay. The 25-plex Antibody Beads is supplied as a 1X and is ready to use for the assay without further dilution. The fluorescent beads are light-sensitive. Protect antibody conjugated beads from light during handling**.**

The experimental outline for using the Human Cytokine 25-Plex

Summary

- Prewet wells
- Add beads
- Wash
- Add incubation buffer, standard, and samples then incubate for 2 hours
- Wash
- Add detection antibody, then incubate for 1 hour, Wash
- Add streptavidin-RPE, then incubate for 30 minutes, Wash

Re-suspend and acquire

Assay Procedure

Method of Washing

1. To wash beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum (**do not exceed** 5 mm Hg). Excessive vacuum can cause the membrane to tear, resulting in antibody bead loss. Prevent any vacuum surge by opening and adjusting the vacuum on the manifold before placing the plate on the manifold surface.

2. Stop the vacuum pressure as soon as the wells are empty. Do not attempt to pull the plate off the vacuum manifold while the vacuum is still on or filter plate damage may occur. Release the

vacuum prior to removing the plate.

3. If solution remains in the wells during vacuum aspiration, **do not detach the bottom of the 96 well filter plate.** In some cases, minor clogs in the filter plate may be dislodged by carefully pressing the bottom of the plate under the clogged well with the pointed end of a 15 mL plastic conical tube.

4. After all wells are empty, lightly tap or press the filter plate onto clean paper towels (hold the plate in the center for tapping) to remove excess fluid from the bottom of the filter plate. **Do not invert plate.**

5. Following the last aspiration and plate taps, use a clean absorbent towel to blot the bottom of the filter plate before addition of next liquid phase or data acquisition step.

6. **Do not leave plate on absorbent surface when adding reagents**.

Analyte Capture

1. Use an adhesive plate cover to seal any unused wells. This will keep the wells dry for future use.

2. Pre-wet the designated assay wells by adding 200 µL of Working Wash Solution into designated wells. Incubate plate 15 to 30 seconds at room temperature.

3. Aspirate the Working Wash Solution from the wells using the vacuum manifold.

4. Vortex the bead solution for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay.

5. Pipette 25 µL of the bead solution into each well. Once the beads are added to the plate, **keep the plate protected from light**.

6. Add 200 µL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds.

7. Aspirate the Working Wash Solution from the wells with the vacuum manifold. Repeat this washing step.

8. Blot the bottom of the filter plate on clean paper towels to remove any residual liquid.

Note: Place the filter plate on a plate cover or non-absorbent surface before all incubations.

9. Pipette 50 µL Incubation Buffer into each well.

10. To wells designated for the standard curve, pipette 100 µL of appropriate standard dilution.

11. To the wells designated for the sample, pipette 50 µL Assay Diluent followed by 50 µL sample to each well or 50 µL in-house controls, if used.

12. Cover filter plate containing beads with an aluminum foil-wrapped plate cover. Incubate the plate for 2 hours at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (500-600 rpm).Larger radius shakers will need a lower speed and smaller radius shakers will typically handle higher speeds without splashing.

13. Ten to fifteen minutes prior to the end of this incubation, prepare the biotinylated detector antibody, and then proceed to

Analyte Detection, Step 1.

Preparing 1X Biotinylated Antibody

To prepare a 1X Biotinylated Antibody stock, dilute 10 µL of 10X Biotinylated Antibody in 100 µL of Biotin Diluent per assay well.

Each well requires 100 µL of the diluted Biotinylated Antibody.

Note: Dilution factor is 1:11 for extra pipetting volume.

Analyte Detection

1. After the 2 hour capture bead incubation, remove the liquid from wells by aspiration with the vacuum manifold.

2. Add 200 µL of Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step.

3. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.

4. Add 100 µL of prepared 1X Biotinylated Detector Antibody to each well and incubate the plate for 1 hour at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500-600 rpm).

5. Prepare the LuminexR 100™ or 200™ instrument during this incubation step. Refer to the Luminex Instrument Quick Reference card provided in kit. Refer to the Technical Data Sheet for all bead regions and standard concentration values.

6. Ten to fifteen minutes prior to the end of the detector incubation step, prepare the Streptavidin-RPE, and then proceed with Assay Reading, Step 1.

Preparing Streptavidin-RPE

The Streptavidin-RPE is supplied as a **10X concentrate and must be diluted prior to use.**

Protect Streptavidin-RPE from light during handling.

To prepare a 1X Streptavidin-RPE stock, dilute 10 µL of 10X Streptavidin-RPE in 100 of µL Streptavidin-RPE Diluent per assay well. Each well requires 100 µL of the diluted Streptavidin-RPE.

Note: Dilution factor is 1:11 for extra pipetting volume.

Assay Reading

1. Remove the liquid from wells by aspiration with the vacuum manifold.

2. Add 200 µL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step.

3. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.

4. Add 100 µL of prepared 1X Streptavidin-RPE to each well and incubate the plate for 30 minutes at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500-600 rpm).

5. Remove the liquid from wells by aspiration with the vacuum manifold.

6. Wash beads by adding 200 µL Working Wash Solution to the wells; allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step 2 additional times for a total of 3 washes.

7. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.

8. Add 100 µL of Working Wash Solution to each well. Shake the plate on an orbital shaker (500-600 rpm) for 2 to 3 minutes to re-suspend the beads.

Note: If the plate cannot be read on the day of the assay, cover and store the plate in the dark overnight at 2 to 8°C for reading the following day without significant loss of fluorescent intensity. Aspirate Working Wash Solution from stored plates and add 100 µL fresh Working Wash Solution. Place the plate on an orbital shaker for 2 to 3 minutes at 500-600 rpm prior to analysis.

9. Uncover the plate and insert the plate into the XY platform of the LuminexR 100™ or 200™ instrument, and analyze the samples.

10. Determine the concentration of samples from the standard curve using curve fitting software. It is recommended to use the five parameter algorithm with a weighted function (1/y2), depending on the software package used.

Instrument Setup

Helpful guides for Luminex 100™and 200™ users.

1. Assign the appropriate **Bead Region** (refer to the kit-specific technical data sheet) to each analyte.

2. We recommend that the user **count 100 events/bead region**.

3. Set **Minimum Events** to 0.

4. Set **Sample Size** to 50 µl.

5. **Set Flow Rate** to 60 µl/minute.

6. For Invitrogen kits we recommend an initial **Double Discriminator (DD)** gate setting of 7,800-15,200. (This setting may vary among instruments and must be determined by the user.)

7. Collect **Median RFU**.

Note: All Invitrogen Multiplex Luminex Kits are qualified at low PMT setting.

Performance Characteristics and Limitations of the Procedure

Refer to analyte specific Technical Data Sheet for performance claims.

Procedure Limitations

1. Do not extrapolate the standard curve beyond the highest or lowest standard point; the dose-response and data collected in these regions may be non-linear and should be considered inaccurate. Note: In some cases, further dilution of the standard beyond 7 points may be possible to extend the low end of the standard curve.

2. Dilute samples that are greater than the highest standard with Assay Diluent or appropriate matrix diluent; reanalyze these samples and multiply results by the appropriate dilution factor.

3. Samples are diluted in the assay 1:2 (50 µL of sample and 50 µL of diluent) relative to the standards. Be sure to account for this dilution factor during sample calculations.

4. The influence of various drugs, aberrant sera (hemolyzed,hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum, plasma and tissue culture supernatant samples have not been thoroughly investigated.

5. The rate of degradation of analytes in various matrices may not have been investigated.

The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Troubleshooting Introduction Refer to the table below to troubleshoot problems encountered with the use of Invitrogen's Multiplex Bead Kits on the LuminexR platform.

To troubleshoot problems with the LuminexR instrument, refer to the manual supplied with the instrument.

Appendix 3: Ethical Approval of the study

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **October 01, 2013**

TO: **KEVIN CAIN (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. STEPHEN MUNGA**
THE ACTING DIRECTOR, CGHR
KISUMU

9/10/2013
FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Sir,

RE: SSC PROTOCOL No. 2343- REVISED (*1st AMENDMENT*): IMPROVING TB DIAGNOSIS IN CHILDREN WITH AND WITHOUT HIV IN KENYA.

Thank-you for attending the 219th KEMRI ERC meeting that was held on 24th September 2013.

The committee noted the following amendments:

1. New diagnostic procedures to be performed on controls and cases
2. Changes in the ICD to include information on new diagnostic tests
3. New diagnostics include HIV, malaria PCR and host genetic analysis
4. Changes also include conducting some tests outside the country
5. Other changes are for clarity of processes
6. Change in the enrolment criteria

This is to inform you that the Ethics Review Committee (ERC) determines that the issues raised during the meeting have been adequately addressed. The Committee therefore concluded that the amendments are justified and do not alter the risk/benefit status of the study and therefore granted approval for implementation. You are required to submit any further requests for changes to this version of the protocol to the SSC and ERC for review and approval prior to implementing any additional changes.

Yours faithfully,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES
Office of the Dean

Our Ref: PG/MSC/109/2011

Private Bag, MASENO, KENYA
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Email: sgs@maseno.ac.ke

Date: 11th August, 2014

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR JEREMIAH KHYAYUMBI—PG/MSC/109/2011

The above named is registered in the Master of Science in Biomedical Science & Technology in the School of Public Health & Community Development, Maseno University. This is to confirm that his research proposal titled "Multiple Cytokine Responses to Mycobacterium Tuberclosis Antigen in Bacille Calmette Querrin Vaccinated Infants in Boro Siaya County" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

MASENO UNIVERSITY DIRECTOR 12 AUG 2014 SCHOOL OF GRADUATE STUDIES

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